Activation of Sterol Ester Hydrolase of Bovine Corpus Luteum by $N^6,O^{2'}$-Dibutyryl Cyclic Adenosine 3':5'-Phosphate

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ABSTRACT

The direct activation of sterol ester hydrolase (E.C. 3.1.1.13) in homogenates of bovine corpus luteum by $N^6O^{2'}$-dibutyryl cyclic adenosine 3':5'-phosphate, (dibutyryl cAMP), adenosine triphosphate (ATP), and Mg^{2+} has been demonstrated. Variability in the extent of activation by the additions was minimized by homogenization of the tissue in 5 mM Mg^{2+}. Baseline sterol ester hydrolase activity was primarily associated with the 105,000 x g soluble fraction, and significant activation of the enzyme preparation preincubated with dibutyryl cAMP, ATP and Mg^{2+} occurred within the first 15 min, prior to addition of substrate. A requirement for protein kinase in the system was demonstrated by blocking the cofactor-dependent enzyme activation with commercial protein kinase inhibitor.

INTRODUCTION

Steroidogenic tissues, such as the adrenal cortex and the corpora lutea of the ovary, contain high levels of cholesteryl esters located largely in lipid inclusion droplets (1,2). The cholesteryl esters of adrenal cortex are markedly depleted during conditions of prolonged steroidogenesis, such as stress or adrenocorticotropic (ACTH) hormone administration (3-5). These esters have, therefore, been considered an important reserve of the free cholesterol substrate required for steroidogenesis (3-5). It is now evident that the activity of the sterol ester hydrolase (SEH) in the adrenal is under direct tropic hormone control (3-5), that the enzyme is activated by cyclic AMP-dependent protein kinase in vitro (6,7), and that activation involves phosphorylation of an inactive precursor (8,9).

The cholesteryl esters of the corpus luteum are similarly depleted by administration of luteinizing hormone (LH) to rats, and it has been suggested that this is a direct effect of LH on the sterol ester hydrolase of this tissue (10-12). Thus, while there is in vivo evidence for the presence of a hormone-sensitive sterol ester hydrolase in corpus luteum (10-12), the involvement of cyclic AMP and protein kinase in the activation process has not yet been demonstrated (11,13). The present communication provides evidence for the involvement of cyclic AMP in the activation of the sterol ester hydrolase of bovine corpora lutea and that the activation may be protein kinase-dependent.

MATERIALS AND METHODS

Bovine ovaries, obtained from Pel-Freez Biologicals, Rogers, AR, had been frozen on dry ice upon removal from the animals, and were maintained at -20 C. Corpora lutea (4-5 g) were dissected from the interstitial tissue, minced, and homogenized in 0.10 M phosphate buffer, pH 7.5, to give a tissue concentration of 15% by weight. In certain studies as indicated, the buffer also contained 5 mM Mg^{2+}. The homogenate was centrifuged at 105,000 x g (Beckman Model L 3-50) for 60 min at 4 C, the floating lipid cake was removed, and the remaining supernatant (S_{105}) was desalted by Sephadex G-25 gel filtration (elution with 0.10 M phosphate buffer, pH 7.5 at 4 C). It has been shown that during centrifugation of bovine adrenal homogenates, endogenous sterol esters are quantitatively floated and thereby eliminated from interference with hydrolase assays (6). With the exception of the studies on subcellular distribution of the enzyme activity, all studies reported here were conducted with the S_{105} fraction.

To 1 ml of the desalted S_{105} preparation (ca. 3.0 mg protein) was added 0.10 M phosphate buffer containing 1.0 mM theophylline, 0.1 mM dithiothreitol (DTT), 0.05 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N'-tetraacetate), and combinations of 0.3 mM adenosine triphosphate (ATP), 1 mM Mg^{2+} and 20 μM N^6,O^2-dibutyryl cyclic adenosine 3':5'-phosphate, (dibutyryl cAMP). The total incubation volume was 2.2 ml. Enzyme activation was carried out by preincubation with cofactors at 37 C in air and with shaking for periods indicated in the text. Following addition of cholesterol [1-^{14}C]oleate (0.5 μc, 6.3 μg) in 50 μl acetone, incubations were carried out at 37 C for up to 3 hr. At 15 min intervals, 0.2 ml of the incubation mixture was extracted with 1 ml chloroform-methanol-benzene (2:2.4:1, v/v), as described by Khoo and Steinberg (14). The extent of hydrolysis of the

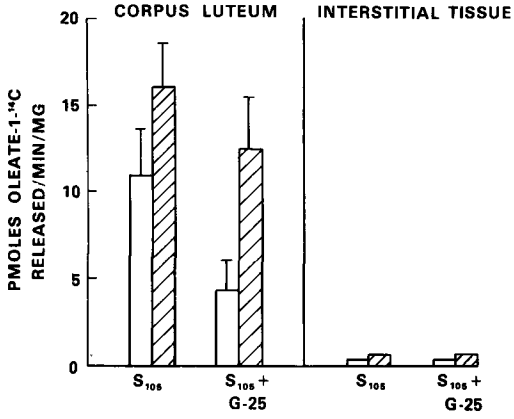

FIG. 1. Sterol ester hydrolase activity in the corpus luteum and interstitial tissue of bovine ovary. Enzyme activities were determined in the 105,000 x g soluble fraction (S_{105}) before and after chromatography on Sephadex G-25, and in the absence □ and presence ▨ of dibutyryl cAMP and ATP-Mg^{2+}.

labeled cholesteryl oleate was determined by a modification of the procedure of Pittman et al. (15). Total protein was estimated by the method of Lowry et al. (16). Results are expressed as pmoles [1-^{14}C] oleic acid released/min/mg protein.

As in the case of studies with hormone-sensitive lipase (14,17) and adrenal sterol ester hydrolase (6,7), all preparations of bovine corpora lutea contained varying levels of sterol ester hydrolase activity. This activity determined in the absence of any exogenous additions is designated baseline. Baseline activity varied between experiments, and various approaches used earlier to reduce these levels (6) proved unsuccessful in the present studies.

RESULTS AND DISCUSSION

The conditions for assay of sterol ester hydrolase activity, and the concentrations of cofactors chosen were based on our earlier studies (6,9) with the hormone-sensitive enzyme in bovine adrenal cortex. In the present study, baseline SEH activity was predominantly (56%) located in the 105,000 x g soluble fraction (S_{105}). This was also the only fraction in which activation of the hydrolase by dibutyryl cAMP, ATP-Mg^{2+} was demonstrable. The data in Figure 1 show the levels of the baseline and cofactor-dependent hydrolase activities in the S_{105} fraction of the corpus luteum and in the remaining interstitial tissue of the ovary. It is apparent that, under these conditions, the nonluteal tissue of bovine ovary contains little or no SEH activity, either in the absence or presence of ATP, Mg^{2+} and dibutyryl cAMP. Hydrolase activity in the S_{105} fraction of corpus luteum was increased by about 65% in the presence of dibutyryl cAMP, ATP and Mg^{2+}. When the S_{105} fraction was chromatographed on Sephadex G-25 (S_{105}-G25), which removes the small molecular weight cofactors (14), baseline SEH activity decreased. However, with these preparations, addition of dituryryl cAMP, ATP and Mg^{2+} resulted in a 287% increase in SEH activity. These findings are comparable to those reported for the hormone-sensitive lipase of adipose tissue (14) and sterol ester hydrolase of adrenal cortex (6).

Initially, the levels of stimulation of enzyme activity by addition of dibutyryl cAMP, ATP and Mg^{2+} were not consistent. Generally, corpora lutea stored frozen for several weeks exhibited higher baseline SEH activity, and activation by added cofactors was either reduced or absent. In addition, it appeared that factitious activation of SEH of corpus luteum occurred during homogenization of the tissue. This resulted in higher baseline levels of activity and a decreased response to added cofactors. Similar findings have been reported for the lipase of adipose tissue (18) and the SEH of bovine adrenal cortex (6). In the present study, it was found that the baseline SEH activity was low when the corpora lutea were homogenized in 0.10 M phosphate buffer containing 5 mM Mg^{2+}. The time required for activation of SEH by dibutyryl cAMP, ATP and Mg^{2+} was determined using the S_{105}-G25 fraction prepared from tissues homogenized in 5 mM Mg^{2+}. Preincubation of the enzyme and the complete cofactor system was carried out at 37 C in air for periods up to 120 min prior to addition of radioactive substrate and subsequent determination of hydrolase activity. As shown in Table I, enzyme activity in tissue preparations obtained following homogenization in 5 mM Mg^{2+} were initially low, and in these preparations, cofactor addition routinely resulted in about a 40% activation, even without preincubation. With a 15 min preincubation prior to addition of substrate, both baseline and cofactor-dependent activities were significantly increased. By paired statistical analysis, it was apparent that activation of SEH by the added cofactors was significantly greater than the increase in baseline activity resulting from the 15 min preincubation period. With increasing periods of preincubation, there was no further effect of added cofactors on SEH activity. However, by 120 min of preincubation, baseline activity was reduced to a level comparable to that with no preincubation. Thus, the difference between cofactor-dependent activity and baseline activities was magnified, e.g., cofactor-dependent

TABLE I

Effect of Preincubation Time on Sterol Ester Hydrolase Activity

Preincubation time prior to assay, min[a]	Sterol ester hydrolase activity[b] additions to preincubation None	Complete	Signif.[c]
0	1.6 ± 0.2[d]	2.2 ± 0.3	< 0.025
15	2.6 ± 0.4	4.0 ± 0.6	< 0.005
60	2.8 ± 0.3	4.5 ± 0.6	< 0.01
120	2.1 ± 0.3	5.1 ± 0.6	< 0.005

[a]Corpora lutea were homogenized in 5 mM Mg^{2+} and the S_{105}-G25 fraction was prepared. Preincubation was carried out at 37 C using 3.0 mg protein of enzyme preparation in the absence or presence of the following components made up to 2.2 ml final volume with 0.10 M phosphate buffer, pH 7.5: 1 mM theophylline; 0.1 mM dithiothreitol; 0.05 mM EGTA; 0.3 mM ATP; 1.0 mM Mg^{2+}; 20 μM dibutyryl cAMP. Following incubation, cholesteryl [1-^{14}C] oleate (6.3 μg) was added in 50 μl acetone for subsequent enzyme assay.

[b]pmoles [1-^{14}C] Oleic acid released/min/mg protein.

[c]Paired analyses between controls and complete system.

[d]Figures represent means from six studies ± SEM.

activity was 240% of baseline activity. In a single study in which preincubation times were extended to 360 min (data not shown), baseline SEH activity continued to decrease, while the cofactor-dependent activity remained elevated. In this study cofactor-dependent activity was 360% of baseline at 240 min of preincubation, and 380% of control after 360 min of preincubation.

Thus, it appears that under these conditions with a crude enzyme preparation, significant activation of the SEH of corpus luteum by dibutyryl cAMP, ATP-Mg^{2+} occurred within the first 15 min and that in the presence of these additions, enzyme activity remained stable for up to 6 hr prior to enzyme assay. These findings are comparable to those obtained with other "hormone sensitive" enzymes. The preincubation time required for maximal activation of adipose tissue lipase by these same cofactors has been reported to be from 2 to 10 min (14,17). With adrenal SEH, maximal activation and phosphorylation occurs during the first half hr of incubation (9) with low levels of cofactors, and within 5 min at high cofactor concentrations (8).

Data on the effect of cofactors, added singly and in various combinations, on the SEH activity of the S_{105}-G25 fraction are shown in Table II. In these studies there was no preincubation period. Additions were made directly to the enzyme assay system containing the S_{105}-G25 fraction (prepared in the absence of Mg^{2+}), theophylline, dithiotreitol DTT, EGTA and cholesteryl oleate. Additions of Mg^{2+} and dibutyryl cAMP, separately or in combination, had no significant effect on SEH activity in these preparations. Addition of 0.3 mM ATP,

TABLE II

Requirements for Adtivation of Sterol Ester Hydrolase

Addition to incubation media[a]	Sterol ester hydrolase activity[b]
None	9.9 ± 0.8
Dibutyryl cAMP (20 μM)	9.9 ± 0.3
ATP (0.3 mM)	12.2 ± 1.3[c]
Mg^{2+} (1.0 mM)	11.9 ± 1.1
Dibutyryl cAMP + Mg^{2+}	11.4 ± 1.0
Dibutyryl cAMP + ATP	12.4 ± 0.3[c]
ATP + MG^{2+}	13.4 ± 1.2[c]
Complete system	16.2 ± 1.1[c,d]
Complete system + protein kinase inhibitor (40 μg)	11.8 ± 1.5[e]

[a]All incubation media contained, in addition to the components indicated, 1 ml (3 mg protein) S_{105}-G25 enzyme preparation; 1 mM theophylline; 0.1 mM dithiothreitol; 0.05 mM EGTA. Total volume was made to 2.2 ml with 0.10 M phosphate buffer, pH 7.5. Incubations were carried out at 37 C in air. Figures represent means ± SEM for four studies.

[b]pmoles 1-^{14}C Oleic acid released/min/mg protein.

[c]$p < 0.05$ from control incubation media with no addition (paired t test).

[d]$p < 0.05$ from system containing ATP + Mg^{2+} (paired t test).

[e]$p < 0.05$ from complete system (paired t test).

alone or in combination with Mg^{2+} or dibutyryl cAMP, resulted in a significant increase in SEH activity compared to baseline levels. However, neither Mg^{2+} nor dibutyryl cAMP additions to the ATP incubations gave any additional stimulation over that observed with ATP alone. This effect of ATP addition on enzyme activation in crude systems has also been observed with bovine adrenal SEH (9). Although addition of dibutyryl cAMP, alone or with

Mg^{2+}, had no significant effect on SEH activity, a significant increase in enzyme activity was observed in incubations including ATP and Mg^{2+} (complete system).

Attempts to demonstrate a protein kinase requirement in crude preparations were unsuccessful. Up to 96 μg rabbit muscle protein kinase (Sigma Chemical Co. St. Louis, MO) was without further effect on the activation observed in the complete cofactor system. This is most likely due to the presence of endogenous protein kinase in the S_{105} fraction (9,19). However, as shown in Table II, addition of 40 μg of protein kinase inhibitor (beef heart, Sigma Chemical Co.) resulted in a significant decrease in level of activation observed in the absence of the inhibitor. These data indicate that the activation of the SEH of bovine corpus luteum may require protein kinase, and imply that activation involves enzyme phosphorylation, as has been shown for adipose tissue lipase (17) and adrenal SEH (8,9). Since significant activation occurred in the presence of only ATP, it is also possible that activation of SEH may involve both cyclic AMP-dependent and cyclic AMP-independent systems. The presence of cyclic AMP-dependent and independent protein kinase activities in bovine corpus luteum has been reported (19). The role(s) of these kinases in the activation of sterol ester hydrolase remains to be determined.

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Occurrence of Plant Sterols in Aquatic Vertebrates

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ABSTRACT

Plant sterols were found by gas liquid chromatography in the sterols of five species of aquatic vertebrates; mackerel (*Scomber japonicus*), rainbow trout (*Salmo gairdnerii*), smelt (*Osmerus dentex*), sardine (*Sardinops melanosticta*) and chimera (*Chimera phantasma*). The sterols of chimera liver, sardine flesh and sardine viscera contained 9.0, 2.4 and 3.1% of C_{28} and C_{29} sterols in addition to 86.7, 96.6 and 95.2% of cholesterol. The occurrence of norcholestandienol, campesterol, β-sitosterol and C_{28} stanol was shown by combined gas chromatography-mass spectrometry. Sperm whale (*Physeter catodon*) sterols consisted of more than 99% cholesterol with only traces of 22-dehydrocholesterol.

INTRODUCTION

It was once accepted that higher animals and most of lower animals contained cholesterol with minor components of C_{27} derivatives, e.g., cholestanol, 7-dehydrocholesterol and 7-cholestenol, while plants and some of lower animals contained a variety of C_{26}-C_{29} sterols (1). Recently, plant sterols were found in man and other higher animals as minor components, and their physiologically important roles are now recognized (2-6).

It is well known that the main sterol of fish is cholesterol. However, minor sterols in fish have not been reported. This study was carried out to investigate the minor components in fish sterols, and we now report the occurrence of C_{28} and C_{29} plant sterols in fish.

MATERIALS AND METHODS

Commercial samples of the crude head and body oils from a sperm whale captured in the Antarctic Ocean were obtained. The head oil was used in the gas liquid chromatographic (GLC) analysis of the wax esters (7). The flesh samples of mackerel (*Scomber japonicus*), sardine (*Sardinops melanosticta*), rainbow trout (*Salmo gairdnerii*), and smelt (*Osmerus dentex*) were obtained commercially at Hakodate in November and December 1977. A chimera (*Chimera phantasma*) captured in the deep sea off New Zealand was obtained from a fishery company.

Extractions of lipids were carried out by the Bligh and Dyer method (8). The unsaponifiables were obtained by ether extraction of the saponified lipids in the ordinary way. The sterol fraction was separated by preparative thin layer chromatography using Merck Kiesel Gel G (Type 60) plates and n-hexane-ether-acetic acid (60:40:1) for development.

GLC analyses of sterols were carried out with a Shimadzu gas chromatograph Model 6AM equipped with dual hydrogen flame detectors on 1 m x 3 mm ID and 2 m x 3 mm ID glass columns packed with 2% OV-1 on 80-100 mesh Chromosorb W AW DMCS and 5% SE-30 on Chromosorb W 100-120 mesh, respectively. The carrier gas was nitrogen.

Gas chromatography-mass spectrometry (GC-MS) analyses of sterols were carried out with a Hitachi EI instrument coupled to a computer. The GLC separations were performed on 1 m x 3 mm ID glass columns packed with 2% OV-1 on 80-100 mesh Chromosorb W. The carrier gas was helium, and the column temperature was programmed from 190 to 280 C at a rate of 5 C/min. The injector, separator, and ion source were kept at 290, 300 and 180 C, respectively. The spectra were taken at an ionizing voltage of 20 eV and accelerating voltage of 3.2 kV. Mass chromatograms and bargraphs were constructed by the computer.

Authentic samples of β-sitosterol, cholesterol and campesterol were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan. Cholesterol containing norcholestandienol, 24-methylenecholesterol and desmosterol (obtained from a crab, *Erimacrus isenbeckii*, cf. Yasuda (9)) was also used as a reference standard.

RESULTS AND DISCUSSION

The typical gas chromatogram of the chimera liver sterols is shown in Figure 1. The retention times of the peaks 1, 3, 4, 5 and 7 were in agreement with those of norcholestandienol, cholesterol, 24-methylenecholesterol, campesterol and β-sitosterol, respectively. The sterol compositions of chimera liver, sardine viscera and sardine flesh are shown in Table I as peak area percents. It is noteworthy that they contain significant amounts of plant sterols. In particular, the plant sterols in the chimera liver reach almost 10%. Such high percentages of plant sterols in vertebrate sterols have not

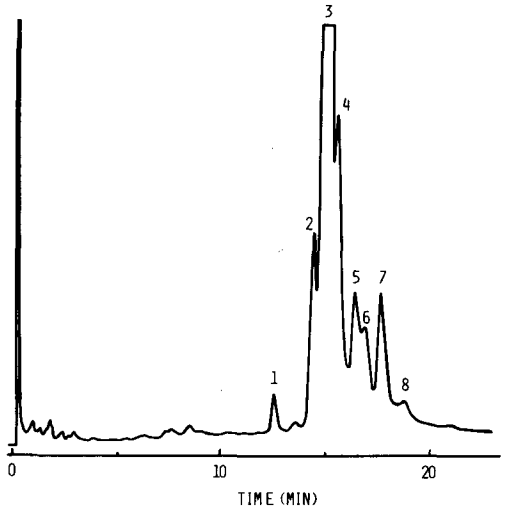

FIG. 1. Gas chromatogram of chimera liver sterols. Hitachi GC-MS EI, 1 m x 3 mm ID glass column packed with 2% OV-1 on 80-100 mesh Chromosorb W. Detector: Total ion collector. Column temperature was programmed from 190 to 280 C at 5 C/min.

hitherto been reported. In the other fish, the minor sterol components which exceed 0.1% were 22-dehydrocholesterol and campesterol under the conditions of this experiment, and the cholesterol contents were more than 98%. The sterols of sperm whale head and body oils contained more than 99% of cholesterol, and C_{28} and C_{29} sterols were not found in them. Their sterol compositions are shown in Table II.

The typical GC-MS spectrum of the plant sterols from the chimera liver is shown in Figure 2. GLC peaks 1-8 showed the following major molecular ions: 370, norcholestandienol, for peak 1; 384, 22-dehydrocholesterol, for peak 2; 386, cholesterol, for peak 3; 398, 24-methylenecholesterol, for peak 4; 400, campesterol, for peak 5; 402, C_{28} stanol, for peak 6; 414, β-sitosterol, for peak 7; and 400, C_{28} monounsaturated sterol, for peak 8. The mass spectrometry (MS) patterns were compared with those described in the literature (10).

The molecular ion peaks were the base peaks as expected from the mild operating conditions

TABLE I

Compositions of Sterols in Chimera Liver, Sardine Viscera and Flesh (GLC Peak Area %)

Peak number	Sterol	Chimera liver	Sardine	
			Viscera	Flesh
1	Norcholestandienol	0.6	0.2	T[a]
2	22-dehydrocholesterol[b]	3.7	1.5	1.0
3	Cholesterol[c]	86.7	95.2	96.6
4	24-methylenecholesterol	2.5	T	---
5	Campesterol	2.1	2.6	2.1
6	C_{28} stanol[d]	1.5	0.4	0.3
7	β-sitosterol	2.5	0.1	T
8	C_{28} sterol[d]	0.4	T	T

[a]Trace, less than 0.1%.
[b]Presumed by its retention time and GC-MS data.
[c]GC-MS data suggest the overlapping of cholestanol peak.
[d]Presumed by GC-MS data.

TABLE II

Compositions of Sterols in Some Aquatic Vertebrates (GLC Peak Area %)

Animal	Part	22-dehydrocholesterol	Cholesterol	Campesterol
Mackerel	White muscle	0.8	98.9	0.3
	Dark muscle	1.3	98.2	0.5
	Viscera	0.7	99.2	0.1
	Head	T	99.8	0.2
Rainbow trout	Whole	---	99.7	0.3
Sperm Whale	Body	0.5	99.5	---
	Head	0.8	99.2	---
Smelt	Whole	---	98.8	1.2

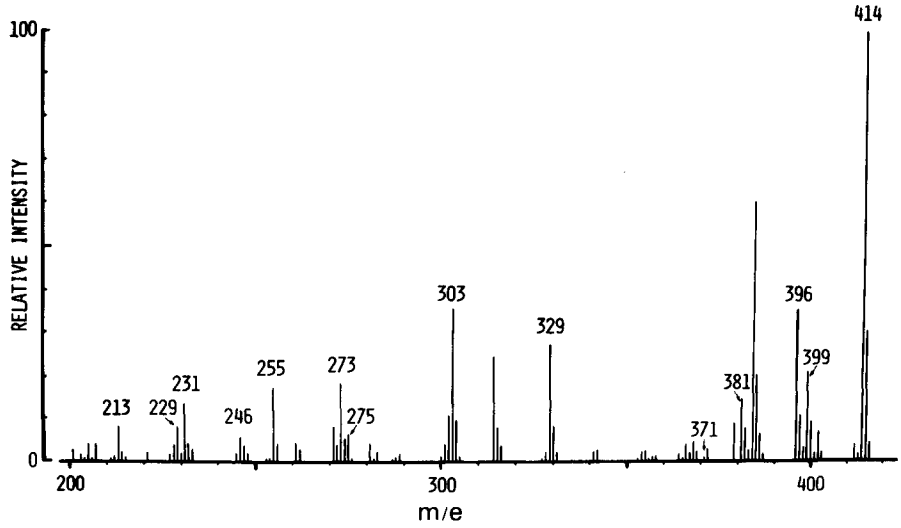

FIG. 2. GC-MS spectrum of GLC peak 7 (β-sitosterol) of chimera liver sterols. Operating conditions as given in text.

for the ionization; the low ionizing voltage (20 eV) and the low temperature of the ion source (180 C) reduce fragmentation. Exceptionally, the molecular ion peak was not the base peak in the spectra for the GLC peak 3, but it was one of the main peaks. The peak of m/e 388 accompanied by the molecular ion peak of cholesterol at m/e 386 suggests overlapping of the cholestanol peak with peak 3 in the gas chromatogram. The fragment ion peaks M^+-15 (CH_3), M^+-18 (H_2O), M^+-15-18 followed the molecular ion peaks as an ordinary pattern in MS. The ion peaks m/e 271 (M^+-R-2H, R = side chain) and adjacent peaks m/e 273 (M^+-R) were common in the spectra for all GLC peaks. They indicated the presence of the monounsaturated sterol ring. These facts and the ion peak 400 accompanied by the molecular ion peak 402 for the GLC peak 6 suggested the tailing of campesterol peak into peak 6.

The presence of an unsaturated C_7H_{13} and C_8H_{15} side chains in the peak 1 and 2 sterols was supported by their intense fragment ions at m/e 97 and 111 (11). The location of a double bond at 22:23 in the side chain was supported by the intense fragment ion at m/e 300, which was derived from the allylic cleavage with one hydrogen transfer (12).

Mass chromatograms of the chimera liver sterols are shown in Figure 3. The presence of peaks 1-7 is in harmony with the interpretation for the peak components given by the GLC retention times. Similar results were obtained with sterols of sardine flesh and viscera. The expected m/e fragment ions were detected

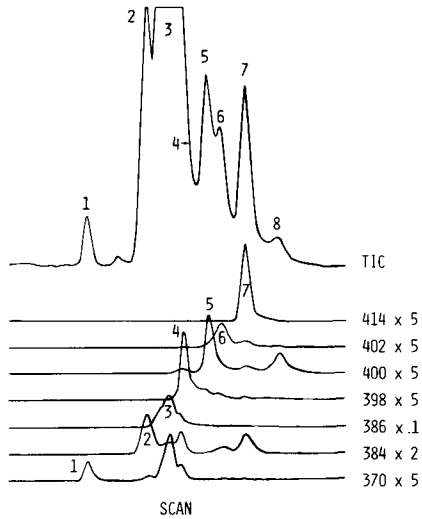

FIG. 3. Mass chromatograms of chimera liver sterols. TIC: Chromatogram by a total ion collector. Relations between peak numbers and components are shown in Table I.

along with those of the respective molecular ions in the same scans.

The results presented here clearly establish that some kinds of fish contain small amounts of plant sterols with a major component cholesterol. Further work is proceeding in our laboratory to determine the origin and the effect of plant sterols in fish.

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Isolation and Partial Characterization of the Neutral Glycosphingolipids and Gangliosides of the Human Heart

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ABSTRACT

The glycosphingolipids (GSL) of the human heart muscle have been isolated from total lipids by column and thin layer chromatography and their sugars and fatty acids analyzed by gas liquid chromatography. Hearts from traffic victims were obtained at autopsy between 12 and 16 hr after death and dissected into parts (left and right ventricular walls, intraventricular septum and papillary muscle). The neutral GSL content for those parts of the hearts of two males aged 22 and one female aged 14 ranged from about 90 to 160 nmoles/g wet weight. Trihexosyl ceramide and globoside were the most abundant neutral GSL. Total ganglioside content was about 50 nmoles/g wet weight, and the most abundant gangliosides were partially characterized as GM_3 and GM_1; other mono-, di- and trisialogangliosides were also present. Differences in the content and composition of neutral GSL and gangliosides between the heart and other human tissues are discussed. It is concluded that the patterns of these two GSL fractions of the heart are more complex than those of most of the extraneural human tissues.

INTRODUCTION

Neutral glycosphingolipids (GSL) and gangliosides are present as minor constituents of the lipid fraction in most, if not all, types of extraneural mammalian organs (1). Even so, the lipids of the human heart muscle have not been examined for glycosphingolipid composition. The presence of these lipids and their characterization in human skeletal muscle has been reported by Svennerholm et al. (2). Qualitative analysis of the ganglioside fractions in the various tissues of pig, rabbit and rat indicated that in these species the pattern of heart gangliosides are different from those of the skeletal muscle (3). In order to investigate further the possible role of GSL in heart muscle function, it was found necessary to report first their qualitative and quantitative analysis in the various parts of the human heart.

MATERIALS

Hearts were taken at autopsy between 12 and 16 hr after death from traffic victims who died suddenly. Hearts I and II were from normal males aged 22 and heart III from a female aged 14. Parts of the left and right ventricles, the intraventricular septum and the papillary muscle were freed of visible fat, washed blood free and kept at -20 C until extracted.

Chemicals were of analytical grade and solvents were distilled before use. Hexamethyldisilazane and trimethylchorosilane were obtained from BDH Chemicals Ltd., England. Neuraminidase (0.6 u/mg) was obtained from Boehringer Mannheim. Standards of neutral GSL and gangliosides were prepared from human kidney and brain as described below.

N-acetyl neuraminic acid 90%, bovine brain gangliosides type IV and silicic acid (special for lipid chromatography) were obtained from Sigma Chemicals Co. St. Louis, Mo. DEAE-Sephadex-A25 was obtained from Pharmacia Fine Chemicals, Sweden.

Precoated Silica Gel G plates and Silica Gel H for column chromatography were purchased from Merck Darmstadt. Silica gel (with no binder) plates used in preparative chromatography of neutral GSL were from Analtech Inc., Newark, DE.

Gas liquid chromatography (GLC) was carried out on a Pye-Unicam gas chromatograph.

METHODS

Extraction of Lipids

The lipids were extracted by homogenizing the tissue (10-40 g) with a series of chloroform-methanol mixtures (1:1, 2:1 and 1:2, v/v). The pooled extracts were dried, taken up in water and chloroform methanol (2:1, v/v). Partition and washing was done as described by Folch et al. (4)

Preparation of Neutral and Acidic Glycosphingolipids

The lower phase lipids were subjected to mild alkaline hydrolysis and GSL isolated by column chromatography on silicic acid as described (5) and used previously (6,7). Neutral and acidic GSL were separated by chromatography on DEAE-Sephadex-A25 (8,9). Individual neutral GSL (mono-, di, trihexosyl ceramide and aminoglycolipid) were isolated by

preparative thin layer chromatography TLC. The monohexosyl ceramide fraction was separated into glucosyl ceramide and galactosyl ceramide (10). The GSL were revealed by spraying with water. The dried silica gel was transferred into columns (0.4 cm i.d.) and washed with 10 ml of chloroform. The GSL was eluted with 20 ml chloroform-methanol (1:1, v/v). Possible water soluble contaminants were washed by partition according to Folch et al. (4). The recovery of separated GSL was calculated as a percentage of total GSL applied on the TLC plate. Purified GSL were characterized in terms of their sugar and sphingosine composition. Fatty acid esters were recovered from the methanolysis mixture by extraction with redistilled hexane and separated into normal and 2-hydroxy fractions by TLC (6). The acidic fraction of GSL was taken down to a small volume dialyzed against water and freeze dried. Hematoside was the only GSL constituent of this fraction. It was isolated by preparative TLC and added to the gangliosides of the upper phase.

Gangliosides

The whole procedure for obtaining total gangliosides from the combined upper phases (dialyses, hydrolysis in sodium methoxide) has been described previously (6). Purification of total gangliosides from fatty acids and other contaminants was performed as described (8) by column chromatography on silicic acid. Total gangliosides are given in nmoles of sialic acid, corrected for recovery (70%). This recovery was estimated by running in parallel samples of bovine brain and human kidney gangliosides, similar in weight to those used for the heart. The recoveries for bovine brain and human kidney gangliosides were 70% and 75%, respectively.

Preparation of Ganglioside Fractions

Total gangliosides were separated into three major fractions exactly as described (2) as follows: Gangliosides were dissolved in chloroform-methanol (9:1, v/v) and applied to a washed Silica Gel H column, which was then eluted with two portions of $CHCl_3$-CH_3OH-H_2O (65:25:4, v/v/v). The first portion contained mostly GM_3. The second portion contained GD_3, GM_2 and some GM_3. The remaining gangliosides were eluted with chloroform-methanol-H_2O (60:35:8, v/v/v). These fractions were used for the purification of the major gangliosides to homogeneous spots by TLC on washed and reactivated Silica Gel G plates. The bands were located with the aid of iodine vapors, and the gangliosides were extracted three times with a mixture of chloroform-methanol water (60:35:8, v/v/v). Purified gangliosides were partially characterized in terms of their carbohydrate and sialic acid composition. Neuraminidase treatement was also used to assist in characterization.

Analytical Procedures

Total lipids were estimated by weighing, to a constant weight, small aliquots of the chloroform phase. Lipid phosphorus was determined by the method of Bartlett (11). Glucose content of neutral GSL was determined by GLC, using mannitol as an internal standard. The results of GSL were expressed in nmoles calculated from the area of the glucose peaks (6,7). Sphingosine was determined (12) as described previously (13). The sialic acid content of the gangliosides was determined by the modified (14) method of Svennerholm (15). TLC was performed using Silica Gel G plates. The bands of neutral GSL were detected by spraying with orcinol reagent (0.2% in 25% sulfuric acid) and heating for 10 min at 100 C Gangliosides were detected with the resorcinol: HCl reagent (15). The solvent for neutral GSL was chloroform-methanol-H_2O (65:25:4, v/v/v). Reference standards of neutral GSl were run for identification. The solvent for gangliosides were: A) chloroform-methanol-NH_4OH 2.5 M (60:40:9, v/v, three developments), b) n-propanol-water (3:1, v/v), and c) n-propanol-conc. ammonia-water (6:1:2, v/v/v); reference standards of bovine brain, human kidney and brain gangliosides were run on the same TLC plates. The percentage distribution of gangliosides was estimated as described by Suzuki (16) in several samples of total gangliosides after separation in TLC system (a). Gangliosides which were not well separated in this system were recovered and their sialic acid content determined following separation in a more appropriate TLC system.

Methanolysis of neutral GSL and gangliosides was performed in HCl 0.8N and 0.5N HCl in dry methanol, respectively. N-Acetylation was performed as described (17). The recovery of N-acetyl galactosamine (NAcgal) was corrected on the basis of the analysis of globoside from human kidney. Preparation of trimethyl-silylderivatives (TMS) derivatives was performed as described (6). TMS derivatives of O-methyl monosaccharides and sialic acid were analyzed by GLC (17) on 3% SE-30 or 3% OV-1 columns (6 ft x 1/4 in.) at a temperature programmed at 2 C/min between 160 C and 225 C. Fatty acid esters were analyzed isothermally at 230 C on the same columns.

TABLE I

Neutral Glycosphingolipid Concentration of Various Parts of the Adult Human Heart[a]

Heart		L.V.		R.V.		I.S.	Papillary
I	nmole/g tissue[b]	134	117	122	135	89	139
	nmole/mg lipid[c]	4.7	4.2	2.5	2.7	5.2	7.7
II	nmole/g tissue	110	124	117	130	94	110
	nmole/mg lipid	4.9	6.3	4.5	4.7	3.6	6.4
III	nmole/g tissue		124	124	160	117	- - -
	nmole/mg lipid		4.4	4.2	5.5	7.7	- - -

[a]GSLs were isolated from the chloroform soluble lipids and estimated in duplicate by GLC as described in the text. Data for the left ventricle (L.V.) are GSL content of parts of the anterior and posterior walls. Data for the right ventricle (R.V.) are GSL content of the anterior and anterolateral walls. Data for the intraventricular septum (I.S.) are GSL content of the lower part of the septum.

[b]nmoles/g tissue are nmoles of GSL per g wet weight.

[c]nmoles/mg lipid are nmoles of GSL per mg total lipid.

TABLE II

Neutral Glycosphingolipid Composition of the Human Heart[a]

	Molar Ratio[b] glu/gal/N Acgal	nmoles/g wet tissue I	II	III
Glucosyl ceramide	1.0:0.0:0.0	1.6	5.3	1.2
Galactosyl ceramide	0.0:1.0:0.0	1.0	5.0	1.0
Lactosyl ceramide	1.0:1.1:0.0	4.1	7.7	11.6
Trihexosyl ceramide	1.0:1.8:0.0	32.0	37.0	43.7
Globoside	1.0:1.9:0.9	43.0	28.0	36.0
Total		81.7	83.0	93.5
Recovery		70%	67%	75%

[a]GSL fractions were separated, as described in Methods, from the posterior wall of each of the hearts presented in Table I. The percentage recovery was calculated from the total GSL of each part shown in Table I. The percentage recovery of each GSL, tested separately, was in the range of 75%.

[b]Determined in pooled samples. The ratio of glucose sphingosine for all GSL fractions ranged between 1.2 and 1.0.

Neuraminidase Treatment

Aliquots of purified gangliosides were dried in small conical tubes, and 0.05 ml of sodium acetate buffer pH 5.5 containing 0.075 units neuraminidase was added to the dry residue. The mixture was incubated at 37 C for 24 hr, extracted and partitioned (4). The lower and upper phases were analyzed by TLC.

It is known (18) that following neuraminidase treatment sialic acid is split off from GM_3 and GD_3. These gangliosides are transformed into lactosyl ceramide. One sialic acid residue is split off from GD_{1a}, GD_{1b}, and GT, and these then yield GM_1. The sialic acid residue of the GM_1 and GM_2 gangliosides is not attacked by neuraminidase.

RESULTS

Total lipids from the various parts of the hearts ranged from 17-50 mg/g wet weight, phospholipids being about 50-70% of the total. In accordance with a previous report (19), the present results showed that regional differences in total lipid and phospholipid content were only occasional, like, for example, the high lipid content of the right ventricules of heart No. I.

Neutral glycosphingolipids

Total neutral GSL are shown in Table I. In all three hearts examined, the content in nmoles/g wet weight of the various parts (see footnote of Table I) did not differ significantly. Some individual variations were found in GSL content of certain parts relative to total lipid. In this respect the GSL content found in the right ventricle of heart No. I was significantly lower than in other parts.

The various parts of the hearts had similar patterns of neutral GSL as measured by TLC analysis. They included mono-, di-, tri- and tetrahexosyl ceramide. Their content and car-

TABLE III

Fatty acid Composition of the Neutral Glycosphingolipids of the Human Heart[a]

Fatty acid	Per cent of total			
	Monohexosyl ceramide	Lactosyl ceramide	Trihexosyl ceramide	Globoside
16:0	13.3	11.0	7.5	1.0
18:1	10.0	7.4	3.1	2.0
18:0	8.3	3.7	2.7	1.5
20:0	10.0	5.9	9.1	2.4
22:0	36.8	28.6	34.6	36.0
23:0	3.3	3.7	13.5	10.0
24:1	6.7	20.6	13.5	39.6
24:0	11.6	19.1	16.0	7.5
22:0-24:0	58.4	72.0	77.6	93.1

[a]Methyl esters of normal fatty acids were prepared as described in Methods and analyzed by GLC on a OV-1 3% column at 230 C. Other fatty acids, 16:1,17:0, 19:0, 20:1, which summed up to less than 3% of total, were not included in the Table. Monohexosyl ceramide is a mixture of glucosyl ceramide and galactosyl ceramide.

TABLE IV

Ganglioside Content and Composition of the Human Heart[a]

No. of heart	I	II	III
Total sialic acid nmoles/g wet weight	50.5	43.5	52.5
Gangliosides	Total sialic acid weight (%)		mole (%)
GM_3	23.0		31.1
GM_2	13.0		17.6
GD_3	22.0		15.0
GM_1	16.0		21.8
GD_{1a}	11.0		7.5
GD_{1b}	5.0		3.4
GT	10.0		4.5

[a]Gangliosides were isolated from the combined upper water phases of the Folch partition. To this fraction GM_3 isolated from the lower phase was added. Total ganglioside sialic acid was determined in triplicate samples. Sialic acid content in the various gangliosides was determined after separation by TLC on system (a) and for GM_1 and GD_3 also on system (c). The gangliosides GM_3, GD_3, GM_1, GD_{1a} and GT were further characterized by GLC. The molar ratio of glucose/galactose/Nacetyl-galactosamine/sialic acid was for GM_3 1.0:0.9:0.0:0.8, for GD_3 1.0:1.2:0.0:1.7, for GM_1 1.0:1.8:1.1:0.85, for GD_{1a} 1.0:1.7:1.1:2.2, and for GT 1.0:1.8:0.9:2.6.

bohydrate composition are shown in Table II. It can be seen that monohexosyl ceramide was a mixture of glucosyl and galactosyl ceramide. Dihexosyl ceramide was characterized as lactosyl ceramide. The possible structure of trihexosyl ceramide was gal-lac-ceramide. Tetrahexosyl ceramide was partially characterized as globoside. The most abundant neutral GSL were trihexosyl ceramide and globoside.

Fatty Acid Composition of the Neutral GSL

Table III shows the fatty acid composition of the neutral GSL isolated from pooled samples of the three hearts. The results indicate that C_{22} fatty acid was major constituent of all GSL fractions. It is also evident that as the carbohydrate chain of the GSL increased so did the percentage of the fatty acid with chains C_{22}-C_{24}. The 2-hydroxy fatty acids were minor constituents of all GSL fractions and were not further analyzed.

Acidic GSL of the Chloroform Phase

The acidic fraction of the chloroform soluble GSL comprised only GM_3 characterized by its chromatographic properties and color reactions. This was isolated by preparative TLC and added to the ganglioside fraction. Spots corresponding to sulfatides of the galcer and/or laccer structure could not be detected by TLC. In this case the analysis was performed with material corresponding to 50 g of fresh tissue. The amount of heart GSL needed to produce a TLC chromatogram with four spots corresponds to about 0.5 g of wet tissue. It is thus concluded that if sulfatides are present they should account for less than 1 % of total GSL.

Gangliosides

Analysis of the gangliosides of the various parts of hearts I and II showed similar patterns. Subsequent examination was therefore performed on pooled samples. Total ganglioside content (Table IV) was in the range of 50 nmoles of sialic acid per g wet tissue. The percentage distribution of the gangliosides for pooled samples of the three hearts are shown in Table IV. It can be seen that CM_3 comprised 23.0% of total sialic acid or 31.1% on a molar basis. This ganglioside appeared on TLC system (a) as a single spot. The results of carbohydrate

and sialic acid composition are shown in Table IV. This ganglioside after treatment with neuraminidase yielded a chloroform soluble GSL with the chromatographic mobility of lactosyl ceramide. The GM_2 ganglioside migrated as multiple band on TLC system (a) in the position of GM_2 of human brain. The sialic acid residue of the material of this band was not split off following neuraminidase treatment. The second largest ganglioside, GM_1, run in system (a) at the position of GM_1 of bovine brain, was not easily distinguished from GD_3 of kidney gangliosides. It was, therefore, isolated from plates developed in system (a) and rerun in system (c) where it was separated into GM_1 and into a ganglioside which in this solvent system runs ahead of GM_1 in the position of human kidney GD_3. Further characterization of GM_1 and GD_3 was based on the molar ratio of their sugar and sialic acid content as shown in Table IV. Ganglioside GM_1 was not affected by neuraminidase treatment. Ganglioside GD_3 on neuraminidase treatment yielded a chloroform soluble GSL with the chromatographic migration of lactosyl ceramide. The gangliosides designated as GD_{1a} and GT_1 had the expected composition of monosaccharides and sialic acid and the expected mobility in TLC system (b). These gangliosides, as well as GD_{1b} after neuraminidase treatment, yielded a ganglioside corresponding in chromatographic migration to GM_1.

DISCUSSION

In this work an attempt has been made to analyze separately the neutral GSL and gangliosides of various parts of the heart. As indicated in the results, the amount and composition of the neutral GSL and the patterns of gangliosides of the two ventricles and the septum were very similar. The neutral GSL content of the ventricles of the adult human myocardium estimated in the present work ranged from 90 to 160 nmoles/g wet tissue. This content is higher than reported for the gastrocnemius muscle of the adult male where GSL content was only 35 nmoles/g wet tissue (2). The procedure applied here for the isolation of neutral GSL is only slightly different from that used for gastrocnemius muscle (2). It is reasonable, therefore, to believe that the large difference in total GSL content of the two tissues is not due to differences in methodology.

Interestingly enough, the pattern of the neutral GSL of the heart was more complex than that found in the gastrocnemius muscle. Thus, while trihexosyl ceramide and globoside were the predominant neutral GSL of the heart, in the gastrocnemius muscle (2) and several other human tissues (13,20-22), the GSL consisted mostly of lactosylceramide. However, the neutral GSL of the heart are similar to those of the kidney (23).

The fatty acid composition of the neutral GSL was found to be similar to that reported for GSL from other extraneural tissues with the exception that for heart GSL the percentage of the C_{22} fatty acid was the same or even higher than the C_{24} fatty acids.

The acidic fractions of heart GSL were carefully examined for the presence of sulfatides, and it was concluded either that they are not present or that they constitute a very minor component. This is not surprising since sulfatides have not been detected in a variety of extraneural human tissues including skeletal muscle (2,13,20-22). They are major components of the human kidney (24) and the thyroid (7).

The value of total ganglioside sialic acid 40-50 nmoles/g wet weight is similar to that found in the human gastrocnemius muscle (2). Lower values have been reported for skeletal and cardiac muscle of various animal species (3). The predominant gangliosides of the human heart were GM_3 and GM_1. In addition, a GD_3 ganglioside similar to that found in human gastrointestinal mucosa (25) and kidney (26) has been detected. This ganglioside was a minor component of gastrocnemius muscle (2). In the heart the galactosamine containing gangliosides made up about 55% of total (on a molar basis). Theses gangliosides are present in lower proportions, 20-30%, in a variety of human extraneural tissues including skeletal muscle (2,25-28). Similar observations have been derived from qualitative examination of the ganglioside pattern of other mammalian species (3). On this account, it becomes evident that heart muscle and skeletal muscle, which have cytologically similar sarcolemma, have a different composition of neutral GSL and gangliosides. Recent observations have stimulated interest in the role of gangliosides as membrane receptors (29-32). They may also play a part in Ca^{++} binding (33) and in mechanisms involved in transmembrane potential (34). These functions are important for the performance of the heart. It therefore would be of interest to check whether those ganglioside species, which are found in higher proportions in the heart than in other extraneural tissues, are implicated in mechanisms specific to heart function such as contractile force and rate.

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Biosynthesis of Unsaturated Fatty Acids in the Diatom *Phaeodactylum tricornutum*

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ABSTRACT

The biosynthesis of fatty acids in the diatom *Phaeodactylum tricornutum* was studied. The diatom was incubated with sodium [1^{14}C]acetate and the acids [1-^{14}C]palmitic, [1-^{14}C]stearic, [1-^{14}C]linoleic and [1-^{14}C] α-linolenic. The distribution of radioactivity in the products was determined by gas liquid radiochromatography. The diatom synthesized "de novo" not only saturated and monounsaturated fatty acids, but also linoleic, α-linolenic and other fatty acids including the highly polyunsaturated 20:5ω3 and 22:6ω3. When labeled acetate, stearic, α-linolenic or even linoleic acid were incubated with the diatom, the polyunsaturated C_{20} fatty acids synthesized belonged predominantly to the ω 3 family. The existence of Δ9, Δ6, Δ5, Δ4, ω6 and possibly ω3 desaturases in *P. tricornutum* is suggested.

INTRODUCTION

The fatty acid composition of the organisms which occupy different trophic levels in the marine food chain generally show qualitative similarities. It is probable that phytoplankton, the first link of the chain, is able to synthesize all the fatty acids de novo. Some phytoplanktonic species (diatoms, dinoflagellates, etc) contribute considerably to the marine ecosystem with high concentrations of the 20:5ω3 and 22:6ω3 acids (1) that are generally considered the typical fatty acids of marine life. Diatoms are accorded the most important place because of the number and diversity of their species (2). Therefore, it is important to determine the biosynthetic routes used by these organisms. In the present work, the biosynthesis of fatty acids in *Phaeodactylum tricornutum* was studied by incubation of the diatom with labeled acetate, palmitic, stearic, linoleic and α-linolenic acid. *P. tricornutum* was chosen since it is ubiquitous, and the morphology and physiology of this alga has been intensively studied (3,4).

MATERIALS AND METHODS

Fatty acids

[1^{14}C]Palmitic (57.7 mCi/mmol, 98% radiochemically pure), [1^{14}C]stearic (48.4 mCi/mmol, 98% radiochemically pure). [1^{14}C]linoleic acid (56 mCi/mmol, 98% radiochemically pure, 2% *trans* isomer), [1^{14}C]α-Linolenic acid (57 mCi/mmol, 99% radiochemically pure, 1% *trans* isomer) and [1^{14}C]acetic acid sodium salt (59 mCi/mmol, 99% radiochemically pure) were purchased from Amersham-Searle (Amersham, England).

Cells

Axenic cultures of *P. tricornutum* (Nitzchia closterium f. minutissima) (Indiana University Culture Collection of algae No. 646) were used. The composition of the incubation medium was similar to the ASP-M medium of McLachlan (5). However, glycyl-glycine buffer was substituted for Tris buffer, and vitamins were not added since they are not necessary for this diatom (6). The diatoms were incubated at 15 ± 2 C in a chamber illuminated with 3200 lux provided by five fluorescent tubes, 40 w each. The flasks were gently agitated. In all experiments when the diatoms reached the last part of the logarithmic phase, they were incubated with the labeled substrates. The cell concentration was 5 x 10^6 cells/ml.

Incubation of Radioactive Fatty Acids

The ammonium salts of radioactive fatty acids and the sodium acetate were each dissolved separately in growth medium. The concentration of test fatty acids in the medium was 0.18 - 0.24 μM. The concentration of sodium acetate was 0.4 μM. Cells of *P. tricornutum* harvested by centrifugation were suspended in 150 ml of radioactive growth medium containing 1.5 mCi of ^{14}C fatty acids or 4 μCi of ^{14}C acetate and incubated for determined

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TABLE I

Fatty Acid Composition of *P. tricornutum*

Fatty acid	This experiment Rt/18:0[b]	Composition (%)	Others[a]
14:0	0.31	4.7	2.3 - 12.5
14:2	---	---	0.0 - 1.0
15:0	0.42	0.5	---
16:0	0.56	12.5	9.2 - 30.9
16:1ω9	0.66	23.1	16.1 - 49.2
16:2ω7	0.80	1.3	0.0 - 4.7
X(16:2ω4)	0.87	1.1	1.4 - 8.7
16:3 18:0	1.00	3.8	0.7 - 10.5
18:1	1.15	6.4	0.9 - 4.7
16:4ω1	---	---	0.0 - 5.4
18:2ω6	1.42	3.4	0.5 - 1.3
18:3ω6	1.66	0.1	0.3 - 1.2
18:3ω3	1.90	0.2	0.2 - 2.0
20:1ω9	2.01	0.2	---
18:4ω3	2.23	0.7	0.0 - 1.4
20:3ω6	---	---	0.0 - 1.0
20:4ω6 20:3ω3	3.33	0.7	---
22:1ω9	3.55	0.6	0.1 - 3.9
20:5ω3	4.48	26.4	8.6 - 20.0
22:4ω6 22:3ω3	5.75	3.5	---
22:6ω3	8.80	10.8	0.6 - 4.2

[a]Taken from references 17 to 21. Range is given.

[b]Rt/18:0 relative retention times vs. 18:0. Acids present in proportion less than 0.1% are not reported.

periods of time in a chamber at 15C. Eight tubes of cells were incubated for each fatty acid and were pooled in groups of 4. Each tube contained 700 to 800 x 10^6 cells, equivalent to 120-150 mg of cells. After the incubation, cells were harvested by centrifugation and washed three times with fresh growth medium with centrifugation each time. Finally, the cells were filtered out with a Whatman GF/C fiberglass filter. Total lipids were extracted with chloroform-methanol (2:1, v/v) (7). The lipids were saponified and the nonsaponifiable matter extracted with petroleum ether. The solution was acidified, the free acids extracted with petroleum ether (bp 30-60 C) and converted to methyl esters (8). The radioactivity distribution in the different fatty acids was measured by gas liquid radiochromatography at 180 C in a Pye apparatus equipped with a proportional counter (8).

The fatty acid composition of the lipids of the diatom was determined by gas liquid chromatography in a Pye instrument with an argon ionization detector (9). The column was packed with 15% diethylene glycol succinate on Chromosorb W (80-100 mesh). The identification of fatty acids in the chromatogram was done by comparison of their retention times with standards by using Ackman's graphic procedure (10) of the retention times vs. chain length, Ackman's separation factors I and II (11-13), and Haken's factor (14). To confirm the chain length of unsaturated fatty acids, the samples were rechromatographed after hydrogenation (15).

RESULTS AND DISCUSSION

Fatty Acid Composition and de novo Biosynthesis

The fatty acid composition of *P. tricornutum* showed in Table I is similar to the composition of other photosynthetic diatoms but remarkably different from Chlorophyceae and higher plants (1). The outstanding differences are high proportions of saturated and unsaturated fatty acids of 16 carbons, of 20:5ω3 and 22:6ω3 acids, and the low content of fatty acids of 18 carbons.

The fatty acid compositon of *P. tricornutum* has been already investigated by Williams (16), Kates and Volcani (17), Ackman et al. (18), Chuecas and Riley (18) and Hinchcliffe and Riley (20). Although the fatty acid composition found by the four groups is similar, some differences exist in the content of 16:0, 16:1, 20:5ω3 and 22:6ω3 acids. These differences are likely due to the different culture conditions used.

Since the fatty acid composition illustrated in Table I corresponds to cells cultured in a medium deprived of fatty acids, it proves that this alga is able not only to synthesize saturated, monounsaturated and linoleic acids as plants generally do, but also the higher polyunsaturated acids of the ω3 family (20:5ω3 and 22:6ω3) that are typical of marine animals. These results were confirmed when the diatom was incubated with labeled acetate (Table II) since labeling was found in major and nearly all of the minor components shown in Table I, from myristic to 20:5ω3 acid. However, unexpectedly 70% of the radioactivity was accumulated in the 18:1 peak, whereas this peak represents only 6.4% of total fatty acids (Table I).

After a 7 hr incubation, no measurable radioactivity was found in the peak of 22:6ω3 acid in spite of its high concentration (Table I). This may be explained by the lack of sufficient time to reach this last step in the biosynthesis of fatty acids and also by the flatness of the peaks in the last part of radiochromatogram that makes it very difficult to detect low radioactivities.

Transformation of Saturated Fatty Acids

Incubation of the diatom with labeled 16:0 acid converts the substrate to fatty acids of 18 carbons. Table III shows that [1-^{14}C]16:0 acid after 3 hr incubation is desaturated to 16:1 or elongated to 18:0, but mainly is converted to 18:1, consequently confirming the results shown in Table II with [1-^{14}C] acetate incubation. No labeling was found in higher homologs in this case. The high labeling of the 18:1 acid peak is difficult to explain. It may correspond to an intermediate step of slow farther transformation. However, since the chromatographic peak of 18:1 acid was not studied by ozonolysis, we cannot ignore the possibility that, besides 18:1ω9, which is the 18:1 isomer synthesized from 18:0 acid (Table III), the 18:1 peak may also contain 18:1ω7 acid. The 18:1ω7 would be produced from 16:1ω7 by elongation. Therefore, any additional attempt at explanation would be very speculative.

When [1-^{14}C]stearic acid was incubated, a small proportion was converted to acids of molecular weight lower than stearic acid. This is considered to be a measure of the oxidation of [1-^{14}C]stearic to labeled acetate and its use in de novo synthesis of fatty acids. However, the main proportion of [1-^{14}C]stearic acid was elongated and desaturated to fatty acids of 18, 20 and 22 carbons. Oleic, 18:2ω6 and 20:5ω3 acids were predominantly synthesized, but many other unsaturated acids of ω3 and ω6 structure were also labeled (Table III). There-

TABLE II

Labeling Distribution in *P. tricornutum* after Incubation with Sodium [1-^{14}C] Acetate[a]

Fatty acids	% Radioactivity 3 hr	7 hr
X	—	0.6 ± 0.2
14:0	6.2 ± 0.5	4.0 ± 0.5
15:0	—	1.8 ± 0.1
16:0	77.0 ± 1.2	5.1 ± 0.6
16:1ω7	6.2 ± 0.8	7.2 ± 1.0
16:2	—	4.2 ± 0.4
16:2	—	0.9 ± 0.1
18:0		
16:3	—	1.6 ± 0.2
18:1	10.6 ± 0.5	70.0 ± 1.8
18:2ω6	—	0.7 ± 0.3
18:3ω6	—	1.3 ± 0.3
18:3ω3	—	0.6 ± 0.2
18:4ω3	—	1.1 ± 0.7
20:4ω3	—	0.4 ± 0.1
20:5ω3	—	0.5 ± 0.1

[a]Results are the mean of two pools of 4 tubes each ± SEM incubated during 3 or 7 hr in the conditions described in the text. The chromatogram was run until 22:6ω3 peak. The radioactivity incorporated in total fatty acids per flask containing 150 ml of medium and 750 x 10^6 cells was 0.4 μCi and 1.2 μCi for 3 and 7 hr incubation.

TABLE III

Radioactivity Distribution in the Fatty Acids of *P. tricornutum* after Incubation with [1-^{14}C]16:0 and [1-^{14}C]18:0

Labeled fatty acids	[1-^{14}C]16:0	[1-^{14}C]18:0
14:0	—	0.1
16:0	46.0	1.7
16:1ω7	6.1	0.1
16:2	—	0.1
16:2	—	0.1
18:0		
16:3	1.3	45.6
18:1	46.6	15.0
18:2ω6	—	21.7
18:3ω6	—	2.5
18:3ω3	—	2.8
18:4ω3	—	0.5
20:3ω3		
20:4ω6	—	1.0
20:4ω3	—	2.3
20:5ω3	—	6.1
22:3ω3		
22:4ω6	—	0.1
22:4ω3	—	0.1
22:5ω3	—	0.1
22:6ω3	—	0.1

[a]Results expressed as percent of total recovered radioactivity are the mean of four samples incubated during 3 hr. The chromatogram was run until 22:6ω3 peak; The radioactivity incorporated in total fatty acids per flask containing 150 ml of medium and 750 x 10^6 cells was 0.105 μCi and 0.113 μCi for 3 hr incubation of [1-^{14}C]16:0 and [1-^{14}C]18:0 acids, respectively.

TABLE IV

Radioactivity Distribution in the Fatty Acids of the Alga after Incubation with [1-^{14}C]18:2 and [1-^{14}C]α18:3[a]

Labeled fatty acids	[1-^{14}C]α18:3ω3	[1-^{14}C]18:2ω6
14:0	0.6	2.2
16:0	1.3	8.1
16:1ω7	---	8.3
16:2	---	1.0
18:0		
16:3	---	1.8
18:1	1.2	27.2
18:2ω6	0.5	26.1
18:3ω6	---	2.4
18:3ω3	70.9	2.2
18:4ω3	3.5	3.2
20:2ω6	---	1.7
20:3ω6	---	1.3
20:3ω3		
20:4ω6	4.3	1.2
20:4ω3	4.7	3.2
20:5ω3	13.0	10.1

[a]Results expressed as percent of total recovered radioactivity are the mean of four samples incubated during 3 hr. The chromatogram was run until 22:6ω3 peak. The radioactivity incorporated in total fatty acids per flask containing 150 ml of medium and 750 x 10^6 cells was 0.122 μCi and 0.119 μCi for the incubation of [1-^{14}C]18:2 and [1-^{14}C]α18:3 acids, respectively.

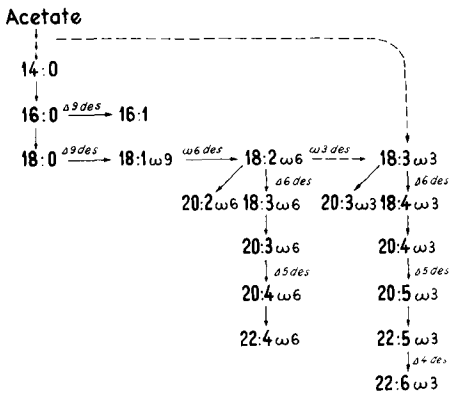

FIG. 1. Suggested routes in the biosynthesis of fatty acids by *P. tricornutum*. Note that 18:2ω6 → 18:3ω3 route is optional.

fore, the results of Table II and III suggest that very probably *P. tricornutum* has the following enzymes: a fatty acid synthetase that synthesizes saturated fatty acids from acetate, a Δ9 desaturase that converts 16:0 to 16:1 and 18:0 to 18:1, an ω6 desaturase that converts 18:1ω9 to 18:2ω6, a Δ6 desaturase that converts 18:2ω6 to 18:3ω6 and 18:3ω3 to 18:4ω3, and ω3 desaturase that synthesizes 18:3ω3 from 18:2ω6, as well as elongating enzymes and Δ5 and Δ4 desaturases that can synthesize 20:5ω3 and 22:6ω3 acids.

The Δ9 desaturase is an enzyme occurring widely in the animal and vegetable kingdoms, as well as in algae. Specifically, Erwin et al. (21) have shown that a Rhodophyceae, a euglenid, and several Chrysophyceae synthesize oleic acid from stearic acid. Brett et al. (22) have also carefully studied the specificity of Δ9 desaturase for different fatty acids in *Chlorella vulgaris*.

The ω6 desaturase is considered as a specific enzyme of plants and Harris and James (23) have demonstrated that it is present in the alga, *C. vulgaris*, converting oleic acid to linoleic acid.

On the other hand, the Δ6 desaturase is an enzyme considered typical of animals. It converts linoleic to α-linolenic acid and 18:3ω3 to 18:4ω3 acid. However, its presence has been suggested by Nichols and Appleby (24) and Pollero et al. (25) in protista and phytoplankton.

Transformation of Linoleic and α-Linolenic Acid

The labeled incubation products of *P. tricornutum* with [1-^{14}C]linoleic acid and [1-^{14}C] linolenic acid are shown in Table IV. These fatty acids evoke two different patterns of transformation.

In the case of [1-^{14}C]α-linolenic acid incubation, only a small part (3.5%) of label was present in 14:0, 16:0, 18:1 and 18:2 acids, whereas the highest portion (70.9%) was still found in the substrate (18:3ω3) together with substantial amounts in the higher homologs of ω3 series, 18:4ω3, 20:3ω3, 20:4ω3, and preferentially 20:5ω3.

Therefore, these results suggest that α-linolenic acid would be directly desaturated by a Δ6 desaturase of the alga to 18:4ω3. Besides, a Δ5 desaturase is apparently also present since the 20:5ω3 acid is also labeled.

In the case of [1-^{14}C]linoleic acid incubation, a remarkable proportion (48.6%) of radioactivity is found in 14:0, 16:0, 16:1, 16:2 18:0 and 18:1 fatty acids. These cannot be considered as directly synthesized from the substrate but instead products of β-oxidation of the substrate and synthesis from the labeled acetate so formed. The rest of labeling is mainly found in linoleic and 20:5ω3 acids with smaller percentages in peaks for 18:3ω6, 18:3ω3, 18:4ω3, 20:3ω3 or 20:4ω6 and 20:4ω3.

The labeling of 18:3ω6 from 18:2ω6 again suggests the existence of a Δ6 desaturase, an enzyme of the animal type that contributes in that kingdom to the synthesis of arachidonic acid. The labeling of 20:3ω6 suggests the

presence of an elongating enzyme that convertes 18:3ω6 to 20:3ω6. However, the peak that corresponds to arachidonic acid shows low radioactivity, especially when it is compared to 20:5ω3 peak. Therefore, it is necessary to propose the existence of some mechanism that makes arachidonic acid synthesis unfavorable, and induces reactions in the direction of 20:5ω3 synthesis. In consequence, these results confirm the data of Tables I and II that showed that 20:5ω3 acid was preferentially synthesized by the diatom instead of 20:4ω6. Mechanisms that would evoke a preferential synthesis of 20:5ω3 instead of 20:4ω6 can be (a.) preferential incorporation of 20:5ω3 in special lipids of the *P. tricornutum*; (b.) higher activity of the ω3 desaturase that converts linoleic acid to α-linolenic acid than the Δ6 desaturase that converts the same substrate to α-linolenic acid; (c.) high oxidation of linoleic acid and synthesis of α-linolenic acid from short chain of fatty acids by Stumpf mechanism (26-28) as will be discussed later; (d.) higher activity of the Δ5 desaturase for 20:4ω3 acid than for 20:3ω6, and higher velocity in the elongation of 18:4ω3 than 18:3ω6.

Since α-18:3ω3 is also synthesized from linoleic acid as well as 20:4ω3, the sequence of reactions that were already proposed for 20:5ω3 biosynthesis in the preceding part when 18:3ω3 was incubated is also consistent with these labeling results.

Radioactive α-linolenic acid, shown in Table IV, could be produced by an ω3 desaturation of linoleic acid. However, the presence of this type of enzyme has been questioned by Stumpf et al. (26-28). They studied 18:3ω3 acid biosynthesis in spinach leaves. Chlorophyceae and yeast. They showed that 12:0 acid synthesized in these cells could be converted to a 12:3 acid with double bonds in Δ3, Δ6, Δ9 and then elongated to α-linolenic acid. Since Table IV shows that incubation of *P. tricornutum* with [1-^{14}C] linoleic acid evokes abundant formation of fatty acids of lower molecular weight than 18:2ω6, it is pertinent to think that Stumpf's route may be a possible mechanism or one of the mechanisms of 18:3ω3 biosynthesis in the alga.

Comparing all the results collected in the present work, it is pertinent to suggest that *P. tricornutum* very possibly synthesizes fatty acids by the routes outlined in Figure 1. These routes use reactions of either "plant" or "animal" type. Besides, the biosynthesis of 22:6ω3 acid is very probably evoked by elongation and desaturation of 20:5ω3 acid using the types of reactions already suggested in animals.

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Separation and Purification of Lecithins by High Pressure Liquid Chromatography

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ABSTRACT

Ten different synthetic lecithins have been analyzed by reverse-phase high pressure liquid chromatography. An empirical lecithin "carbon number" that depends on the total number of carbons and double bonds in the fatty acyl chains is a useful index in predicting retention volumes of lecithins on a nonpolar octadecyl fatty acid column. Commercial egg lecithin is separated into its components by this technique.

INTRODUCTION

The separation and identification of lecithins with different fatty acyl groups has been the subject of extensive investigation (1). The "classic" method for determining the structure of fatty acyl groups of a particular lecithin or mixture of lecithins involves partial ester hydrolysis with phospholipase A_2 coupled with total ester hydrolysis using hydroxide ion. The free fatty acids generated by these hydrolytic methods are then analyzed by standard chromatographic techniques. By the use of these laborious hydrolytic techniques coupled with argentation chromatography, mixtures of natural lecithins can be separated into molecular species and identified.

Recently, reports of attempted separation of phospholipids by high pressure liquid chromatography (HPLC) have appeared in the literature (2-4). These publications have emphasized separating *classes* of phospholipids by HPLC (i.e., separation of phosphatidic acids from phosphatidylethanolamines and phosphatidyl cholines), and little has been reported concerning the separation of individual phospholipids within a class. Because of the importance of lecithins (*1*) in membrane structure and function, and the potential importance of a straightforward method for separation and analysis of various lecithins, we undertook a study directed toward developing HPLC methods of lecithin separation.

We report here results that suggest that HPLC of lecithins promises to greatly simplify the analysis of phospholipid mixutres of biological importance.

MATERIALS AND METHODS

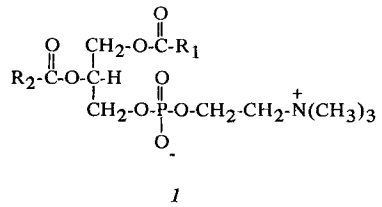
Fatty acids were obtained from NuCkek Prep (Elysian, MN) and used without further purification. Egg and soybean lecithins were obtained from Sigma Biochemical Co. (St. Louis, MO).

Lecithin Synthesis

Lecithins *1*(a-j) were prepared by the method reported by Gupta et al. (4). Thus, defatted egg lecithin was converted to glycerophosphorylcholine which was then diacylated to the symmetrically substituted lecithin. Snake venom hydrolysis followed by reacylation gave the "mixed" lecithins. These synthetic lecithins were characterized by ^1H and ^{13}C nuclear magnetic resonance (NMR) and were pure by thin layer chromatography (TLC) and HPLC (*vide infra*).

HLPC conditions

Two reverse-phase columns were used for lecithin separations. The columns and the solvent conditions used were: (a) μ-Bondapak C-18 (Waters Assoc., Milford, MA), CH_3OH-H_2O-$CHCl_3$ (100:10:10, v/v) and (b) FATTY



1

	R_1[a]	R_2
1 a	14:0	14:0
1 b	16:0	16:0
1 c	16:0	18:2
1 d	16:0	18:1
1 e	18:1	18:1
1 f	16:0	18:0
1 g	18:0	16:0
1 h	18:0	18:1
1 i	18:0	20:4
1 j	18:0	18:0

[a]14:0 = myristic, 16:0 = palmitic, 18:0 = stearic, 18:2 = linoleic, 18:1 = oleic, 20:4 = arachidonic acid.

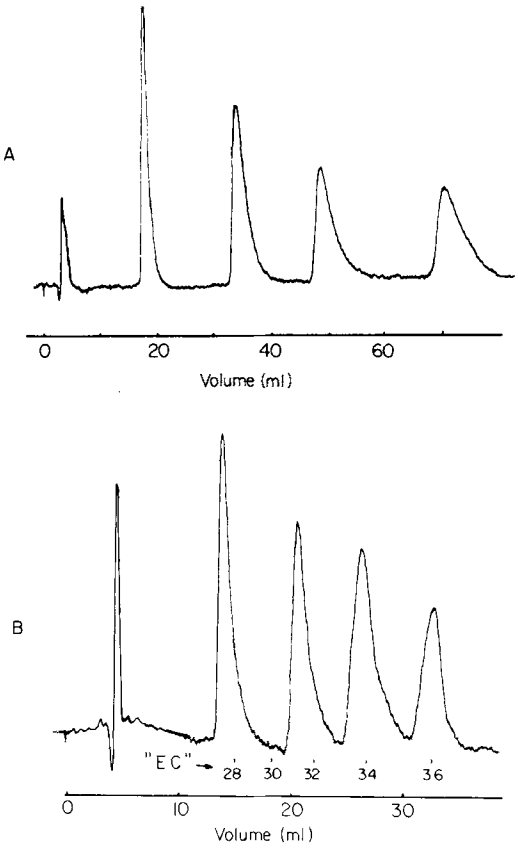


FIG. 1. Chromatograms for separation of a mixture of saturated lecithins: a) C-18 column; b) FA column.

ACID ANALYSIS (FA), (tradename for a reverse-phase column used primarily for the separation of fatty acids, Waters Assoc.) CH_3-OH-H_2O-$CHCl_3$ (70:19:10, v/v). Flow rates were 2-4 ml/min. Detection was by refractive index and .50 μmole of lecithin (in a .01 *M* solution) could be readily detected. Refractive index detection compares favorably with UV detection in that there are no solvent limitations with refractive index, whereas many solvents absorb significantly in the UV range of detection (203-214 nm) suitable for lecithins (3).

TABLE I

Retention Volumes for Lecithin HPLC

Lecithin No.	Fatty Acid, ml[a]	μ-C-18, ml[a]
1 a	13.7 ± 0.5	18.3 ± 0.7
1 b	20.7 ± 0.6	34.7 ± 0.7
1 c	(20.7)[b]	29.1 ± 0.6
1 d	23.1 ± 0.1	35.5 ± 0.8
1 e	26.5 ± 0.2	35.5 ± 0.8
1 f	26.5 ± 0.2	49.6 ± 1.0
1 g	26.5 ± 0.6	49.6 ± 1.0
1 h	29.8 ± 0.0	52.1 ± 0.8
1 i	30.0 ± 0.3	39.0 ± 1.8
1 j	33.5 ± 0.7	71.5 ± 1.2

[a]Error is standard deviation from the average. Numbers are for tops of peaks.

[b]Only one run of 16:0, 18:2 was made, and its retention time was identical to that of *1* b.

RESULTS

The ten synthetic phosphatidyl cholines prepared in this study were analyzed by HPLC using two different reverse-phase columns, C-18 and FATTY ACID ANALYSIS (FA). Typical chromatograms of a mixture containing the saturated lecithins *1* a, b, f, and j are presented in Figure 1 for chromatography on both columns. There is a close correspondence of total carbons in the fatty acyl chains and retention volume on both of the columns. Thus, the order of elution corresponds directly with the number of carbon atoms in the acid constituents (14:0, 14:0; 16:0, 16:0; 18:0; 18:0). Under our conditions of analysis, the separation of saturated lecithins is somewhat better by the use of the C-18 column as compared to the FA column.

Table I presents the retention volume found for each of the synthetic lecithins on both the C-18 and FA columns. Table II presents a chart which lists pairs of lecithins that were difficult (or impossible) to separate on one of the

TABLE II

Separations of "Difficult" Lecithin Pairs

Lecithin pairs	Separated by FATTY ACID?	Separated by μ-C-18?
1 b & c	NO	YES
1 b & d	YES	NO
1 b & e	YES	NO
1 d & e	YES	NO
1 e & f, g	NO	YES
1 f & g	NO	NO
1 f, g & h	YES	NO
1 h & i	NO	YES

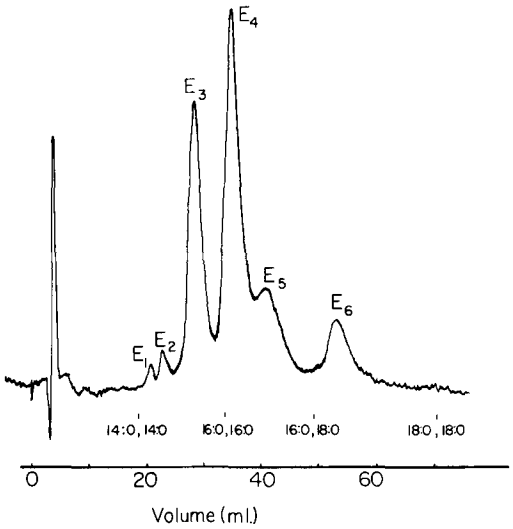


FIG. 2. Chromatogram for commercial egg lecithin on C-18 with conditions as described in Methods.

TABLE III

"Effective carbon" number of Synthetic Lecithins

	Lecithin	Predicted E.C.	Observed E.C.
1 a	14:0, 14:0	28	28
1 b	16:0, 16:0	32	32
1 c	16:0, 18:2	32	32
1 d	16:0, 18:1	33	33
1 f	16:0, 18:0	34	34
1 g	18:0, 16:0	34	34
1 e	18:1, 18:1	34	34
1 i	18:0, 20:4	34	35
1 h	18:0, 18:1	35	35
1 j	18:0, 18:0	36	36

columns. All lecithin pairs not included in Table II could be separated by either column.

Egg lecithin was chromatographed on both columns, and a typical chromatogram for the C-18 separation is shown in Figure 2. The retention volumes of the saturated synthetic lecithins are indicated in the Figure for orientation. Six peaks are observed with retention volume and integrated intensities as follows: peak, ret. vol. (ml), integration %. E_1, 21.3, .7; E_2, 23.3, 1.7; E_3, 29.1, 30.7; E_4, 35.4, 44.9; E_5, 41.3, 11.9; E_6, 54.2, 10.0. The major fractions E_3-E_6 were reinjected on the FA columns as described in the Discussion.

DISCUSSION

The analysis of fatty acyl components in lecithins is a tedious, time-consuming process. Selective and total hydrolysis of the acyl ester linkage followed by gas chromatography of the fatty acids is required for analysis. Even after this effort, results may still be ambiguous since information is only available about total fatty acid and glycero-C-2 substituted fatty acids. For determination of molecular species present in mixtures of lecithins, tedious argentation chromatography of the derivative 3-acetyl triglycerides followed by hydrolysis steps is required (5,6). HPLC techniques would seem to be an ideal approach for solution of this difficult problem, and some preliminary reports of phospholipid separations have appeared (2-4). The two reverse-phase columns used in this study offer the possibility of direct analysis of lecithin mixtures and may make tedious hydrolytic degradation of lecithins unnecessary.

All of the synthetic lecithins with the exception of *1* f and *1* g could be separated by a combination of chromatography on the two columns (Tables I and II). Thus, if the FA column did not separate a pair (i.e., *1* b and *1* c), the C-18 column would. In fact, all pairs that were inseparable by one column were readily separated on the other (with the exception of the isomer *1* f and g lecithins). The complementary nature of the two columns would appear to make possible, at least in theory, the total resolution of natural lecithin mixtures.

A useful relationship can be derived for retention volume vs. structure of individual lecithins on the FA column. An "effective carbon" can be defined that relates the total carbon atoms and number of double bonds in the fatty acyl groups with the retention volume on the FA column. Thus: E.C. = "Effective carbon number" = total number of acyl carbon atoms *less* the number of double bonds in the lecithin; e.g., E.C. for 1-palmitic, 2-oleic = 16 + 18 - 1 = 33.

Table III shows the predicted "zone" of retention volume vs. the observed "zone," with these "zones" being defined by the fully saturated lecithins. With the exception of *1* i, which has an observed E.C. one unit larger than expected, all of the lecithins studied have observed E.C. numbers equal to those predicted. This structure-chromatography index may prove to be a useful guide in predicting the structure of unkown phospholipids.

The relationship between structure and retention volume can be further developed in a quantitative way. Quantitative relationships between retention volume and structure are common in gas liquid chromatography (GLC) and other chromatographic separations of lipids (7). Thus, for our FA column: .042 x "E.C." = log (retention volume). Theoretical retention

TABLE IV

Egg Lecithin Fatty Acid Composition

Fatty acid	Reported[a]	Reported values corrected[b]	mole %[c]
16:0	32	(35)	38
18:0	16	(13)	11
18:1	30	(33)	27.5
18:2	17	(19)	21.5
20:4	4	---	---

[a]Reference 8.
[b]Corrected by assuming that all 18:0, 20:4 had been autoxidized.
[c]Calculated from our chromatography analysis.

volumes calculated by this equation compare favorably with the observed values (Fig. 1B). A significantly better correlation could not be obtained using the retention volume corrected by subtraction of the retention volume of unretained compound.

Commercial egg lecithin was analyzed to illustrate the potential utility of coupled chromatography on C-18 and FA columns. The crude lecithin was prepurified by alumina chromatography, and it is probable that significant oxidation of highly unsaturated fatty acyl groups had occurred during shipment.

Thus, we expected to observe lower levels of the highly unsaturated lecithins in this commercial sample than have been reported in analyses of freshly prepared egg lecithin (8,9). The first large component of egg lecithin (E_3, Fig. 2) eluted with the same retention volume as *1* c (16:0, 18:2). Collection of E_3 and reinjection on the FA column gave one peak with E.C. = 32. E_3 is thus assigned the structure of *1* c (16:0, 18:2). E_4 elutes with the same retention volume on the C-18 column as either *1* d or *1* e. Collection of E_4 from the C-18 chromatography followed by reanalysis on the FA column gave one component with E.C. = 33. E_4 is thus *1* d, (1-palmitic, 2-oleic lecithin), and it is the major component of commercial egg lecithin. Similar series chromatography of E_5 and E_6 on the C-18 and FA columns suggested that these components were, respectively, 1-stearic, 2-linoleic and 1-stearic, 2-oleic lecithin. The polar trace components E_1 and E_2 did not correspond to any of the synthetic lecithins (E.C. ~ 29) and may well be oxidation products of unsaturated lecithins. This analysis of commercial egg lecithin thus gives the following composition:

Lecithin R_1, R_2	Mole %
16:0, 18:1	45
16:0, 18:2	31
18:0, 18:2	12
18:0, 18:1	10
unknown polar (E_1 & E_2)	2

If this mixture of lecithins were to be hydrolyzed, the mole percents of fatty acids shown in Table IV would result. Further, if one assumes that 18:0, 20:4 was 8% of the original lecithin (8) mixture but that this component was oxidized in the commercial sample before analysis, the corrected fatty acid composition shown in Table IV can be calculated. The correlation of the corrected literature values and the values obtained from this work suggests that this straightforward chromatographic technique may be a useful tool for the analysis of natural lecithins.

One final comment should be made about the potential of the HPLC technique as applied to lecithins. Analysis of a sample of *1* c that had been stored for more than a month at $<0^{\circ}$ showed a polar impurity at E.C. ~ 27 in addition to the expected peak at E.C. = 32. This impure sample showed only one spot by conventional thin layer analysis. After separation of the two components by HPLC, both samples were shown to be phospholipids as evidenced by their blue stain with molybdenum blue after TLC. (The more polar HPLC impurity ran *very slightly* slower on TLC than *1* c.) Thus, the HPLC technique is far superior to TLC for assessing the purity of synthetic or natural lecithins.

The HPLC separations and analyses of lecithins reported here are by no means exhaustive or complete. Many other synthetic phospholipids need to be prepared and studied, and naturally occurring mixtures of lecithins need to be rigorously analyzed. The studies reported here, however, suggest that this chromatographic technique is potentially a powerful tool for the direct separation and analysis of phospholipids.

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Analysis of Subcellular Phosphatidyl Choline in Developing Rabbit Lung

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ABSTRACT

Phosphatidyl choline is a major lung surfactant. Insufficient development of the surfactant in neonates is often associated with the Respiratory Distress Syndrome. The concentration and fatty acid composition of phosphatidyl choline have not been studied in the subcellular organelles of the developing lung. This study has investigated the development of the concentration and fatty acid composition of phosphatidyl choline in subcellular fractions of 28-day and 30-day fetal and maternal New Zealand rabbit lungs. The concentration of total phospholipids in lamellar bodies increased four to five fold from 28-day fetus to 30-day fetus which, in turn, was similar to the maternal level. Total phospholipid content increased only about 50% in mitochondria and microsomes. The percentage of phosphatidyl choline among total phospholipids in lamellar bodies increased successively from 60% at 28 days gestation to 84% at 30 days gestation and leveled at 84% in maternal lamellar bodies. Microsomal PC increased steadily from 52% in the 28-day fetus to 65% in the adult. Analysis of the fatty acid composition of phosphatidyl choline in lamellar bodies confirmed 16:0 as the major fatty acid, and its content remained constant from 28 days gestation to adult. In contrast, the content of 16:0 of the microsomal phosphatidyl choline decreased with increasing gestation. Changes of several unsaturated fatty acid components were observed in both lamellar bodies and microsomes in the developing lungs. Maturational development of phosphatidyl choline is reflected in an increase in the concentration of this surfactant, particularly in lamellar bodies, and possibly in remodeling of fatty acid composition in both lamellar bodies and microsomes.

INTRODUCTION

A major lung surfactant is phosphatidyl choline (1,2-diacyl-*sn*-glycero-3-phosphocholine) which is stored in the osmiophilic lamellar inclusion bodies of type II alveolar epithelial cells, from which it is secreted to line the air-alveolar interface and stabilize respiration (1,2). Insufficient development of lung surfactant in neonates is often associated with the Respiratory Distress Syndrome. The nature of the fatty acid groups of phosphatidyl choline also to some extent determines the surface-active properties of this phospholipid. The concentration and fatty acid composition of phosphatidyl choline in mammalian lungs has been extensively studied. However, most studies were conducted with either lung tissue slices (3-10) or tracheal washings (3,4), or focused on adult lung subcellular fractions (11,12). Previous results showed that the amount of phosphatidyl choline, either isolated from the developing whole lung tissues, or from the tracheal efflux, increases with increasing gestational ages. It is generally believed that these increases of phosphatidyl choline are directly related to the maturation of the lamellar bodies in type II cells. Development of lamellar bodies in animal lungs was well studied morphologically (13,14); but, the development of phosphatidyl choline concentration and its fatty acid composition from isolated lamellar bodies and other subcellular organelles has not been previously investigated.

To examine the evolution of fatty acid moieties of phosphatidyl choline in the developing lungs, we have analyzed the fatty acid composition of phosphatidyl choline in 28-day, 30-day, and maternal rabbit lung lamellar bodies and microsomes. We have also determined the concentrations of total phospholipids and phosphatidyl choline in the developing subcellular fractions of lamellar bodies, microsomes, mitochondria, and crude homogenates.

MATERIAL AND METHODS

Animals and Preparation of Lung Subcellular Fractions

At 28 and 30 days timed gestation (± 4 hours), pregnant New Zealand rabbit does were anesthetized with 20-30 mg/kg of intravenous sodium pentobarbital. The fetuses were removed through an abdominal incision, and their tracheas were clamped immediately to prevent respiration. After fetal exsanguination by transaortic incision, the

fetal lung tissue was removed, rinsed repeatedly with ice cold saline and briefly stored on ice. The doe was sacrificed by exsanguination as lung tissue was perfused in situ with ice cold normal saline via the pulmonary artery. During this perfusion the lungs were repeatedly inflated to improve removal of blood. After removal of bronchial tissues, both maternal lung and fetal lung were separately homogenized in three volumes of 0.33 M sucrose-0.01 M Tris-HCl buffer (pH 7.4) (15). Subcellular fractions of lamellar bodies, mitochondria, and microsomes, were isolated by the method of Page-Roberts (16), and the purity of these fractions has been detailed elsewhere (15).

Protein and Phospholipid Analyses

Protein concentrations of each fraction were determined by the method of Lowry et al. (17), using a standard of bovine serum albumin (Fraction V, Miles Laboratories, Inc., Kankakee, IL). Lipids of each fraction were extracted by the solvent mixture of chloforom-methanol (1:2) as described by Bligh and Dyer (18). From an aliquot of the extract, phosphatidyl choline and phosphatidyl ethanolamine were isolated by thin layer chromatography (TLC) on a precoated Silica Gel 60 glass plate (E. Merck, Darmstadt, Germany) with a developing solvent of chloroform-methanol-water (65:25:4 v/v). The spots of phospholipids were visualized by brief exposure to iodine vapor and identified by comparison of their R_f values with those of standards. From these spots the concentrations of phosphatidyl choline and phosphatidyl ethanolamine were determined in the presence of silica gel, and total phospholipids were determined from aliquot of the original lipid extract by the method of Chen et al. (19) with slight modification (15).

Analysis of Fatty Acid Composition of Phosphatidyl Choline

The lipid extract of lamellar bodies or of microsomes, containing no less than 200 nmoles of phosphatidyl choline, was applied as a band to the TLC plate under nitrogen. After development of the TLC plate in the solvent as described above, phosphatidyl choline was identified by spraying with a 0.05% solution of Rhodamine 6G (20) (Supelco, Inc., Bellefonte, PA) and visualized under ultraviolet light. Phosphatidyl choline was then extracted twice from silica gel with chloroform-methanol-water-acetic acid (97:97:4:1 v/v). The extract and silica gel were separated by centrifugations at 2,000 x g for 20 min. The pooled extract was evaporated to dryness under nitrogen. Phosphatidyl choline was redissolved in 0.2 ml benzene and 0.5 ml 1.5N HCl in methanol (prepared from 25 ml anhydrous methanol and 2 ml acetyl chloride) in a 3 ml screw-capped vial with a Teflon septum. Fatty acid moieties of phosphatidyl choline were transmethylated at 80 C for 30 min. After cooling, 1 ml water was added to the solution, and fatty acid methyl esters were extracted twice with 1 ml hexane. The volume of hexane extract was reduced to an approapriate volume just prior to subjection to the fatty acid composition analysis.

The fatty acid composition of the methyl ester derived from phosphatidyl choline was determined by gas liquid chromatography (GLC) with a flame ionization detector (210 C) (Packard Instrument Co., Inc., Downers Grove, IL) and a glass column of 10% diethylene-glycol succinate on 80:100 mesh chromosorb W/AW (Supelco, Inc.) at 180 C. Each peak was quantified by a linear integrator (Linear Instruments Corp., Irvine, CA).

RESULTS

Attempts to extend this study to earlier gestational ages were unsuccessful, as isolation of lamellar bodies from a pool of thirty-two 26-day fetuses yielded only trace amounts of lamellar bodies, indicating that the 26-day fetal lung contains very little osmiophilic lamellar bodies, and confirming that the rabbit fetal lung is significantly more mature at 28-days gestation, although lamellar inclusion bodies can be observed histologically at 26-day gestational age (13).

Expressed in relation to protein content, the concentration of total phospholipids in the crude homogenate of lung increased with increasing gestational age (Table I), maternal concentration being about three-fold that of the 28-day gestation fetus and two-fold that of the 30-day gestation fetus. Among the subcellular fractions, the most marked increase of phospholipid concentration was found in lamellar bodies. The maternal phospholipid in lamellar bodies was five-fold higher than that of 28-day gestation fetus, but only 20% higher than that of 30-day gestation fetus. Concentrations of microsomal and mitochondrial

phospholipids of 28-day gestation and 30-day gestation were ca. 30% to 50% lower than that of the adult value.

The concentration fo the major lung surfactant, phosphatidyl choline, and a minor surface active component, phosphatidyl ethanolamine, was also determined (Table I). Expressed as a percentage of total phospholipids, phosphatidyl choline increased with increasing gestational age, while the percentage contributed to the total by phosphatidyl ethanolamine remained unchanged in all subcellular fractions. Specifically, the percentage of the phosphatidyl choline component among total phospholipids in lamellar bodies increased from the 28-day fetus to the 30-day fetus with no further increment from the 30-day fetus to the adult. A marginal increase of microsomal phosphatidyl choline was also observed between the 28-day fetus and the 30-day fetus, with a further, larger increment to the maternal levels. The percentage of phosphatidyl choline among total phospholipids in mitochondria did not increase significantly from 28-day fetus to the adult.

Analysis of fatty acid composition of phosphatidyl choline in lamellar bodies and microsomes of 28-day and 30-day fetal and maternal lungs showed that 16:0 was the major fatty acid component in both fetus and adult (Table II). Both maternal and fetal lamellar body phosphatidyl cholines contained 55% of their fatty acids as 16:0, and this remained constant from the 28-day fetus to the adult. Some variations of other fatty acids in the developing lamellar bodies were observed. The percentage of 14:0 in the 28-day and 30-day fetuses were one and one-half times that of the adult. For 16:1, 28-day and 30-day fetal values were twice that of the adult. In contrast, the percentage of lamellar body 18:0 decreased from 28 to 30 days, but rose sharply again in the adult. The percentage of 18:2 in the 28-day and 30-day fetal lamellar bodies was also somewhat lower than maternal.

The contribution of 16:0 to fetal lamellar body fatty acid methyl esters of phosphatidyl choline was indistinguishable from the adult. However, the contribution of 16:0 to fatty acid residue in microsomal phosphatidyl choline showed a decrease from the 28 to the 30-day fetus and an overall trend downward to the adult level. Additionally, the 28-day fetal 16:1 concentration in microsomes tended to be higher than adult level, as observed in lamellar

TABLE I

Phospholipid Concentration of Subcellular Fractions in Developing Rabbit Lung[a]

Subcellular fractions	Total phospholipids (μmole/mg protein)			% of total phospholipids					
				PC			PE		
	28 day fetus (28)	30 day fetus (20)	Doe (5)	28 day fetus	30 day fetus	Doe	28 day fetus	30 day fetus	Doe
Crude homogenate	0.16 ± 0.03	0.24 ± 0.03	0.50 ± 0.09	46.56 ± 7.69	63.52 ±5.97	62.25 ± 10.13	17.54 ± 1.18	18.79 ± 2.73	15.61 ± 3.58
Lamellar bodies	1.73 ± 1.10	7.89 ± 1.35	9.38 ± 2.10	59.69 ± 3.77	83.66 ±3.26	83.60 ± 14.48	6.96 ± 4.81	6.22 ± 0.73	6.81 ± 2.23
Mitochondria	0.65 ± 0.24	0.48 ± 0.26	0.87 ± 0.29	54.85 ± 3.53	55.36 ± 3.30	56.88 ± 4.27	23.77 ± 3.12	20.44 ± 2.40	20.92 ± 2.08
Microsomes	0.46 ± 0.26	0.49 ± 0.16	0.94 ± 0.18	51.95 ± 2.86	55.83 ± 2.60	64.92 ± 2.74	18.24 ± 0.99	21.65 ± 2.18	21.70 ± 4.15

[a]Values are means ± SD from duplicates of two different experiments. Numbers in the parentheses represent fetuses and does used for the experiment. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

TABLE II

Fatty Acid Composition of Phosphatidyl Choline in Lamellar Bodies and Microsomes of Fetal and Maternal Rabbit Lungs[a]

Fatty acid methyl ester	Lamellar bodies % of total fatty acids			Microsomes % of total fatty acids		
	28 day fetus (28)	30 day fetus (30)	Doe (5)	28 day fetus (28)	30 day fetus (20)	Doe (5)
14:0	5.06 ± 1.60	5.62 ± 1.22	3.49 ± 0.50	1.82 ± 0.64	1.40 ± 0.85	1.70 ± 0.42
14:1	1.08 ± 0.19	1.32 ± 0.41	Trace[b]	1.50 ± 0.25	1.20 ± 0.10	2.19 ± 0.79
16:0	56.73 ± 3.19	53.95 ± 7.81	56.60 ± 4.67	47.77 ± 6.50	38.05 ± 1.77	34.51 ± 3.34
16:1	11.53 ± 1.09	14.32 ± 2.53	6.37 ± 0.92	4.47 ± 0.29	3.90 ± 0.28	3.79 ± 0.42
18:0	5.04 ± 0.70	3.36 ± 0.31	4.20 ± 1.45	9.75 ± 1.89	15.10 ± 0.14	12.54 ± 1.26
18:1	13.08 ± 2.81	10.20 ± 1.56	17.89 ± 4.84	19.02 ± 4.83	22.00 ± 1.41	18.73 ± 2.62
18:2	7.08 ± 0.78	7.62 ± 0.97	9.16 ± 0.76	10.22 ± 0.55	10.55 ± 0.78	15.03 ± 0.89
18:3	Trace[b]	2.00 ± 0.29	2.24 ± 0.30	2.52 ± 0.55	Trace[b]	2.51 ± 0.88
20:4				6.41 ± 1.33	7.70 ± 0.57	7.20 ± 0.53

[a]Values are means ± SD, from duplicates of two different experiments. Numbers in the parentheses represent the animals used for the experiment.

[b]Less than 1%.

bodies, while 18:0 and 18:2 were lower than that of the adult. Fatty acid composition of 30-day fetal, microsomal phosphatidyl choline was the same as the adult, except for fetal microsomal 18:0 which was 30% lower.

DISCUSSION

In this work we have isolated subcellular fractions of lamellar bodies, microsomes, and mitochondria from 28-day and 30-day fetal and maternal lungs to study the maturational development of the concentration and fatty acid composition of phosphatidyl choline (PC) in each fraction. The fatty acid composition of PC in alveolar was not done; however, Jobe et al. (12) have indicated that alveolar wash PC composition closely parallels that of the lamellar bodies.

Although fetal lamellar bodies become well formed at 28-days (13), their concentration of phospholipid per milligram protein is only 1/5 that of the adult (Table I). The marked difference in the ratio of phospholipid concentration to protein concentration between 28-day fetal and maternal lamellar bodies indicates that the development of lamellar body proteins and phospholipids are occurring at relatively different rates. Not surprisingly, at 30-days gestation, the concentration of phospholipids increases nearly to the maternal levels as the 31-day term approaches. Concentrations of phospholipids in both 28-day and 30-day fetal mitochondria and microsomes were about 30-50% that of the adult, suggesting further development of these subcellular phospholipids must occur after 30 days' gestation.

A study comparing the concentrations of phosphatidyl choline and a minor surface active phospholipid, phosphatidyl ethanolamine, revealed that of all phospholipids only the percentage of phosphatidyl choline among total phospholipids increased with increasing gestational ages in both lamellar bodies and microsomes, supporting the well established observation that the formation of phosphatidyl choline is turned on at late gestational ages during fetal lung development (1,2).

Among the molecular species of phosphatidyl choline, dipalmitoylphosphatidyl choline is the most abundant and important lung surfactant (1,2). Lamellar bodies and microsomes were analyzed as to their relative composition of fatty acid moieties (Table II). For the most part, our data of the fatty acid composition of phosphatidyl choline in maternal lamellar bodies and microsomes are similar to those of Rooney et al. (11) and Jobe et al. (12), except the percentage of 18:0 was higher than that of 18:2. Others have observed this high 18:0-low 18:2 composition at 1- and 2-positions of lamellar body phosphatidyl choline (21), or in phosphatidyl choline from whole lung tissue (5,8,10,21) and alveolar wash (4). Although 28-day fetal lamellar bodies had a lower concentration of phosphatidyl choline than that of adult, the relative percentage of 16:0 was the same as the maternal value. Clearly, this observation cannot be interpreted to indicate that 28-day fetal and maternal lamellar bodies have the same percentage of dipal-

mitoylphosphatidylcholine. Nonetheless, Soodsma et al. (22) reported that the proportion of dipalmitate in fetal rabbit lung tissues increased with gestation from 23.5 days to 30.5 days. Since it has been observed that 24-day gestation fetal rabbit lung rarely contains lamellar inclusion bodies (13), and, furthermore, that the amount of dipalmitate of 28.5-day and 30 .5-day fetal lung tissues were almost the same (22), our data, along with those of Soodsma et al. (22), suggest that 28-day and 30-day fetal lamellar bodies may have the same percentage of dipalmitoylphosphatidyl choline among total phosphatidyl choline, as does the maternal lamellar bodies. However, if the data presented in Tables I and II are extended to express the PC and fatty acid composition in quantities of picamoles/milligram protein rather than on a percentage basis, then the expected increases in absolute amounts are seen: five to seven-fold increases in lamellar bodies from 28 to 30 days' gestation; one to two-fold increases in lamellar bodies from 30 days to adult and one to three-fold increases in mitochondria at each stage.

In addition to the major molecular species of dipalmitate, other molecular species may be essential to surfactant properties. Some remodeling of these molecular species are probably involved during the lung development. It is believed that the endoplasmic reticulum of the microsomal fraction is the major site of phosphatidyl choline synthesis (1,2). The presented data show 48% of palmitate in 28-day fetal microsomes, compared with 38% of adult (Table II). Again, we cannot say from this study whether 28-day fetal microsomes have a higher percentage of dipalmitate than that of the adult. However, with the differences in relative percentage of 16:1, 18:0, and 18:2 in the 28-day fetal microsomes and maternal microsomes, some remodeling of phosphatidyl choline by this subcellular fraction is suggested during lung development. The mechanisms of phosphatidyl choline remodeling in both lamellar bodies and microsomes are unknown; but, the deacylation-reacylation and deacylation-transacylation pathways have been suggested (1,2) or a transfer protein exchanging phosphatidyl choline between microsomes and lamellar bodies (23) may also play a role in this remodeling.

In summary, maturation of the lung surfactant phosphatidyl choline in lamellar bodies is controlled by the quantitative synthesis of this surfactant with perhaps significant remodeling of some of its unsaturated fatty acid moieties. Further analysis based on the molecular species of phosphatidyl choline in the developing lamellar bodies and microsomes will be necessary to provide insight into the development of this lung surfactant.

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Diet-induced Alterations in the Discoid Shape and Phospholipid Fatty Acid Compositions of Rat Erythrocytes

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ABSTRACT

For eight weeks young male rats were fed diets rich in 18:2 (stock diet, or 10% corn oil, CO) or those devoid of 18:2 (fat free, FF, or 10% hydrogenated coconut oil, HCNO). The CO and HCNO diets were fed in the absence or presence of eicosa-5,8,11,14-tetraynoic acid (TYA). When 18:2 was excluded, an increase in the level of 16:1, 18:1 and 20:3 and a decrease in 18:2 was observed in the fatty acids of red cells. On feeding TYA, an increase in 18:2 and in the case of the HCNO+TYA diet, a decrease of 12:0 and 14:0 was also observed. In all cases the levels of 20:4 in erythrocyte fatty acids were similar. Saturated fatty acids were predominant in phosphatidyl choline (PC), lysophosphatidyl-choline, (LPC) and sphingomyelin whereas unsaturated acids were predominant in phosphatidyl ethanolamine (PE), (PS), and phosphatidyl inositol (PI). Acids containing three or more double bonds comprised about 90% of the total acids in PI. In all the phospholipids, the characteristic changes in the composition of fatty acids were observed due to the exclusion of 18:2 from the diet. However, changes due to the feeding of TYA were found only in PC and LPC. In rats fed the 18:2-rich diet, about 60% of the red cells were discocytes. In those fed the 18:2-free diet, the level of discocytes decreased to about 23%, and the levels of echinocytes II and III increased. The exclusion of 18:2 for even a few days decreased the proportion of discocytes. The loss of discoid shape was reversed in a few days by feeding an 18:2-rich diet. Fatty acid analysis of erythrocytes of rats on the various dietary manipulations showed that the change in the proportion of discocytes followed the change in the level of 18:2.

INTRODUCTION

Several investigations have reported the fatty acid composition of total lipids of rat erythrocytes (1). However, to our knowledge, neither the composition of fatty acids in their individual phospholipids nor their alterations due to diet fat have been reported. In the present study, we determined the fatty acid compositions of erythrocyte phospholipids of rats which were fed various diets that are known to affect the tissue levels of 18:2 and 20:4. Furthermore, we examined the morphological structure of red cells using scanning electron microscopy and observed the importance of dietary 18:2 for the normal discoid shape of erythrocytes. A preliminary report of this study has already appeared (2).

MATERIALS AND METHODS

One month old Sprague Dawley male rats (100 g) were obtained from Hilltop Animal Supplier, Chatsworth, CA. They were divided into six groups of four each. Each group was fed for eight weeks one of the following six diets: 1) stock diet (Wayne Lab Blox; Allied Mills, Chicago, IL) which contained 4.5% fat and 45.5% 18:2 in the total fatty acids; 2) a high carbohydrate, 10% Mazola Corn Oil (CO) diet; 3) a CO diet containing 0.033% eicosa-5,8,11,14-tetraynoic acid (TYA); 4) a high carbohydrate, 10% hydrogenated coconut oil (HCNO) diet; 5) a HCNO diet containing 0.05% TYA, and 6) a high carbohydrate fat free (FF) diet. The composition of the CO, HCNO and FF diets was given previously (3).

In some experiments, rats (100 g) were fed for a week a stock diet and fed for three, five and seven days a FF diet. A group of rats which were fed the FF diet for five days were then fed the CO diet for three or seven days.

Hydrogenated coconut oil (Cobee 92) was a generous gift from PVO International, Inc., Richmond, CA. The fatty acid methyl ester standards were obtained from Applied Science Laboratories, State College, PA, and Supelco, Bellefonte, PA.

Rats were anesthetised by an intraperitoneal injection of sodium pentobarbital (50mg/ml/300 g rat) and exsanguinated using heparin-washed syringes and needles. Whole blood was transferred without pressure into heparinized vacutainer tubes. Immediately after the blood was obtained, a 0.5 ml sample was fixed in 0.5% glutaraldehyde in standard incubation mixture (SIM), which contained NaCl (141 mM), KCl (10 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.3 mM), NaH_2PO_4 (0.8 mM) and Na_2HPO_4 (5 mM).

Whole blood was centrifuged at about 22 C at 2500 rpm for 7 min, and the plasma and buffy layer were removed. Red cells were

washed three times by suspension in SIM. Hemoglobin determinations were carried out using the cyanmethemoglobin method. The packed red cell volumes were measured in duplicate with a Phillips-Drucker hematocrit centrifuge and read on an IEC microcapillary reader.

The fresh blood samples fixed in glutaraldehyde were washed three times in SIM, sedimented on glass slides, dehydrated in a graded alcohol series and critical point dried (4). After being coated with gold-palladium, they were examined and classified using an ETEC Autoscan scanning electron microscope (5).

Total lipids from erythrocytes were extracted as described by Rose and Oklander (6). Lipid extracts were protected from the oxidation of unsaturated fatty acids by adding butylated hydroxytoluene (BHT) (0.02%) and storing under N_2. During the gas liquid chromatographic (GLC) separation of methyl esters, BHT elutes with methyl myristate. Since the HCNO diet contains 14:0, BHT was not added during the analysis of the total fatty acid composition of red cells. We have found that the absence of BHT in the lipid extracts did not reduce the level of polyunsaturated fatty acids. In the solvents used for the separation of phospholipids and methyl esters by thin layer chromatography (TLC), BHT was added. Therefore, the values for 14:0 were not obtained from the analysis of phosphoglycerides.

Phospholipids were separated by TLC, their fatty acids were converted to methyl esters and purified from dimethylacetal derivatives as described by Pullarkat et al. (7,8). Analysis of fatty acids by GLC was carried out at 180 C in a Varian Aerograph Model 2740 using a flame ionization detector and a stainless steel column (6' x 1/8") packed with 5% diethylene glycol succinate on H/P Chromosorb G. Analysis of the methyl esters from red cells of rats fed the HCNO diet was carried out at 150-180 C at 10 C/min. Areas of peaks and percent composition of fatty acid methyl esters were computed using a Varian Chromatography Data System.

RESULTS AND DISCUSSION

In the erythrocyte lipids from various species, 20:4 is a major fatty acid. In rats maintained on linoleate-rich diets, as much as 30% of the total fatty acids in red cells is 20:4 (9). In the present study, rats were fed various diets that influence the tissue levels of 18:2 and 20:4. These were diets rich in 18:2, free of 18:2 and those which contained TYA – an inhibitor of the synthesis of 20:4 from 18:2 (10,11).

When 18:2 was excluded from the diets, the rat growth was somewhat depressed. Rats fed stock and CO diets for 8 weeks weighed about 527 g and 512 g, respectively, while those fed the HCNO and FF diets weighed 481 g and 479 g, respectively Dietary TYA caused a further reduction in their weights. Rats fed the CO + TYA diet, weighed 486 g while those fed the HCNO + TYA diet, weighed 400 g. The blood samples of all rats had a similar packed red cell volume (43.0 – 44.9%) and hemoglobin content (15.1 – 15.7g%). Since the amount of blood in rats has been estimated to be 7.6 ± 0.2 ml/100 g body weight (12), it would appear that it is somewhat decreased by the exclusion of 18:2 or the inclusion of TYA in the diet.

As observed previously (9,13), the composition of total fatty acids in rat erythrocytes was altered when 18:2 was omitted from the diet (Table I). The 16:1 and 20:3 levels and the values for the ratio 18:1/18:0 were enhanced while the levels of 18:2 were decreased. In the present study, the 20:4 level in the total fatty acids of erythrocytes was not decreased. A marked reduction in the level of 20:4 has been reported in red cells when weanling rats were fed an 18:2 deficient diet for 6 months (13). When TYA was included in the diet, a significant decrease in values for the ratios 16:1/16:0 and 18:1/18:0 has been observed in the lipids of liver and plasma of rats (14). In the erythrocyte lipids, such a decrease in the relative levels of monounsaturated fatty acids was small (Table I). As in the case of the liver and plasma lipids (14), when HCNO+TYA diet was fed, the levels of 12:0 and 14:0 were decreased in the erythrocyte lipids (Table I). As previously observed with several other tissues (10,11,14), the level of 18:2 was increased and the proportion of 20:4 to 18:2 was decreased in erythrocytes due to dietary TYA.

A detailed study of the fatty acid composition of individual phospholipids of rat erythrocytes has not been carried out (1). In some previous investigations, not all the phospholipid species were analyzed, while in others several fatty acids were grouped together (1). We have determined the fatty acid compositions of various individual phospholipids in erythrocytes of rats and their changes due to different diets (Table II–VII). Each phospholipid species exhibited a characteristic fatty acid composition which was altered by the dietary manipulations. The phosphatidyl choline (PC), lysophosphatidyl choline (LPC), and sphingomyelin contained relatively more saturated acids. In PC and LPC, 16:0 and 18:0 were predominant, while in sphingomyelin, 16:0 and 24:0 were major saturated acids (Table II–IV). On the

TABLE I

Fatty Acid Composition of Erythrocytes of Rats Fed Different Diets[a]

Fatty acid	Stock diet[b]	CO diet[b]		HCNO diet[b]		FF diet
		-TYA	+ TYA	-TYA	+TYA	
12:0	0	0	0	0.7 ± 0.1	0.3 ± 0.05	0
14:0	0.7 ± 0.1	0.8 ± 0.07	T	1.5 ± 0.1	0.6 ± 0.04	0.6 ± 0.05
16:0	18.9 ± 1.3	16.4 ± 0.3	18.5 ± 0.3	19.4 ± 0.5	19.3 ± 0.5	20.0 ± 0.5
16:1	0.7 ± 0.1	T	T	2.4 ± 0.1	1.6 ± 0.04	2.6± 0.1
18:0	21.0 ± 1.2	21.3 ± 0.4	21.2 ± 0.3	17.9 ± 0.2	19.3 ± 0.3	19.1± 0.2
18:1	10.6 ± 0.6	9.5 ± 0.3	8.2 ± 0.2	13.8 ± 0.2	13.3 ± 0.3	16.2± 0.4
18:2	10.3 ± 0.5	12.5 ± 0.2	18.9 ± 0.5	1.6 ± 0.1	3.6 ± 0.1	1.6± 0.1
18:3	T	T	T	---	---	---
20:3W9	---	---	---	7.2 ± 0.3	7.5 ± 0.3	6.2± 0.2
22:0	T	T	T	1.1 ± 0.1	3.1 ± 0.1	1.0± 0.1
20:4	26.5 ± 1.0	28.8 ± 0.3	26.1 ± 0.4	28.0 ± 0.3	26.4 ± 0.7	27.8± 0.6
22:4[c]	4.6 ± 0.6	5.6 ± 0.2	4.2 ± 0.2	3.3 ± 0.2	2.5 ± 0.2	2.7± 0.1
22:5	1.9 ± 0.3	1.7 ± 0.1	0.8 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.8± 0.2
22:6	3.9 ± 0.5	3.3 0.1	1.3 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.6± 0.1
16:1/16:0	---	---	---	0.12 ± 0.01	0.08 ± 0.003	0.13± 0.004
18:1/18:0	0.51 ±0.02	0.45 ± 0.02	0.38 ± 0.02	0.77 ± 0.01	0.69 ± 0.02	0.85 ±0.02
20:4/18:2	2.57 ± 0.19	2.3 ± 0.06	1.4 ± 0.04	17.7 ± 0.9	7.3 ± 0.3	18.1 ±0.8

[a]Percent of total fatty acids is given as Mean ± SE of analysis with erythrocytes from four rats in each diet group. Duplicate determinations were carried out with erythrocytes from each rat. Values 0.5% or less are given as T.

[b]The stock diet contained the fatty acids: 14:0, 3.3%; 16:0, 18.2%; 16:1, 2.5%; 18:0, 4.1%; 18:1, 23.3%, 18:2, 45.5% and 18:3, 3.1%. The corn oil (CO) diet contained the fatty acids: 16:0, 10.7%; 18:0, 1.8%; 18:1, 24.8%; 18:2, 61.9%; and 18:3, 1.0%. The hydrogenated coconut oil (HCNO) diet contained the fatty acids: 6:0, 1.0%; 8:0, 11.9%; 10:0, 11.9%; 12:0, 18.6%; 14:0, 20.9%; 16:0, 16.7%; 18:0, 18.6%, and 18:1, 0.4%.

[c] Included 24:0 and 24:1.

TABLE II

Fatty Acid Composition of Phosphatidylcholine from Erythrocytes of Rats Fed Different Diets[a]

Fatty acid	Stock diet	CO diet		HCNO diet		FF diet
		-TYA	+TYA	-TYA	+TYA	
16:0	35.6 ± 2.2	40.6 ± 0.4	37.4 ± 0.7	35.4 ± 0.6	34.0 ± 0.8	36.8 ± 1.2
16:1	0.9 ± 0.1	T	T	3.7 ± 0.2	3.0 ± 0.1	3.8 ± 0.5
18:0	27.6 ± 1.8	20.3 ± 0.8	21.1 ± 1.0	18.2 ± 0.8	20.4 ± 0.6	15.4 ± 0.4
18:1	8.3 ± 0.5	7.1 ± 0.5	6.8 ± 0.7	24.5 ± 0.8	22.0 ± 1.0	24.3 ± 0.8
18:2	12.3 ± 0.6	15.0 ± 0.5	20.3 ± 0.3	1.5 ± 0.1	5.8 ± 0.2	1.4 ± 0.2
18:3	1.1 ± 0.2	T	T	---	---	---
20:3W9	---	---	---	8.0 ± 0.5	7.5 ± 0.3	9.1 ± 0.8
20:4	13.8 ± 0.6	17.2 ± 1.6	13.2 ± 0.4	8.6 ± 0.6	7.3 ± 0.4	9.3 ± 0.5

[a]Percent of total fatty acids given are Mean ± SE of values from three determinations with PC isolated from red cell lipids of separate rats in each diet group.

other hand, in phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI), unsaturated acids were present in relatively large levels. In these lipids the 20:4 level varied from 42 to 60%. The level of 18:0 was greater in PS than in PE or PI. The composition of PI was unique in that acids containing three or more double bonds comprised about 90% of the total fatty acids.

The fatty acid compositions of various phospholipids were altered due to the dietary manipulations. When 18:2 was omitted from the diet, 16:1 and 20:3 levels were increased and the 18:2 and 20:4 levels were decreased in PC and LPC. In these lipids relatively more 18:1 was present than 18:0. As observed by Watson previously (15), relatively more of 24:1 than 24:0 was found in the sphingomyelin fraction of erythrocyte lipids of rats fed the 18:2-free diets (Table IV). Upon feeding the

TABLE III

Fatty Acid Composition of Lysophosphatidyl Choline from Erythrocytes of Rats Fed Different Diets[a]

Fatty acid	Stock diet	CO diet		HCNO diet		FF diet
		-TYA	+TYA	-TYA	+TYA	
16:0	33.1	39.0	37.1	36.9	39.9	35.8
16:1	1.4	1.1	0.5	3.7	2.4	3.2
18:0	40.6	29.8	36.2	24.2	28.6	24.9
18:1	8.1	11.9	8.4	20.4	15.7	21.8
18:2	6.9	6.7	9.3	T	T	T
18:3	1.4	T	T	---	---	---
20:3W9	---	---	---	7.6	6.4	6.1
20:4	7.9	11.2	8.4	7.2	6.8	8.2

[a]Percent of total fatty acids given are means of closely agreeing values from duplicate determinations with the pooled lysophosphatidyl choline fractions of red cell lipids in each dietary condition.

TABLE IV

Fatty Acid Composition of Sphingomyelin from Erythrocytes of Rats Fed Different Diets[a]

Fatty acid	Stock diet	CO diet		HCNO diet		FF diets
		-TYA	+ TYA	-TYA	+TYA	
16:0	30.5 ± 2.1	26.1 ± 2.8	26.4 ± 3.6	30.1 ± 1.3	27.6 ± 1.8	28.0 ± 1.5
18:0	7.3 ± 0.6	7.1 ± 1.1	6.7 ± 0.8	6.0 ± 0.7	6.9 ± 1.0	7.6 ± 0.6
18:1	8.0 ± 0.8	6.0 ± 0.7	6.1 ± 0.4	3.9 ± 0.2	2.5 ± 0.5	6.3 ± 0.2
20:0	1.5 ± 0.2	2.1 ± 0.4	1.7 ± 0.5	2.2 ± 0.4	1.4 ± 0.2	2.0 ± 0.4
22:0[b]	7.9 ± 0.7	8.2 ± 0.8	9.2 ±1.0	9.1 ± 0.8	8.6 ± 0.5	8.0 ±0.5
24:0	25.9 ± 0.8	28.9 ± 2.7	28.7 ± 1.3	17.1 ± 0.7	18.3 ±0.5	15.9 ± 0.6
24:1	11.3 ± 0.6	12.6 ± 0.8	13.7 ± 0.6	30.3 ± 0.4	33.2 ± 1.3	30.7 ± 1.6
24:2	7.2 ± 1.1	8.8 ± 0.7	7.3 ± 0.9	1.1 ± 0.2	1.3 ± 0.2	1.3 ± 0.2

[a]Percent of total fatty acids given are Mean ± SE of values from three determinations with Sphingomyelin isolated from red cell lipids from separate rats in each group.

[b]Contained small amounts of 22:1.

TABLE V

Fatty Acid Composition of Phosphatidyl Ethanolamine From Erythrocytes of Rats Fed Different Diets[a]

Fatty acid	Stock diet	CO diet		HCNO diet		FF diet
		-TYA	+ TYA	-TYA	+ TYA	
16:0	8.9 ± 0.6	9.6 ± 0.5	12.2 ± 1.2	9.1 ± 0.4	9.1 ± 0.7	11.5 ± 0.8
16:1	T	T	T	2.5 ± 0.1	2.0 ± 0.1	2.5 ± 0.2
18:0	7.5 ± 0.5	9.0 ± 0.7	12.4 ± 0.9	10.8 ± 0.2	13.5 ± 0.5	8.8 ± 0.5
18:1	14.5 ± 1.5	17.4 ± 1.6	15.6 ± 0.5	17.6 ± 0.1	15.1 ± 0.9	20.1 ± 0.4
18:2	8.7 ± 0.5	10.6 ± 0.5	10.3 ± 0.4	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
18:3	T	T	T	---	---	---
20:3W9	---	---	---	13.8 ± 0.8	13.6 ± 0.3	10.6 ± 0.8
20:4	52.4 ± 2.2	46.6 ± 1.0	44.9 ± 1.1	43.2 ± 0.5	42.4 ± 0.8	42.0 ± 1.2
22:4	4.0 ± 0.2	3.7 ± 1.1	2.9 ± 0.1	1.6 ± 0.2	1.8 ± 0.4	2.3 ± 0.1
22:5	1.6 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.2
22:6	1.8 ± 0.1	1.9 ± 0.1	0.9 ±0.2	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.2

[a]Percent of total fatty acids given are Mean ± SE of values from three determinations with PE isolated from red cell lipids of separate rats in each diet group.

diets devoid of 18:2, the level of 18:2 decreased and of 20:3 increased in PE, PS and PI. However, only in PI did the relative level of 18:1 to 18:0 increase. Dietary TYA did not alter the fatty acid compositions of sphingomyelin, PE, PS and PI, but did cause an increase in the level of 18:2 in PC and LPC. Earlier studies with red cell ghosts have shown that all

TABLE VI

Fatty Acid Composition of Phosphatidyl Serine from Erythrocytes of Rats Fed Different Diets[a]

Fatty acid	Stock diet	CO diet		HCNO diet		FF diet
		-TYA	+ TYA	-TYA	+TYA	
16:0	6.1 ± 0.4	6.0 ± 0.4	5.6 ± 0.4	6.5 ± 0.6	7.4 ± 0.7	6.2 ± 0.2
16:1	T	T	T	0.8 ± 0.1	T	T
18:0	29.6 ± 2.6	26.3 ± 0.4	27.9 ± 0.8	24.4 ± 0.7	25.3 ± 1.2	22.7 ± 1.0
18:1	6.5 ± 0.5	6.3 ± 0.5	6.5 ± 0.4	9.5 ± 0.4	8.3 ± 0.5	10.4 ± 0.9
18:2	3.7 ± 0.4	7.2 ± 0.9	6.9 ± 0.9	T	T	T
18:3	T	T	T	---	---	---
20:3W9	---	---	---	7.2 ± 0.8	7.7 ± 0.5	6.6 ± 0.6
20:4	50.4 ± 2.4	51.3 ± 0.8	50.7 ± 1.3	49.1 ± 0.9	49.7 ± 0.6	50.1 ± 1.3
22:4	1.7 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	1.4 ± 0.2
22:5	1.7 ± 0.3	1.5 ± 0.2	1.1 ± 0.2	1.4 ± 0.3	T	1.7 ± 0.1

[a]Percent of total fatty acids given are Mean ± SE of values from three determinations with PS isolated from red cell lipids from red cell lipids from separate rats in each diet group.

TABLE VII

Fatty Acid Composition of Phosphatidyl Inositol from Erythrocytes of Rats Fed Different Diets[a]

Fatty acid	Stock diet	CO diet		HCNO diet		FF diet
		-TYA	+ TYA	-TYA	+ TYA	
16:0	3.4 ± 0.4	4.3 ± 0.4	3.8 ± 0.5	3.6 ± 0.3	3.4 ± 0.5	3.9 ± 0.4
16:1	T	T	T	0.8 ± 0.1	T	0.8 ± 0.1
18:0	2.1 ± 0.5	1.6 ± 0.2	1.1 ± 0.1	1.3 ± 0.2	1.7 ± 0.2	1.4 ± 0.2
18:1	2.3 ± 0.4	1.9 ± 0.2	1.2 ± 0.1	4.4 ± 0.4	3.8 ± 0.3	4.9 ± 0.4
18:2	2.0 ± 0.6	1.6 ± 0.1	1.4 ± 0.4	---	---	---
18:3	0.8 ± 0.1	T	T	---	---	---
20:3W9	---	---	---	19.6 ± 0.6	20.2 ± 0.8	19.6 ± 0.6
20:4	51.2 ± 1.3	59.0 ± 0.8	58.8 ± 1.5	60.0 ± 1.0	59.5 ± 1.9	58.9 ± 1.0
22:4	17.7 ± 1.4	19.4 ± 0.7	20.3 ± 1.2	4.0 ± 0.2	3.8 ± 0.4	4.0 ± 0.2
22:5	9.6 ± 0.8	6.0 ± 0.6	6.1 ± 0.6	2.5 ± 0.6	2.9 ± 0.1	2.7 ± 0.1
22:6	10.5 ± 0.4	6.2 ± 0.2	7.1 ± 0.5	3.9 ± 0.1	4.5 ± 0.4	3.6 ± 0.4

[a]Percent of total fatty acids given are Mean ± SE of values from three determinations with PI isolated from red cell lipids from separate rats in each diet group.

TABLE VIII

Morphologic Structure of Erythrocytes of Rats Fed Different Diets[a]

Cell type	Stock diet	CO diet		HCNO diet		FF diet
		-TYA	+ TYA	-TYA	+ TYA	
Discocytes	63.9 ± 0.6	56.8 ± 4.2	57.7 ± 4.7	23.5 ± 4.4	27.6 ± 3.5	16.9 ± 1.5
Knizocytes	---	0.3 ± 0.2	2.1 ± 0.8	---	---	0.3 ± 0.2
Stomatocytes	7.3 ± 0.3	4.0 ± 1.1	4.0 ± 0.6	1.3 ± 0.6	2.4 ± 0.5	1.9 ± 0.6
Echinocytes I	25.8 ± 0.5	31.1 ± 4.6	27.9 ± 2.3	32.6 ± 2.1	33.6 ± 4.7	44.2 ± 3.0
Echinocytes II	2.4 ± 0.2	7.0 ± 0.1	7.6 ± 2.8	34.9 ± 4.7	30.1 ± 3.7	30.7 ± 4.4
Echinocytes III	---	0.7 ± 0.3	0.6 ± 0.2	5.9 ± 1.0	4.4 ± 1.0	5.6 ± 1.5

[a]Percent distribution of the various cell types are given as Mean ± SE of the values obtained by counting 500 cells in duplicate from the blood sample from each rat in the diet group. Samples of blood from four rats in the CO and FF diet groups and three rats in the HCNO and stock diet groups were used.

the lipid present in the red cell resides in the membrane (16,17). Thus, the composition of fatty acids and their changes that were observed in our investigation using red cells must be those of the membranes.

Although the level of 20:4 in the total fatty acids of erythrocytes was essentially similar under various dietary conditions (Table I), exclusion of 18:2 or addition of TYA in the diet resulted in a decrease in the level of 20:4 in

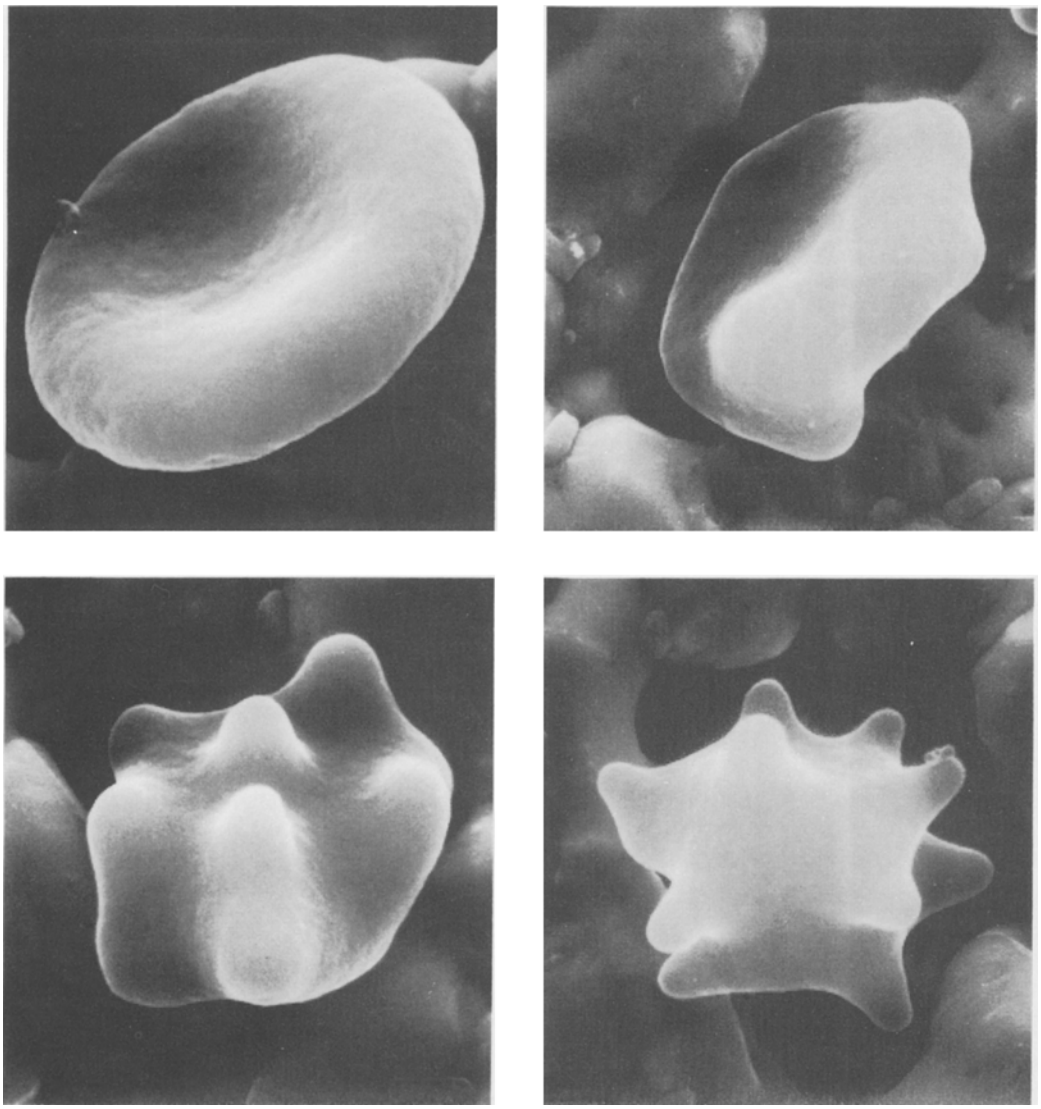

FIG. 1. Scanning electron microscopic examination of rat erythrocytes. Top left, Discocyte (X12,00); top right, Echinocyte I (X13,000); bottom left, Echinocyte II (X13,000); bottom right, Echinocyte III (X13,000).

PC and LPC (Table II, III). Whether this discrepancy is due to the change in the content of individual phospholipids due to diets is not known at the present time. Previously, Farnsworth et al. observed that the relative amount of 'lecithin' increased and 'cephalin' decreased in erythrocytes of rats which were fed a fat free diet as compared to their controls (18). On the other hand, De Gier and Van Deenen (19) reported that the variation in the regimen of rats did not significantly change the proportions of major phospholipids.

Red cells from rats fed an 18:2-deficient diet for 3 months were reported to be morphologically normal (15). However, this conclusion was based on the results of cell sizing with a Coulter Counter. In our studies of the morphology of the erythrocytes using scanning electron micriscopy, alteration of the normal biconcave structure of the discocytes was observed due to the exclusion of 18:2 from the diet (Table VIII). A majority of the cells (60%) from rats fed linoleate-rich diets (stock diet, CO, CO+TYA) are discocytes which are characterized by their biconcave structure, while about 30% of cells are irregularly contoured

TABLE IX

Effect of Exclusion or Addition of Linoleate to Diets Fed to Rats for Short Periods on the Morphologic Structure of Erythrocytes[a]

	Number of days on FF diet[b]			Number of days on CO diet			
Cell type	0	3	7	0[c]	3[c]	7[c]	7[d]
Discocytes	66.4	43.0	9.8	23.2	41.0	63.2	66.8
Knizocytes	6.4	3.8	0.4	1.2	0.0	3.4	0.8
Stomatocytes	15.6	6.1	2.2	4.0	5.9	4.4	3.6
Echinocytes I	11.6	31.9	31.2	32.0	23.4	17.8	20.4
Echinocytes II	0.0	14.5	44.0	36.8	26.8	9.3	7.2
Echinocytes III	0.0	0.6	11.8	2.8	2.9	1.8	0.8

[a]Percent of various types of cells given is mean of values from counting 500 cells in duplicate with samples of blood pooled from two young rats or separate samples from adult rats.

[b]Young rats (100 g) fed a stock diet were then fed a FF diet as indicated.

[c]Young rats fed a stock diet were fed FF diet for five days and then the CO diet as indicated.

[d]Young rats were fed a FF diet for eight weeks and then fed the CO diet as indicated.

TABLE X

Changes in the Fatty Acid Composition of Erythrocytes due to the Exclusion or Addition of Linoleate to Diets Fed to Rats for Short Periods[a]

	Number of days on FF diet			Number of days on CO diet			
Fatty acid	0	3	7	0	3	7	7
16:0	26.2	26.6	27.5	27.7	27.3	28.0	26.1
16:1	T	0.7	1.9	2.1	0.8	0.6	0.5
18:0	15.4	14.0	14.8	13.2	15.2	13.6	16.4
18:1	9.1	12.4	16.5	14.3	13.7	10.2	10.7
18:2	12.5	6.8	2.1	4.4	6.6	10.8	8.3
20:3w9	---	---	2.8	1.2	0.5	0.5	3.1
22:0	T	T	T	T	T	T	0.5
20:4	28.8	28.4	26.9	26.2	25.5	27.2	27.2
24:0)							
22:4)	3.4	4.8	4.6	4.1	5.4	4.8	4.0
24:1)							
22:5	1.2	1.9	1.7	2.9	2.0	2.1	1.2
22:6	3.2	3.8	1.4	3.5	2.7	2.1	1.8

[a]Percent of total fatty acids given are the means of closely agreeing values from duplicate determinations with lipid extracts of erythrocytes of rats used in Table IX. Values 0.5% or less are given as T.

discocytes (Echinocytes I) (Fig. 1). Flat red cells with spicules (Echinocytes II) are few 2-7%), and spherical cells with spicules (echinocytes III) are practically absent (Table VIII, Fig. 1). However, when rats were fed diets devoid of 18:2 (HCNO, HCNO + TYA, FF) the proportion of discocytes decreased to 17-28%, while echinocytes II and echinocytes III increased to 35% and 6%, respectively. These structural changes appear to be associated with the presence of 20:3 and the reduced level of 18:2 in red cells (Table I).

In order to establish the involvement of dietary 18:2 in maintaining the normal structure of erythrocytes, we determined whether the loss of discoid structure could be reversed by feeding a 18:2-rich diet (Table IX). Furthermore, since the life span of red cells is about 60 days, it was not known whether the increased level of echinocytes produced by feeding rats a diet devoid of 18:2 for eight weeks was related to the turnover of the cells. Hence, we determined the fatty acid composition and morphological structure of red cells from rats which were fed a FF diet for only a few days and also from those which were fed a FF diet and then fed a diet rich in 18:2 for a few days (Table IX,X).

A majority of the red cells (66%) in rats fed the stock diet were discocytes. Echinocytes I were few (12%) and echinocytes II and III were practically absent (Table IX, Fig. 2). On feeding

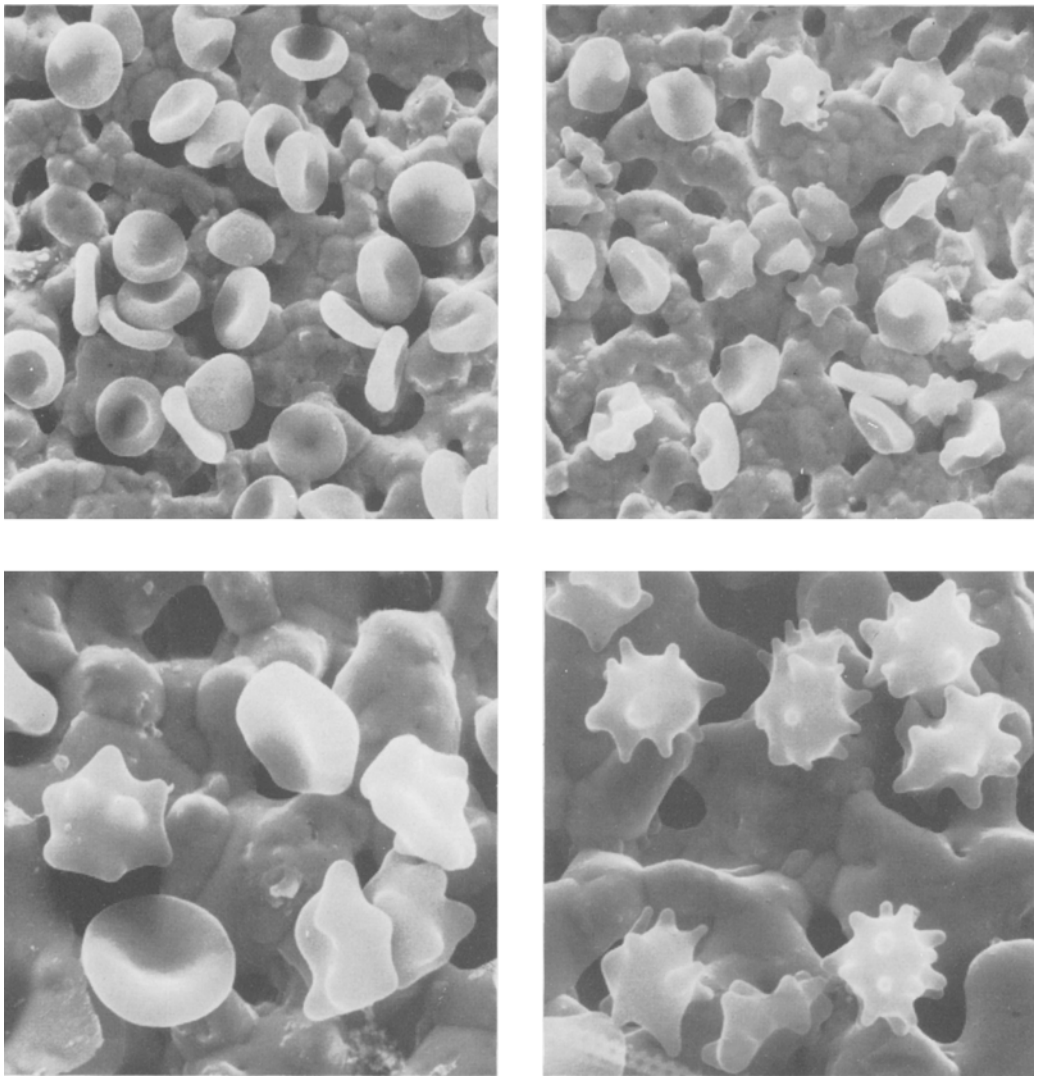

FIG. 2. Scanning electron microscopic examination of erythrocytes from rats fed 18:2-rich or 18:2-free diets. Top left, Erythrocytes were obtained from rats maintained on a stock diet; top right, bottom left and right, erythrocytes were from rats fed a FF diet for one week. Top, X2500 Bottom, X5000.

a FF diet for one week, the level of discocytes decreased to 10%, while the level of echinocytes I, II and III increased to 31%, 44% and 12%, respectively (Table IX, Fig. 2). Even after three days, a significant reduction in discocytes and an increase in echinocytes was observed (Table IX). Thus, in order for the structural changes in erythrocytes to occur, a long term (eight weeks) feeding of an 18:2-deficient diet is not needed.

When rats were fed a FF diet for five days or eight weeks and then fed a CO diet for a week, a reversal in the loss of discocytes occurred (Table IX). An appreciable increase in the number of discocytes and a decrease in echinocytes I and II was found by feeding the CO diet for even three days. Thus, loss of the discoid shape of erythrocytes due to feeding rats an 18:2-free diet is reversed by feeding them an 18:2-rich diet. Since structural changes have occurred in erythrocytes by short term dietary manipulations, it is clear that these changes are not related to the life span of red cells.

In erythrocyte lipids the characteristic changes in the composition of fatty acids due to feeding a FF diet, (presence of 20:3; decrease of 18:2 and relatively high levels of 16:1 and 18:1) were observed in a few days (Table

X). The changes in the composition due to feeding CO diet (increased levels of 18:2; low levels of 20:3; and a decrease in 16:1 and 18:1) also occurred (Table X).

In rats fed a FF diet for eight weeks, the erythrocyte fatty acids contained significant levels (6.2%) of 20:3 and very low levels (1.6% of 18:2 (Table I). When they were then fed a CO diet for one week, their red cells still contained 20:3 (3.1%) while the level of 18:2 increased to 8.3% (Table X). Since the discocyte level was normal (Table IX), it is apparent that the presence of 20:3 may not be related to the loss of discocytes or increase in the number of echinocytes. On the other hand, the loss of discocytes and the concomitant increase in the number of echinocytes is related to the level of 18:2 in erythrocyte fatty acids (Table X). However, it cannot be concluded from these results that the alterations in the composition of fatty acids are responsible for the structural changes of the red cells. Exposure of erythrocytes to plasma which contains free fatty acids or LPC or changes in the ratio of cholesterol to phospholipids in erythrocytes alters the structure of red cells (20,21). Whether the structural modifications seen in our study are due to such changes in the lipid composition of plasma or red cells is not yet known. However, our results do demonstrate the importance of dietary linoleate in maintaining the normal discoid shape of red cells.

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry. IV. Soybean Oil Methyl Esters[1]

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ABSTRACT

An unusual isomeric distribution of hydroperoxides has been found in soybean oil esters oxidized at low levels (peroxide values below 50). The unexpectedly high concentration of the 12-hydroperoxide isomer is in marked contrast to the isomeric composition of oxidized pure linolenate. The different isomeric hydroperoxides observed at low levels of oxidation may contribute through their decomposition to the unique flavor deterioration of soybean oil. Quantitative gas chromatography-mass spectrometry (GC-MS) used in this study provides for the first time an answer to the basic question of which hydroperoxides contribute to the state of oxidation of soybean oil. Results of GC-MS were confirmed by capillary gas chromatography. Analyses of highly oxidized soybean esters (peroxide values 468 and 2352) reveal the same main compounds as those found in oxidized pure linoleate, together with small amounts of oleate and linolenate hydroperoxides.

INTRODUCTION

How different fatty acids in natural fats interact during autoxidation is a subject that has received little attention. Previous studies have dealt with kinetics of oxidation of oleic-linoleic acid or ester mixtures (1,2). However, kinetic data alone cannot be used directly to predict the thermodynamic stability of the hydroperoxides. Analyses of hydroperoxides reflect not only the relative ease of hydrogen abstraction from each unsaturated fatty ester but also the relative disappearance due to decomposition and secondary oxidation (3). These factors would be particularly important in mixtures containing linolenate that is particularly sensitive to oxidative decomposition (4).

Hydroperoxides have been determined by gas chromatography (GC) of the allylic hydroxy esters from oxidized mixtures of oleate and linoleate (5), and by spectrophotometric analysis of "conjugatable oxidation products" from different polyunsaturated fats (6,7). In previous papers of this series (8-10), isomeric hydroperoxides were determined by gas chromatography-mass spectrometry (GC-MS) of the saturated hydroxy esters from autoxidized methyl oleate, linoleate, linolenate, and their mixtures. In this paper the oxidation products from mixtures of unsaturated fatty esters in soybean oil are analyzed by GC-MS. Because flavor deterioration in soybean oil occurs at low peroxide values (PV), samples autoxidized at low levels were included in this study to clarify the relative contribution of isomeric hydroperoxides as precursors of offensive odors and flavors.

EXPERIMENTAL

Commercially refined and deodorized soybean oil was transesterified with $NaOCH_3$, and the methyl esters were obtained by vacuum distillation (fatty acid analysis by GC: 14.8% palmitate, 3.7% stearate, 23.1% oleate, 51.5% linoleate, 6.9% linolenate).

Procedures for autoxidation, analyses $NaBH_4$ reduction, catalytic hydrogenation, silylation, and GC-MS were described previously (8,9,11). To extend the usefulness of the GC-MS method to PVs below 100, it was necessary to decrease the contribution of the unoxidized fatty esters that obscure the MS of the hydroxy ester components. A simple short silicic acid chromatographic column was used to concentrate the saturated hydroxy derivatives. Samples (200-400 mg) hydrogenated immediately after autoxidation were dissolved in 1 ml 5% ethyl ether in hexane and added to a 4-cm column of silicic acid (Mallinckrodt analytical reagent, 100 mesh, activated overnight at 110 C) in a short stem Pasteur pipette. Most of the methyl stearate and palmitate was eluted with 4 ml of 5% ethyl ether in hexane. The tail end of the saturated fatty esters and the hydrogenated oxidation products were eluted in a second fraction with 5 ml each of ethyl ether and methanol. To ensure that all hydroxy esters were included in the second fraction, this chromatographic separation was monitored by GC after silylation, and the

[1]Presented at AOCS Meeting, St. Louis, Missouri, May 14-18, 1978.

[2]Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

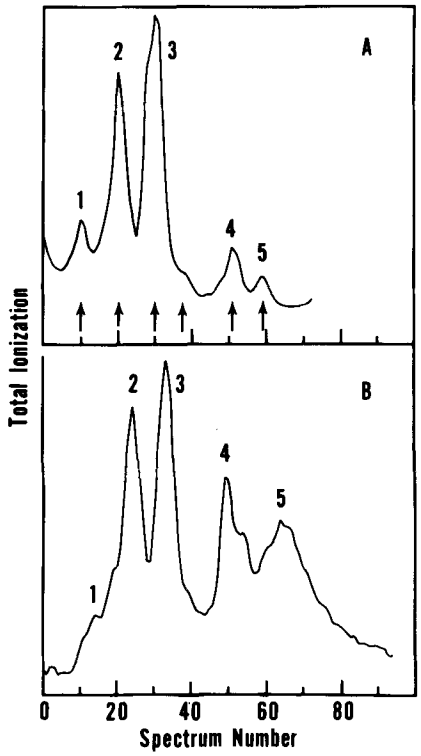

FIG. 1. GC-MS of trimethylsilyl ethers of $NaBH_4$ reduced-oxidized soybean methyl esters: computer traces of MS total ionization vs. spectrum numbers corresponding to full MS scan recorded every 12 sec; A. Peroxide value 468. Arrows indicate position of individual spectra analyzed by GC-MS (Table I); B. Peroxide value 2352. Peak 1 corresponds to the TMS esters of stearic and epoxystearic acids (8). Peak 2 corresponds to the TMS derivatives of the enols from oleate hydroperoxides. TMS derivatives of dienols from linoleate hydroperoxides and trienols from linolenate hydroperoxides emerge in peaks 2 and 3. Peaks 3, 4, and 5 are due to TMS derivatives of epoxyenols, enediols, and enetriols from oxidized methyl linoleate (9).

weight recovery was checked to be quantitative. The second fraction was silylated before GC-MS analysis. Chromatographic concentration of a freshly distilled nonoxidized sample of soybean oil methyl esters (PV = 0.9) gave on GC no evidence for peaks due to trimethylsilyl (TMS) ethers.

Capillary GC was used to confirm the GC-MS identification of isomeric hydroxystearates from hydrogenated-oxidized soybean esters. This qualitative method was based on the TMS ethers of methyl hydroxystearates (12), using a polyphenyl ether column (1800 x 0.02 in.), a Perkin-Elmer 3920 gas chromatograph with flame ionization detector, inlet split ratio of 100:1, gas flow of 13 ml/min (at room temperature), column temperature 190 C, injector temperature 220 C, and detector temperature 200 C.

RESULTS

Qualitative Studies

Figure 1 shows computer-generated gas chromatograms of the oxidation products from two samples of methyl esters of soybean oil (MeSBO) obtained after $NaBH_4$ reduction and silylation. MS analyses reveal that enols from 8-, 9-, 10-, and 11-oleate hydroperoxides emerge in peak 2; dienols from 9- and 13-linoleate hydroperoxides, and trienols from 9-, 12-, 13-, and 16-linolenate hydroperoxides emerge in peaks 2 and 3 (Table I). Peak 1 shows the presence of the TMS ester of stearic acid and 9,10-epoxystearate found in oxidized oleate (8). Other compounds identified from peaks 3, 4, and 5 are those found in oxidized methyl linoleate (9): epoxy-enols, 9,13-enediol, and enetriols.

Figure 2 shows computer-generated gas chromatograms of two samples of autoxidized MeSBO after catalytic hydrogenation of the double bonds and silylation. MS analysis (Table I) shows that peak 2 is due to the expected mixture of 8-, 9-, 10-, 11-, 12-, 13-, and 16-hydroxystearates (TMS ethers) from the hydroperoxides of oleate, linoleate, and linolenate. Peak 1 includes the TMS of stearic acid, keto-, and epoxystearates. Peak 3 is due to a mixture of hydroxyepoxy- and dihydroxystearate. Peaks 4 and 5 are attributed to a complex mixture of di- and trihydroxystearates previously identified in oxidized linoleate and linolenate (9,10). Qualitative identifications in Table I were confirmed by mass chromatography (8,9), an MS computer system involving complete scans of characteristic fragments plotted against retention times (spectrum numbers).

Quantitative Studies

To estimate the different hydroperoxides in oxidized MeSBO, the same computer summation method was used as established for known mixtures of hydroxystearates derived from autoxidized oleate (8), linoleate (9), and linolenate (10). At PVs below 100, the unoxidized fatty esters dominated the mass spectra and interfered with the quantitative analyses of the hydroxystearates. This interference was removed by chromatographically concentrating the oxidation products after catalytic hydrogenation. The gas chromatograms of a sample of oxidized MeSBO (PV 33.5) are compared before and after chromatographic concentra-

TABLE I

GC-MS Analysis of Autoxidized Soybean Methyl Esters (PV 468)

Peak (spectrum number)	Characteristic fragments m/e (relative abundance)	Identification[a] (OTMS-C_{18} methyl esters)	Origin[b]
	Figure 1A $NaBH_4$ reduced-oxidized		
1(10)	341 (70.7) M-15	TMS ester	St
	155 (25.5), 171 (6.5), 199 (6.0)	9,10 epoxy	Ol
2(20)	241 (4.5), 227 (12.7), 271 (5.2), 285 (5.2)	8-,9-,10-,11-enols	Ol
	225 (64.0), 382 (12.9)	9-dienol	Lo
	311 (32.3)	13-dienol/-trienol	Lo,Ln
	223 (1.4), 183 (3.5), 351 (1.2)	9-,12-,16-trienol	Ln
3(30)	225 (72.1), 382 (16.5)	9-dienol	Lo
	311 (48.4)	13-dienol/-trienol	Lo,Ln
	223 (1.3), 183 (1.8), 351 (1.5)	9-,12-,16-trienol	Ln
3(38)	199 (33.1), 285 (24.3)	9,10-/12,13-epoxy-11-enol	Lo
4(51)	173 (100), 259 (73.7)	9,13-enediol	Lo
5(59)	173 (100), 259 (22.0), 301 (28.3)	9,12,13-/9,10,13-enetriol	Lo
	Figure 2A Hydrogenated-oxidized		
1(11)	341 (100), M-15, 356 (17.7) M	TMS ester	St
1(16)	155 (52.1), 185 (25.7), 281 (21.7) M-31	9-keto	Lo
	99 (43.3), 241 (20.5)	13-keto	Lo
	171 (18.4), 199 (21.5)	9,10-epoxy	Ol
2(25)	243 (7.5), 245 (2.22), 215 (8.0), 273 (6.9)	8-, 10-OH	Ol
	229 (83.9), 259 (100)	9-OH	Ol,Lo,Ln
	201 (9.5), 287 (8.6)	11-OH	Ol
	173 (80.8), 315 (71.0)	13-OH	Lo,Ln
	187 (6.9), 301 (4.1)	12-OH	Ln
2(34)	131 (68.5), 357 (24.5)	16-OH	Ln
3(39)	173 (57.1), 301 (10.3)	11-OH-9,10-epoxy/-12,13-diOH	Lo
	259 (51.2), 215 (29.9)	9,10-diOH	Ol,Lo
4(50)	259 (94.6), 215 (6.6)	9,10-diOH	Ol,Lo
	173 (100), 301 (8.2)	12,13-/9,13-diOH	Lo
5(62)	173 (57.6), 213 (39.2), 259 (89.3), 299 (23.5)	9,12,13-/9,10,13,triOH	Lo
	131 (16.6), 187 (83.7), 315 (17.2)	9,10,12-/13,15,16-triOH	Ln

[a]See fragmentation schemes in preceding papers (8-10).
[b]ST = stearate, Ol = oleate, Lo = linoleate, Ln = linolenate.

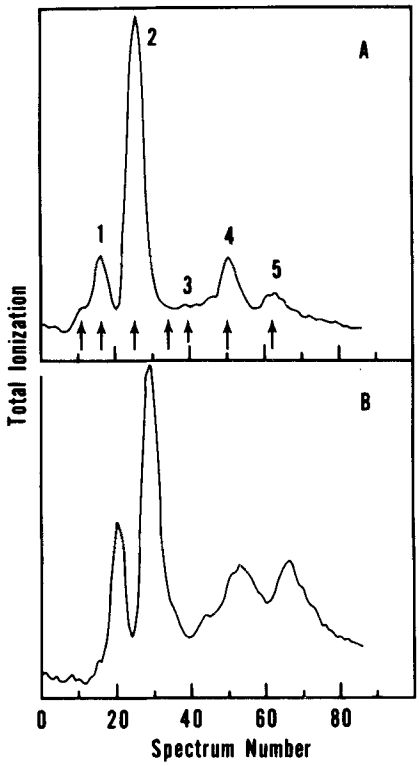

FIG. 2. GC-MS of trimethylsilyl ethers of hydrogenated-oxidized soybean methyl esters; A. Peroxide value 468. Arrows indicate position of individual spectra analyzed by GC-MS (Table I); B. Peroxide value 2352. Peak 1 includes TMS esters of stearic, ketostearic, and epoxystearic acids. Peak 2 is due to the TMS ethers of hydroxystearates from the hydroperoxides of oleate, linoleate, and linolenate. Peak 3 includes the TMS derivatives of hydroxyepoxy- and dihydroxystearates. Peaks 4 and 5 are attributed to the TMS derivatives of di- and tri-hydroxystearates (9,10).

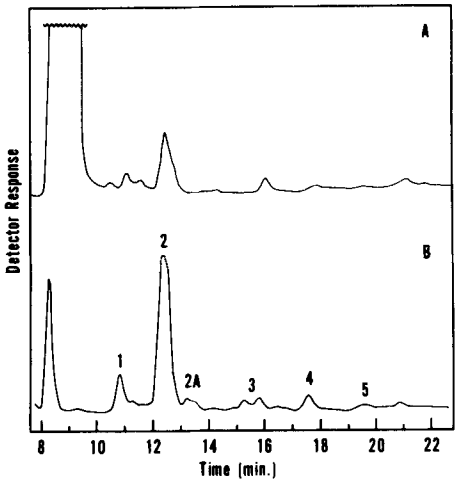

FIG. 3. Gas chromatogram of trimethylsilyl ethers of hydrogenated-autoxidized soybean methyl esters (PV 33.5); A. Before chromatographic concentration; B. After chromatographic concentration. Peak 1 includes TMS esters of stearic, ketostearic, and epoxystearic acids. Peak 2 and 2A are due to the TMS ethers of 8-, 9-, 10-, 11-, 12-, 13-, and 16-hydroxystearates from the hydroperoxides of oleate, linoleate, and linolenate. Peak 3 includes the TMS derivatives of hydroxyepoxy and dihydroxystearates. Peaks 4 and 5 are attributed to the TMS derivatives of di- and trihydroxystearates (9,10).

tion in Figure 3. Quantitative GC-MS analyses are based on the computer summation of all the spectra within peaks 2 and 2A corresponding to isomeric hydroxystearates.

Samples of MeSBO were oxidized to various PVs and at different temperatures to investigate the relative contribution of hydroperoxides from oleate, linoleate, and linolenate. Analyses of the isomeric composition of hydroxy esters show an unexpectedly high proportion of the 12-hydroxy isomers at low peroxide values (Table II). At PVs below 50, the 12-hydroxy isomer is significantly higher than the 16-hydroxy isomer. With increasing PVs there is a marked decrease in the 12- and increase in the 13-isomer, a small increase in the 9- and decrease in the 10- and 16-hydroxy isomers. The initial isomeric ratio is in marked contrast to previous results with autoxidized pure methyl linolenate, where the proportion of the 16-hydroperoxide is much greater than that of the 12-hydroperoxides (10,13).

To verify our methodology, a sample of pure methyl linolenate autoxidized to a PV of 20 was concentrated chromatographically after hydrogenation by the same procedure used for the MeSBO. The isomeric distribution determined by GC-MS was 30% 9-, 13% 12-, 15% 13-, and 42% 16-hydroxy esters. This distribution is the same as that previously determined by GC-MS in highly oxidized methyl linolenate without chromatographic concentration (10).

The GC-MS analysis of hydroxyester was further checked by capillary GC (12). The separation of two mixtures of synthetic hydroxy esters is compared with the isomeric mixture of a sample of hydrogenated-oxidized methyl linolenate (Fig. 4). The same trend was obtained as the GC-MS method, i.e., more 9- and 16-hydroxy esters than 12- and 13-hydroxy esters. However, when this capillary GC method was tested with known weighed mixtures of synthetic 9-, 12-, 13-, and 16-hydroxystearates, the results were not quantitative. Analyses were higher for 12-hydroxystearate (relative deviation: 3.8 to 12.8%) and lower for 16-hydroxy-

TABLE II

GC-MS Analysis[a] of Isomeric Hydroxystearate in Autoxidized Soybean Esters

Run	Oxidation temp., C	Peroxide value	Relative percent						
			8-OH	9-OH	10-OH	11-OH	12-OH	13-OH	16-OH
1[b]	30	4.4	1.4	40.5	3.9	1.5	21.8	24.2	6.8
		11.8	1.2	40.3	5.5	1.1	20.8	24.0	6.9
		16.8	1.1	40.8	3.5	1.0	17.5	28.2	8.2
		23.9	1.9	40.0	4.1	1.6	14.6	30.3	7.5
		35.5	1.9	40.8	2.3	2.2	9.5	35.4	8.2
		52.5	1.5	43.5	2.3	1.6	8.1	36.4	6.5
		85.0	1.4	43.3	2.4	1.5	5.6	38.5	7.2
		220	1.6	45.3	1.6	1.5	3.1	40.6	6.3
2	30	4.0	1.2	41.1	4.0	1.6	17.2	28.8	6.1
		7.0	1.1	39.6	3.4	1.1	13.6	35.4	5.8
		9.9	1.1	39.1	4.5	1.6	9.0	38.4	6.3
		29.1	1.2	40.0	2.1	0.8	4.8	42.5	4.6
		43.6	1.9	44.2	2.0	1.4	2.9	43.3	4.3
3	40	5.9	0.3	40.1	4.5	0.3	20.7	22.9	11.2
		9.3	1.4	39.1	4.4	2.0	17.7	27.8	7.8
		18.8	1.5	41.5	3.5	2.0	12.3	32.3	7.0
		58.6	1.9	42.0	2.5	2.0	6.6	37.4	7.7
4	40	3.4	1.9	35.8	4.6	2.1	17.1	29.8	8.7
		4.5	1.3	37.6	5.1	2.2	13.5	33.8	6.5
		5.8	1.7	40.5	4.0	2.1	10.8	34.4	6.5
		9.0	1.8	40.9	4.0	1.5	9.0	36.6	6.2
		16.9	2.2	42.4	2.9	2.2	5.1	40.7	4.7
		29.5	1.1	44.3	2.4	1.6	3.3	41.8	5.5
		47.5	2.0	39.5	1.7	2.0	3.0	46.6	5.2
5	60	10.0	0.9	33.5	3.8	2.0	17.0	31.2	11.7
		33.5	1.6	42.9	2.1	2.1	9.8	34.4	7.1
		47.6	1.5	44.2	1.7	1.9	7.1	35.4	8.1
		517	2.2	42.5	2.0	1.2	2.9	40.0	9.2

[a]All analyses reported are based on two or more replications.
[b]See Figure 6 for calculated hydroperoxide distribution.

stearate (relative deviation: 1 to 12.9%). The inconsistency of these deviations precluded the use of a correction factor to relate capillary GC and GC-MS analyses.

Figure 5 shows capillary gas chromatograms for three samples of hydrogenated-oxidized MeSBO. It is evident that the 12-hydroxy ester is in relatively higher concentration at the low PV than at the high PVs. Although this result cannot be taken in absolutely quantitative terms, it clearly confirms the same trend as the GC-MS results (Table II).

To calculate the proportion of different hydroperoxides in oxidized MeSBO, two possibilities were considered for the origin of the 12-OH isomer. Either this isomer comes from linolenate by autoxidation (4), or from both linoleate and linolenate by photosensitized oxidation (14,15). In the first case, the proportion of linolenate hydroperoxides can be caluculated as before (10) on the basis of the averaged percentage of 12- and 16-hydroxystearates (56.5%). In the second case, linolenate hydroperoxides can be estimated on the basis of the averaged value for 16-hydroxystearate, assuming it is present in the same proportion as in pure oxidized linolenate (46%). In both cases the oleate hydroperoxides are estimated on the basis of the 8- and 11-hydroxystearate isomers formed only from oleate (53.8%).

If the 12-hydroxy isomer comes from linolenate by autoxidation, linolenate hydroperoxides are the most important products at PVs below 15 (Fig. 6). Linoleate hydroperoxides become more important at higher PVs. Oleate hydroperoxides are minor products at all levels of oxidation studied. If the 12-hydroxyisomer comes from linoleate and linolenate by photosensitized oxidation, linoleate hydroperoxides become the most important products (76 to 81%) at all levels of oxidation over a range in peroxide values from about 4 to 85. Linolenate hydroperoxides vary from 14 to 18% and oleate hydroperoxides from 4 to 6.5%. Hydroperoxide composition was calculated as follows: Ln-OOH = 16-OH ÷ 0.46; 01-OOH = (8-OH + 11-OH) ÷ 0.538; Lo-OOH = 100- (Ln-OOH + 01-OOH).

To ascertain the origin of the 12-hydroperoxide in MeSBO, attempts were made to separate the allylic alcohols derived from hydroperoxides of oleate, linoleate, and linolenate. Such a determination was reported by Wong and Hammond (5) for the allylic alcohols of oleate and linoleate hydroperoxides. How-

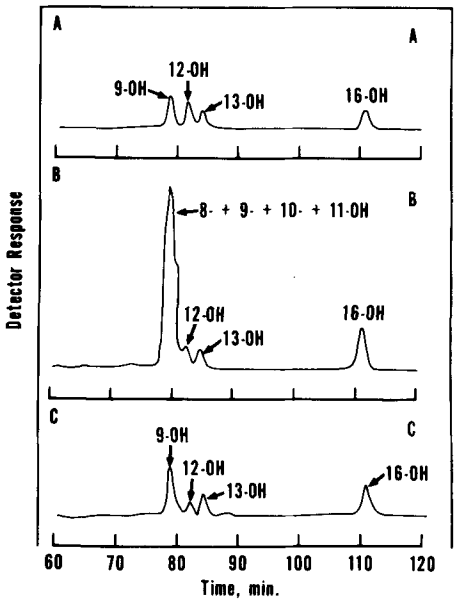

FIG. 4. Gas capillary chromatogram of trimethyl silyl ethers of hydroxystearates; A. Synthetic mixture: 9-OH + 12-OH + 13-OH + 16-OH; B. Synthetic mixture: 8-OH + 9-OH + 10-OH + 11-OH + 12-OH + 13-OH + 16-OH; C. Hydrogenated-autoxidized methyl linolenate (peroxide value 20).

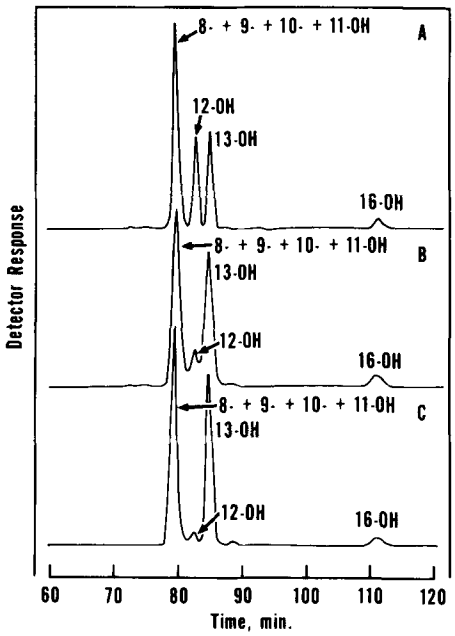

FIG. 5. Gas capillary chromatogram of trimethyl silyl ethers of hydrogenated-autoxidized soybean methyl esters; A. Peroxide value 12; B. Peroxide value 36; C. Peroxide value 220.

ever, the more complex mixture of allylic alcohols from oxidized MeSBO could not be separated, using either a GC system similar to that of Wong and Hammond (5), or capillary GC (12). The high pressure liquid chromatographic method of Chan and Levett (13) was also tried with no success.

DISCUSSION

GC-MS analysis of the highly autoxidized MeSBO reflects the same oxidation products expected from the unsaturated fatty acid constituents. Compounds identified are mainly those found in autoxidized pure *linoleate*: hydroperoxides (9 + 13), hydroxy-epoxy, di- and trihydroxy esters, together with small amounts of oleate hydroperoxides (8 + 9 + 10 + 11) and linolenate hydroperoxides (9 + 12 + 13 + 16). Although GC analyses of $NaBH_4$-reduced samples indicate the origin of oxidation products, they are not quantitative because allylic enols and dienols (from oleate and linoleate hydroperoxides) are poorly separated and allylic dienols and trienols (from linoleate and linolenate hydroperoxides) overlap (Fig. 1, Table I).

Quantitative GC-MS analysis of the saturated hydroxy derivatives has provided new insight on the isomeric hydroperoxides from the

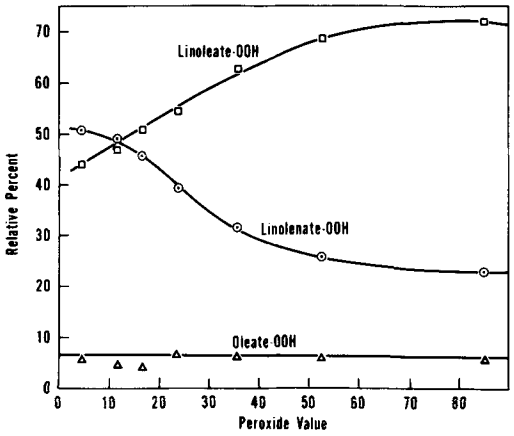

FIG. 6. Effect of peroxide value on hydroperoxide composition of soybean methyl esters autoxidized at 30 C, *assuming* 12-hydroxy isomer comes from linolenate by autoxidation. Hydroperoxide composition calculated as follows: Ln-OOH = (12-OH + 16-OH) ÷ 0.565; 01-OOH = (8-OH + 11-OH) ÷ 0.538; Lo-OOH = 100 - (Ln-OOH + 01-OOH).

ternary mixture of oleate, linoleate, and linolenate in soybean oil. In the previous paper (10), oxidation of an equal mixture of oleate, linoleate, and linolenate produced more linolenate hydroperoxides at PV 114 than linoleate hydroperoxides, and their ratio was reversed at higher

PVs. In the present paper with MeSBO containing only 7% linolenate, an unexpectedly high proportion of the derived 12-hydroxy isomer was found at PVs below 50. This unusual isomeric composition seems unique to MeSBO because pure methyl linolenate oxidized at PV of 20 gave the same isomeric composition as observed at much higher PVs (10). If the 12-hydroperoxide in MeSBO comes from linolenate by autoxidation, the high proportion of this isomer can be rationalized as follows: less cyclization of the 12-linolenate hydroperoxide (10), or more decomposition of the 16-linolenate hydroperoxide, or allylic rearrangement from the 16- to the 12-hydroperoxide (10).

Photosensitized oxidation of linoleate and linolenate is another possible source of the 12-hydroperoxide in weakly oxidized MeSBO. Clements et al. (16) found that minor components in refined soybean oil can act as a sensitizer for the photooxidation of a model diene, 4,7-undecadiene. A singlet O_2 intermediate was postulated that produced a mixture of conjugated and nonconjugated hydroperoxides. More recently, the hydroperoxides from linoleate photosensitized with methylene blue were identified as a mixture of 9-, 10-, 12-, and 13-isomers, and from linolenate as a mixture of the 9-, 10-, 12-, 13-, 15-, and 16-isomers (15). This same mixture was also identified from linolenate oxidized in the presence of erythrosine as photosensitizer (17). Therefore, in the present work, if the 12-hydroperoxide comes from linoleate and linolenate by photosensitized oxidation, then one would expect a higher proportion of the 10-hydroxy isomer as well as some 15-hydroxy isomer from linolenate. The proportion of 10-hydroxy ester was indeed higher than either the 8- or 11-hydroxy esters, a ratio which is not consistent with analyses of autoxidized pure oleate (8). Although the proportion of 10-hydroxy ester was much lower than that of the 12-hydroxy ester, both of those isomers decreased at higher PVs (Table II). The absence of the 15-hydroxy ester that would be expected from linolenate by photosensitized oxidation cannot rule out this process. The 15-hydroperoxide is only one of the six isomers expected from linolenate and would be formed at a level below the limit of detection.

Attempts to determine the allylic alcohols directly by GC-MS were not successful. At a PV of 468 (Table I), the concentration of 12-hydroxy ester was too low to determine whether it is conjugated and derived from linolenate (m/e 183, 3.5%) or nonconjugated and derived from linoleate (m/e 185, 2.3%). At PVs below 50, GC-MS analyses of allylic alcohols in concentrated samples did not prove reliable. As previously established, GC-MS analyses of allylic alcohols are not quantitative and are subject to errors caused by thermal rearrangement of the TMS ethers under GC conditions (10,18). There remains the problem of explaining why the concentration of the 12-hydroperoxide (and to a certain degree the 10-isomer) decreases at higher PVs. Oxygen reportedly quenches triplet sensitizers (19,20), and their effect may become negligible at high levels of oxidation where radical oxidation becomes dominant.

The results of this study have obvious implications on the flavor precursors of oxidized soybean oil. The different isomeric hydroperoxide distribution observed at low levels of oxidation suggests that it may contribute to the unique flavor deterioration of soybean oil. Critical problems for further work include determining whether the unique hydroperoxide composition of MeSBO at low PVs is due to either a different mechanism of linoleate and linolenate oxidation caused by minor oil components acting as photosensitizers, or decomposition of the respective hydroperoxides.

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Lipogenesis in the Developing Brain: Utilization of Radioactive Leucine, Isoleucine, Octanoic Acid and β-Hydroxybutyric Acid

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ABSTRACT

Incorporation of radioactivity from intracranially injected radioactive leucine, isoleucine (ketogenic amino acids), octanoic acid and β-hydroxybutyric acid into the brain lipids of 15 to 16 day-old rats was examined. The results showed that radioactivity from all the above precursors was incorporated into brain lipids. Radioactivity from injected isoleucine was incorporated into odd numbered fatty acids indicating an alternate pathway to α-oxidation for the biosynthesis of these fatty acids in the brain. For some as yet unclear reasons, a substantial portion of the radioactivity from injected octanoic acid was incorporated into free fatty acids. Utilization of these compounds for providing carbon for lipogenesis during development under unstressed normal conditions is discussed.

INTRODUCTION

It is well known that the principal source of lipid carbon in the brain is blood glucose, including metabolites of glucose such as acetate, α-glycerophosphate, and pyruvate. Mannosamine and galactose also are utilized in the formation of complex lipids. During the last few years, alternate sources for energy and lipid synthesis have been discovered. For example, ketone bodies, such as β-hydroxybutyrate and acetoacetate, are utilized by human subjects (1) during severe fasting, and the newborn brain utilizes ketone bodies in significant amounts (2). Edmond (3) has recently shown that 3-hydroxybutyrate was the preferred substrate for sterol and fatty acid biosynthesis and emphasized the major role of ketone bodies in the CNS during myelination. Lipogenesis from branched chain amino acids by homogenates of rat muscle (4) and recently by aorta (5) has been reported. Similarly, incorporation of label from isoleucine into odd numbered fatty acids of rat skin surface lipids (6) has been shown. Smith (7) has studied the uptake and incorporation of leucine and isoleucine into lipids by brain and spinal cord slices and found that there was a considerable degree of crossover as lipid precursors. Recently Cremer et al. (8) have investigated utilization of octanoate, butyrate and leucine by the rat brain in vivo and effects of portocaval anastomosis on metabolism of these compounds. The present study was undertaken to explore sources of lipid carbon in the developing brain. Leucine and isoleucine are known ketogenic amino acids, and octanoic acid occurs in the lipids of milk (9), which is the sole source of nutrition for the suckling rat. Another peculiarity of octanoic acid, a medium chain fatty acid, is its ability to pass directly into the systemic blood bypassing the normal lymphatic absorption of long chain fatty acids. Thus, short chain fatty acids might be transported and made available directly to the brain.

MATERIALS AND METHODS

Isotopes

The following radioactive compounds were purchased from New England Nuclear, Boston, MA: [U-^{14}C]leucine, 270 mCi/mM; [U-^{14}C]-isoleucine, 270 mCi/mM; [3-^{14}C]DL β hydroxybutyrate, sodium salt 1-5 mCi/mM; and [1-^{14}C]octanoic acid, 20-30 mCi/mM.

Animals

Fifteen to sixteen day-old suckling rats (four in a group), average weight 31.4 ± 3.8 g, were used in the study; the first group of rats was given 62.5 μCi of [U-^{14}C]leucine per rat, the second group was given 62.5 μCi of [U-^{14}C]-isoleucine per rat, the third group was given 25 μCi of [3-^{14}C]β-hydroxybutyrate per rat and, finally, the fourth group received 62.5 μCi of [1-^{14}C]octanoic acid per rat. All isotopes were injected directly into the brain (10). All animals were sacrificed 1 hr after injection.

Lipid Extraction and Fractionation

The brain was excised, following decapitation, and lipids were extracted from the pooled tissue by the method of Folch et al. (11). The chloroform phase was washed several times and the upper phase was tested for radioactivity. When no radioactivity was found in the upper phase, the total lipids were assumed to be free from water soluble starting material. In the case of β-hydroxybutyrate, after the above mentioned washing procedure, the total lipids were additionally dissolved in benzene and washed with water. β-Hydroxybutyrate is sparingly soluble in benzene and was thus removed. In the case of octanoate, we did not find any

TABLE I

Radioactivity in Brain Lipids 1 hr After Intracranial Injection

	TRACER			
	[U-^{14}C] Leucine	[U-^{14}C] Isoleucine	[3-^{14}C]β Hydroxy butyrate	[1-^{14}C] Octanoic acid
Total lipids mg/g wet wt	48.3	52.9	54.9	48.5
Percent of dose incorporated[a]	0.97	0.42	0.87	0.48
Ratio of radioactivity				
$\frac{\text{Polarlipids}}{\text{Free sterols}}$	2.08	3.58	2.18	2.85
Percent of total radioactivity				
Neutral lipids				
Free sterols	72.6	67.2	75.3	31.5
Total acyl glycerides	9.9	11.1	8.5	7.9
Free fatty acids	17.5	21.7	16.2	60.6
Total polar lipids				
Choline phosphoglycerides	51.2	49.7	50.7	46.4
Ethanolamine phosphoglycerides	21.2	17.5	18.9	4.6
Serine phosphoglycerides	10.8	9.6	8.4	5.5
Cerebrosides	9.2	10.7	13.3	14.8
Sphingomyelin	3.3	2.3	1.7	1.7
Ceramide	2.4	8.8	5.5	22.7

[a]Dose incorporated = $\frac{\text{Radioactivity in total lipids per g wet wt}}{\text{Dose injected } (\mu\text{Ci})} \times 100$

measurable octanoate radioactive peak on radioanalysis (GLC) and, therefore, it was assumed that the original total lipid extract did not contain any appreciable amounts of this injected material. Another criterion used to estimate contamination of total lipids from starting material was the total amount of radioactivity recovered in various lipid components after chromatography. This ranged from 93-95% of the total lipid radioactivity applied to the GLC plate. Fractionation was achieved by a combination of silica gel column and thin layer chromatography and radioactivity determined as described previously (12). Radio-gas liquid chromatography (GLC) was performed using a Packard GLC Model 824 in combination with a proportional counter Model 894 under conditions described before (10).

RESULTS

Table I shows the percent of the injected radioactivity incorporated into the brain total lipids per g of wet brain by the end of 1 hr. At this time interval, the radioactivity in brain lipid from injected leucine was highest, whereas it was lowest following injection of isoleucine. Radioactivity incorporation from β-hydroxybutyrate was closer to that from leucine and incorporation from injected octanoate was close to that from isoleucine. Since brain lipids consist largely of polar lipids (phospho + sphingolipids) and free sterols, the ratio of total radioactivity of polar lipids to that of free sterols per g wet brain was calculated from specific activity values determined experimentally. The ratio was higher following injection of isoleucine than leucine. This indicated that a large proportion of radioactivity from radioactive isoleucine was incorporated into fatty acids relative to free sterols and vice-versa when radioactive leucine was injected. The ratio following β-hydroxybutyrate injection was closer to that obtained after leucine injection, whereas the ratio after octanoate injection was closer to that after isoleucine injection.

The distribution of radioactivity is given separately for neutral (less polar) and polar lipids in Table I. The radioactivity distribution in various neutral lipid components, following injection of radioactive octanoate, is quite different from that obtained after injection of all the other labeled compounds. The free fatty acid component contained more than half of the total radioactivity. This free fatty acid fraction was collected, methylated and analyzed by radio-GLC (Table II). It was found that 50% of the radioactivity in this fraction was associated with the octadecenoate; palmitate had 35.4% and stearate contained 14% of the total radioactivity. The distribution of radioactivity in fatty acids from total polar lipids, on the other hand, showed that about 65% of the radioactivity was in palmitic acid, and the remaining 35% was about equally associated with 18:0 and 18:1. Since the radioactivity distribution between the carboxyl carbon and the total fatty acids (% RCA) has been used to ascertain

TABLE II

Percent Distribution of Radioactivity in the Free Fatty Acid Fraction and Fatty Acids from Total Polar Lipids of Brains of Rats Injected with [1-^{14}C] Octanoic Acid

Fatty acid	% of Total Radioactivity		% RCA[a]	
	Free fatty acid fraction	Fatty acid from total polar lipids	Free fatty acid fraction	Fatty acids from total polar lipids
16:0	35.4	64.8	13.6	12.6
18:0	14.0	18.7	20.7	18.5
18:1	50.6	16.6	---	---

[a]$\%RCA = \frac{\text{Radioactivity in -COOH}}{\text{Radioactivity in total fatty acid}} \times 100$

TABLE III

Percent Distribution of Radioactivity in Brain Fatty Acids of Total Polar Lipids 1 hr Following Intracranial Injection[a]

Fatty acid	[U-^{14}C] Leucine	[U-^{14}C] Isoleucine	[3-^{14}C]β Hydroxy butyrate	[1-^{14}C] Octanoic acid
14:0	4.0	--	2.7	---
15:0	---	9.0	---	---
16:0	64.4	47.9	58.9	64.8
16:1	---	---	4.7	---
17:0	---	10.7	---	---
18:0	19.8	17.3	25.2	18.7
18:1	11.9	15.0	8.4	16.6

[a]5000 to 7000 cpm were injected into the GLC column radio-GLC settings: 500 cpm to give full scale deflection.

the synthetic pathway, the individual fatty acids were isolated and decarboxylated. However, the % RCA values for fatty acids were the same irrespective of the source. The distribution of radioactivity in various polar lipid components (Table I) showed that choline phosphoglyceride was the most radioactive component irrespective of the tracer injected. Again [1-^{14}C]octanoate injection resulted in a distribution of radioactivity that was distinct from other intracranially administered compounds. Ceramide, for example, contained a rather high amount of radioactivity.

Table III shows the distribution of radioactivity in various fatty acids formed from injected labeled compounds. As before, 16:0 had the largest amount of radioactivity. Injection of isoleucine resulted in an appreciable amount of radioactivity in odd chain fatty acids, 15:0 and 17:0. No other injected radioactive compounds gave rise to radioactivity in the odd chain fatty acids. β-Hydroxybutyrate injection gave rise to radioactivity in palmitoleate, whereas [U-^{14}C]leucine was the only tracer that resulted in distinct radioactivity in the polyunsaturated fatty acids 20:4 and 22:4. In spite of several attempts with amounts up to 10,000 cpm injection, no radioactivity was noticed in the polyunsaturated fatty acid following injections of labeled isoleucine, β-hydroxybutyrate or octanoate.

DISCUSSION

β-Hydroxybutyrate has been shown to provide carbon for lipogenesis during the early stages of brain development (3). Intracerebral injections of labeled compounds, such as the ketogenic amino acids, leucine and isoleucine, as well as octanoic acid, a normal component of rat milk (9), resulted in radioactivity in both polar lipids and free sterols. This indicated that metabolic reactions related to catabolism of leucine, isoleucine and octanoate that provide acetyl CoA for lipogenesis can occur in the brain in situ. Earlier work by Patel and Balazs (13) indicated that systemically administered [U-^{14}C]leucine led to a low incorporation of radioactivity into brain lipids. They also indicated that the incorporation of radioactivity in the whole tissue reached a maximum at about 1 hr after injection, and the radioactivity in "intermediates of leucine catabolism" also reached a high radioactivity at about the same time.

This led us to sacrifice the animals 1 hr after injection. It is interesting to note that incorporation of radioactivity into brain total lipids from injected β-hydroxybutyrate (known to provide carbon for lipogenesis in suckling animals) was, in fact, slightly lower than incorporation from labeled leucine (Table I). It should be recalled that one molecule of CO_2 is lost during catabolism of leucine resulting in the formation of acetoacetate plus acetyl CoA and thus, not all the radioactive carbons from [U-^{14}C]leucine can be utilized for fatty acid synthesis (lipogenesis). Isoleucine is catabolized to form α-methyl β-hydroxy butyryl CoA which results in the formation of propionyl CoA plus acetyl CoA. Propionyl CoA can be a substrate for the synthesis of odd numbered fatty acids (14). Odd numbered fatty acids can also arise by α-oxidation of even numbered acids (15,16). Injection of [U-^{14}C]isoleucine resulted in appreciable amounts of 15:0 and 17:0 as shown in Table III, strongly suggesting that isoleucine was a source of carbon for odd chain fatty acids. The ratio of radioactivity of polar lipids to free sterols per g wet brain was higher after injection of [U-^{14}C]isoleucine, indicating a lower amount of ^{14}C in free sterols possibly due to the fact that neither HMG-CoA nor acetoacetate are formed directly by catabolic reactions of isoleucine. On the other hand, both β hydroxybutyrate and leucine produce HMG-CoA and acetoacetate which, in turn, are substrates for synthesis of free sterols. Octanoic acid injection resulted in a rather surprisingly high radioactivity in the free fatty acid fraction of brain total lipids as compared to when leucine, isoleucine or β hydroxybutyrate was injected. At first, we assumed that this might be unincorporated tracer itself. However, analysis of this fraction by radio-GLC indicated that there was no peak corresponding to radioactive octanoate, and instead the radioactivity was distributed between 18:1, 16:0 and 18:0 in the proportion of 50, 35 and 14% of the total radioactivity. Since this was not the unincorporated starting tracer material, we thought it might be an artifact formed from the total lipids during the work up. However, the radioactivity distribution in the total fatty acids obtained by methanolysis of polar lipids was not similar to the above distribution. When routes of biosynthesis were examined by way of radioactivity distribution between the carboxyl carbon in relation to the radioactivity of the whole molecule (%RCA), it was found to be identical in fatty acid from the two fractions (Table III). Thus, octanoate was activated and catabolized by β oxidation to acetyl CoA which, in turn, led to the synthesis of other fatty acids, which, for unknown reasons, were not incorporated extensively into complex lipids.

The developing brain needs a supply of precursors and some preformed compounds for rapid buildup of lipids. In earlier experiments (17), we found that 1 hr after intracerebral injection of [U-^{14}C]glucose, 0.75% of the injected ^{14}C was incorporated into brain total lipids in 15-day-old rats. The present work indicates that radioactivity from β-hydroxybutyrate and leucine incorporated into brain lipids was not lower than that from radioactive glucose. The intracerebral injection of a radioactive compound can only show metabolic reactions that the neural tissue is capable of performing, but availability of substrate in the circulating blood, transport from the blood into the brain, endogenous levels in the brain, turnover rate in the brain, etc., all play a specific role in the overall metabolism in vivo, and this will influence the observed percent of given dose incorporated into total lipids per g of wet brain. Since the penetration of leucine from the blood into the brain and its endogenous level in the brain is higher in the developing brain than in the adult brain (18), it seems that during development under normal conditions, the brain may have the potential to utilize sources other than glucose for providing a carbon source for lipogenesis.

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A Comparison of the Ganglioside Distributions of Fat Tissues in Various Animals by Two-Dimensional Thin Layer Chromatography

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ABSTRACT

The ganglioside distributions of various fat tissues from human, rabbit, rat, mouse, chicken and frog were compared with pig adipose gangliosides by two-dimensional thin layer chromatography. It was found that there is a remarkable species variation in ganglioside distribution, especially in the composition and relative concentration of complex gangliosides. Differing from pig adipose tissues, those of human, rabbit, rat, mouse, chicken, but not frog, contained GM3 as a most abundant ganglioside. The data for human, rabbit and chicken indicated a simple distribution of only NeuAc-type gangliosides, while those for rat and mouse indicated a rather complicated pattern containing both NeuAc- and NeuGc-type gangliosides. The ganglioside pattern of the frog fat body differed markedly from those of mammalian fat tissues because of the presence of three different, unusual monosialosylgangliosides as major components. In other respects, a substantial amount of disialosyl-gangliosides was commonly found in all animal fat tissues.

INTRODUCTION

In connection with the study on the glycolipid composition of pig adipose tissue (1), this paper is focused on the ganglioside composition of fat tissues in other species of animals. It will present comparative data of the ganglioside composition of fat tissues from human, pig, rabbit, rat, mouse, chicken and frog obtained by two-dimensional thin layer chromatography (TLC) using two solvent pairs.

MATERIALS AND METHODS

Fat Tissues

Fat tissues from individual animals used are as follows: abdominal subcutaneous fat, 28 g, of human (Japanese, female, 75 years old); loin adipose tissue of pig (Yorkshire-middle-type) (1); renal fat, 328 g, from two rabbits (New Zealand White, male, 12 months old); a mixture of epididymal and abdominal subcutaneous fats, 96 g, of two rats (Wistar, male, 26 months old); epididymal fat, 14 g, of mouse (C3H/He, male, 6 weeks old); renal fat, 140 g, of two chickens (White Leghorn, male, 12 months old); and frog fat body, 515 g (*Rana catesbeiana*).

In addition to the rat tissues described above, epididymal, abdominal and back subcutaneous fats (28 g, 30 g and 37 g, respectively) of 12 rats (Wistar, male, 8 weeks old) were used for the analysis of ganglioside variation in various fat tissues within one species.

Reference Substances

The following purified gangliosides from pig adipose tissue (1) were used for TLC as references: GM3 (NeuAc-type), GM3 (NeuGc-type), GM1 (NeuAc-type), GD1a (NeuAc-type), GD1a (containing both NeuAc and NeuGc in a single molecule), GT1 (NeuAc-type), sialosyl-paragloboside (NeuAc-type) (SPG), GlcNAc-(2 mol)-containing ganglioside (NeuAc-(Gal-GlcNAc)$_2$-Gal-Glc-ceramide) (2), fucose-containing gangliosides (fucosyl-GM1) (NeuAc-type and NeuGc-type). A monosialosylganglioside mixture which had been separated from pig adipose glycolipids by DEAE-Sephadex column chromatography was routinely used for two-dimensional TLC as a reference marker, Purified GM1 and a mixture of GD1a and GD1b from bovine brain and GM2 from human brain with Tay-Sacks disease were also used.

Gas Liquid Chromatography

The sialic acid and glucose contents of total glycolipid fractions were determined by gas liquid chromatography (GLC) according to Sweeley and Walker (3), using mannitol as an internal standard. The species of sialic acid also was analyzed according to Yu and Ledeen (4).

Preparation of Ganglioside Fraction from Fat Tissues

The total glycolipids were isolated by hot ethanol extraction from tissue defatted with acetone, as described previously (1). They were dissolved by sonication in a small amount of chloroform-methanol-water (3:7:1, v/v), at 45 C, and then left to stand overnight at 5 C. The insoluble materials containing most of the phospholipids and residual triglycerides were removed by centrifugation, and the clear supernatant was used as a sample of the total ganglioside mixture for the present study.

TABLE I

Amounts of Total Glycolipids(μmol of Glucose/100g of Fresh Tissue) and Gangliosides(μmol of Sialic Acid/100g of Fresh Tissue) from Fat Tissue in Various Animals

	Fat tissue	Amount of total glycolipids: μmol of glucose/100g of fresh tissue	Amount of gangliosides: μmol of sialic acid/100g of fresh tissue
Human	Subcutaneous fat	2.4	2.4
Rabbit	Renal fat	0.62	0.85
Rat	Mixture of epididymal and subcutaneous fats	1.5	1.6
Mouse	Epididymal fat	3.5	5.5
Chicken	Renal fat	1.3	1.6
Frog	Fat body	1.6	1.9
Pig[a]	Subcutaneous fat	1.2	1.5

[a]Previous data (1).

Identification of Gangliosides by Two-dimensional TLC with Two Solvent Pairs

The solvent systems used were: (A) chloroform-methanol-28% aqueous ammonia-water (60:40:3:6, v/v) developed twice in all cases, (B) n-propanol-28% aqueous ammonia-water (75:5:25, v/v) and (C) chloroform-methanol-water (60:40:9, v/v). Small-sized precoated silica gel plates (10 x 10 cm, E. Merck, Darmstadt, Cat. No. 5721) were used in all cases. A sample of the total gangliosides was applied on two silica gel plates (4-5 μg of lipid-bound sialic acid/plate) and separated into individual components by two-dimensional TLC with two solvent pairs of the above three solvent systems; that is, both plates with the same sample were developed simultaneously with the solvent (A) to the first direction, and then one was followed by (B), and the other was followed by (C), to the second direction. After each development the plates were dried over P_2O_5 for at least 2 hr *in vacuo,* and the final solvent was removed by blower. The gangliosides were made visible by spraying the plate with resorcinol reagent (5) and heating it at 120 C for several minutes. Several principal components of a sample on a pair of plates were identified with reference to standard gangliosides of known structure.

RESULTS AND DISCUSSION

Although the site of the fat tissue sample was not always the same with each animal, the crude glycolipid fractions ("protagon") obtained by hot ethanol extraction were around 0.05% (by weight) of the original fat tissues in all animals. These fractions were still contaminated with a considerable amount of phospholipids and residual neutral lipids; the clear supernatants obtained after exhaustively solubilizing them in chloroform-methanol-water (3:7:1, v/v), were therefore used as the sample of total gangliosides.

Both amounts of total glycolipids and gangliosides as determined by GLC analysis of glucose and sialic acid, respectively, were in the range of 1-2 μmoles of glucose or sialic acid/100 g of fresh tissue as shown in Table I. These values suggested that the amount of gangliosides exceeded that of neutral glycolipids in all fat tissues, and they were in good agreement with previous data of pig adipose. Only in the epididymal fat of mouse, both amounts were two or three times higher than those of others, and the amount of gangliosides was similar to that found in rabbit muscle (6) and other extraneural tissues (7,8). In human, rabbit, and chicken fat, most of the sialic acids in the total gangliosides were found in N-acetylated form, while rat, mouse, frog as well as pig fat contained N-glycolylneuraminic acid in rates of 25%, 11%, 7% and 9%, respectively, together with the N-acetylated form of the acid.

On a pair of two-dimensional thin layer chromatograms, most of the pig adipose gangliosides of known chemical structure could be identified by referring to their relative migration rates with the three solvent systems used, as indicated in Figure 1. The sample was applied at the lower right side of chromatograms a and b in Figure 1; these were developed upward, first, with solvent (A), and then to the left followed by (B) and (C), respectively. Each component was designated in the map of spots above chromatograms a and b. The chemical structure of several minor components, especially those having low Rf values, is still

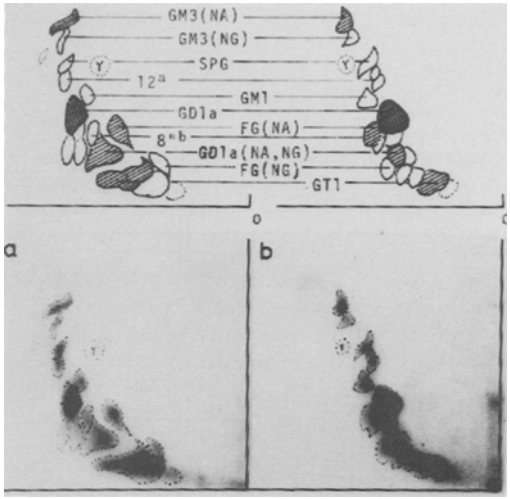

FIG. 1. Two-dimensional TLC with map of spots of total gangliosides from pig adipose tissue. The sample was applied at the lower right side of plates a and b. The same solvent (A) chloroform-methanol-28% aqueous ammonia-water (60:40:3:6) was used for the first development (upwards); then solvent (B) n-propanol-28% aqueous ammonia-water (75:5:25– and solvent (C) chloroform-methanol-water (60:40:9) for the second development (to the left). Note that by comparing the two chromatograms, the basic solvent (B) causes a greater change in the migration of the more acidic di- and trisialosylgangliosides than in that of the monosialosyl components. Abbreviations: GM3(NA), NeuAc-Gal-Clc-ceramide; GM3(NG), NeuGc-Gal-Glc-ceramide; SPG, NeuAc-Gal-ClcNAc-Gal-Clc-ceramide; GM1, Gal-GalNAc-(NeuAc)Gal-Glc-ceramide; GDla, NeuAc-Gal-GalNac-(NeuAc)Gal-Glc-ceramide; GDla(NA,NG), a mixture of NeuAc-Gal-GalNAc-(NeuGc)Gal-Glc-ceramide and NeuGc-Gal-GalNAc-(NeuAc)Gal-Glc-ceramide; FG(NA), Fuc-Gal-GalNAc-(NeuAc)Gal-Glc-ceramide; FG(NG), Fuc-Gal-Gal NAc-(NeuGc)Gal-Glc-ceramide; GT1, NeuAc-Gal-GalNAc-(NeuAc-NeuAc)Gal-Glc-ceramide; (a). Spot No. 12 was estimated as GD3, NeuAc-NeuAc-Gal-Glc-ceramide, on this figure; (b). Spot No. 8" is a GlcNAc-(two moles)-containing ganglioside, NeuAc-Gal-GlcNAc-Gal-GlcNAc-Gal-Glc-ceramide, with close migration to FG(NA).

unknown. Spot No. 12 seemed to be a disialosylganglioside GD3 judging from its chromatographic behavior. The spot designated as Y is negative with resorcinol reagent and is a globoside, a major neutral glycolipid in pig adipose.

The same chromatographic systems were routinely employed for separation and identification of adipose gangliosides in the other animals. A pair of chromatograms with a map of the spots for illustration in Figures 2 to 7 show ganglioside patterns in human, rabbit, rat, mouse, chicken and frog fat tissues. It was found in most animals except for the frog that GM3 ganglioside(s) was dominant, as in organs other than those of the central nervous systems

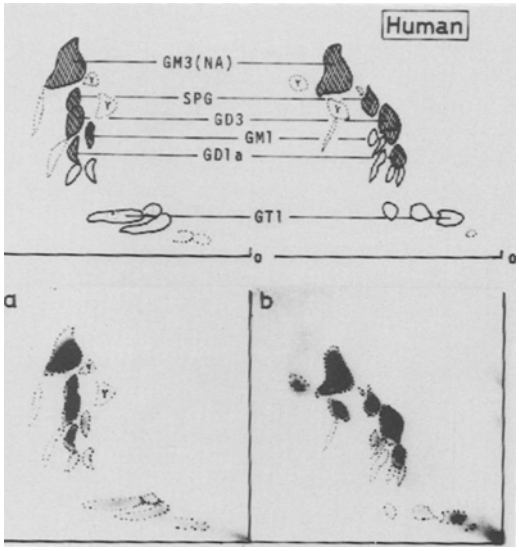

FIG. 2. Two-dimensional TLC with map of spots of total gangliosides from human adipose tissue. The development systems described in Figure 1 were used. Spots "Y" were neutral glycolipids, while all other spots even without designation were resorcinol-positive. See Figure 1 for abbreviations and comments.

(7,9,10), but there were marked species variations in the other small components among the different animals.

In human fat (Fig. 2), more than twelve components of gangliosides were observed. The major GM3 is clearly identified only with the NeuAc-type. The second major component should be a disialosylganglioside GD3 showing characteristically higher migration than that of GM1 in basic solvents (A) and (B). The third spot, which migrated between GM3 and GD3, is probably sialosylparagloboside because its migration is slightly lower than that of GM2 ganglioside (Fig. 2). The fourth one should be a GD1a ganglioside showing typical chromatographic behavior in both systems. GM1 ganglioside was also present in a small amount.

Rabbit gangliosides (Fig.3) seemed to be similar to those of human (Fig.2) and chicken (Fig.6); they contained NeuAc-type of GM3, sialosylparagloboside, disialosylgangliosides, GD3 and GDla, and smaller amounts of lower Rf components. In this case the amount of GDla exceeded that of GD3, contrary to the situation in human fat. A distinct unidentified spot just behind GDla was also found dominantly in chicken and will be discussed later (Fig.6). In contrast to the similarities of the simple ganglioside patterns of human, rabbit and also chicken, the distributions of neutral glycolipids (spots designated as Y) are quite different

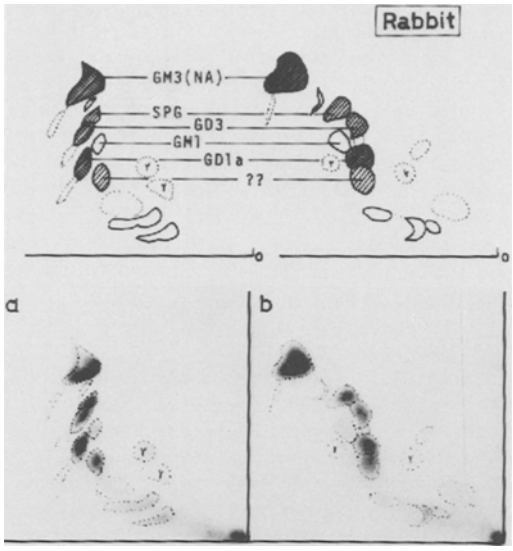

FIG. 3. Two-dimensional TLC with map of spots of total gangliosides from rabbit renal fat.

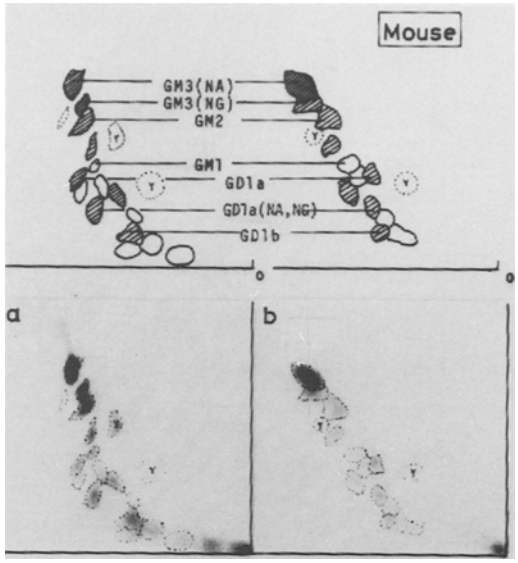

FIG. 5. Two-dimensional TLC with map of spots of total gangliosides from mouse epididymal fat pad.

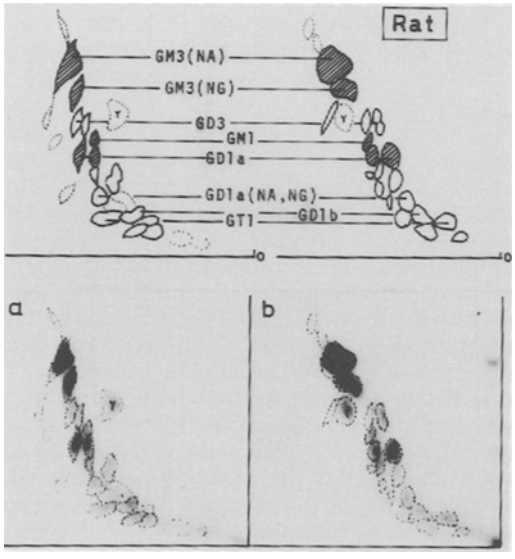

FIG. 4. Two-dimensional TLC with map of spots of total gangliosides from rat fat tissues.

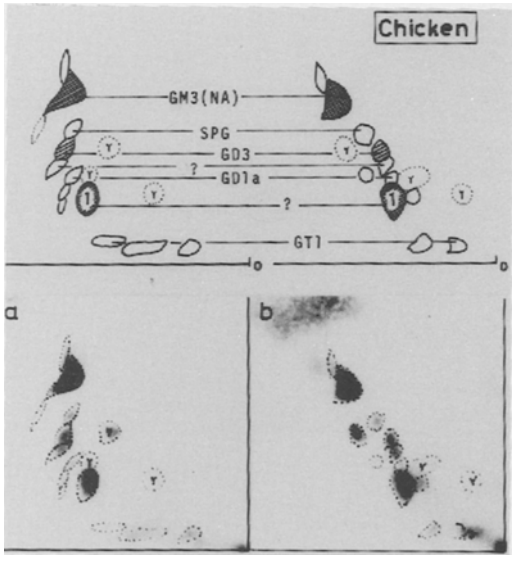

FIG. 6. Two-dimensional TLC with map of spots of total gangliosides from chicken renal fat. The development systems described in Figure 1 were used. The spots "Y" in chicken (not observed in frog) were negative with resorcinol reagent, but were examined further. All other spots even without designation were resorcinol-positive. See Figure 1 for abbreviations and comments.

from each other, suggesting that there is much more species variation such as that seen in erythrocyte membranes (11-14).

In both rat (Fig.4) and mouse (Fig.5), the ganglioside composition was found to be rather complicated, because of the presence of two species of sialic acids, NeuAc and NeuGc. It was determined in the rat that more than twenty species of gangliosides are widely distributed; these consist of GDla as a principal component in addition to a large amount of GM3 (both NeuAc- and NeuGc-type), and each series of mono-, di- and trisialosyl groups that were not precisely identified. Mouse gangliosides are similarly complicated, but in a slightly different

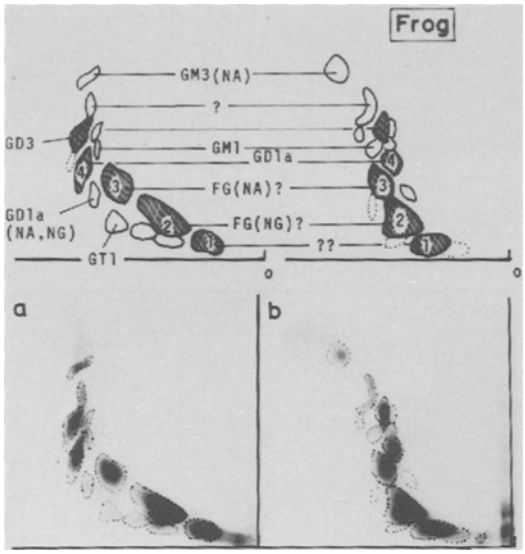

FIG. 7. Two-dimensional TLC with map of spots of total ganglsiosides from frog fat body.

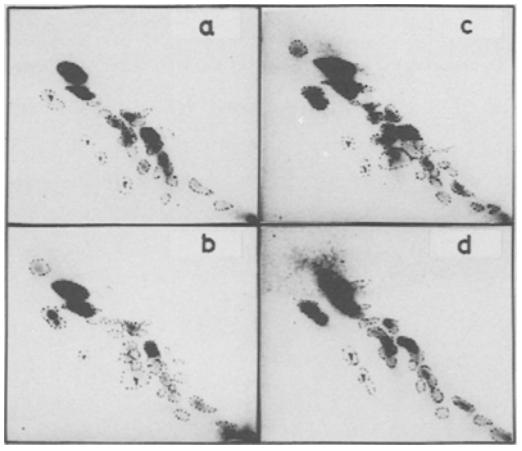

FIG. 8. Two-dimensional TLC of total gangliosides from separate fat tissues in rat. a) Epididymal fat, b) abdominal adipose, c) back adipose, d) same rat sample as shown in Figure 4. Samples were applied to silica gel plates and developed with solvent (A), followed by (C), by developing twice in each dimension. Better resolution of ganglioside species was obtained with this solvent system (cf. Fig. 4).

pattern. A distinct amount of GM2 gangliosides, besides GM3 and no GD3, were found in this case. The presence of GDla was common in the rat. Other components in the mouse could not be resolved clearly due to an insufficiency of the sample.

In chicken (Fig.6), a relatively simple ganglioside pattern was found, which was characterized by the presence of an intense spot of an unidentified component (marked 1 in the map) together with a principal GM3 (NeuAc-type) and about ten species of minor gangliosides. Spot 1 seemed to be a component similar to that present in rabbit gangliosides which has a slightly different migration from fucosyl-GM1 (NeuAc-type), running somewhat faster in the basic solvent (B) and more slowly in the neutral solvent (C) than fucosyl-GM1. Such a migration behavior is quite similar to that of a monosialosylganglioside containing heptasaccharide with a sialic acid residue at an external position (for example, the spot No.8" in Figure 1).

The frog was the only cold blooded animal used in the present experiment. As shown in Figure 7, its ganglioside pattern was strikingly different from those of the other animals. Along with the several major gangliosides, about ten spots of resorcinol-positive small components were clearly resolved. The presence of a certain amount of disialosylgangliosides, GD3 and GDla, and trace amounts of GM1 and GT1 are common to all the animals, but the amount of GM3 was smallest. It is quite interesting that frog fat bodies contain predominantly a series of unusual gangliosides (marked 1, 2 and 3 in the map) which were separated in a row over the low Rf region on thin layer chromatograms. According to their chromatographic behavior, it was suggested that they should be grouped into a monosialosylganglioside, and actually this was supported by DEAE-Sephadex column chromatography in further studies. The large components, 2 and 3, seemed to have mobilities similar to the NeuGc- and NeuAc-types of fucosyl-GM1, respectively, but their distributions were too broad and too large to be identified at present. The lowest one, 1, was the most characteristic component in the frog, and it is probably a novel ganglioside with no references. Studies on the precise chemical structures of these characteristic components 1, 2 and 3, are now in progress and will be reported in the near future.

From these findings, it was thus clearly defined that there are marked species variations in the ganglioside distribution of fat tissues from various animals, but a substantial amount of disialosylganglioside, both GDla and GD3, is commonly present in all animal fats. In addition, GM1, which has been demonstrated to be the receptor for cholera toxin in adipocytes (15,16), was always present in a constantly small amount in all animals except the chicken. Since GM1 was only a small spot on TLC even in the large preparation of whole fat tissue, it is conceivable that no GM1 could have been detected chemically in the isolated fat cells

(17). Recently, however, Pacuszka et al. indicated the presence of the native GM1 (both NeuAc- and NeuGc-types) in rat adipocyte preparations according to their sensitive method (18).

It was also realized from these results that the ganglioside pattern of pig adipose, which had previously been found, was quite exceptional in relation to those of other mammalian (human, rabbit, rat, and mouse) and bird (chicken) fat tissues, in which the most abundant gangliosides were GM3s, followed by other complex gangliosides with a large species variation. The species variation may not always be due to the presence of either acetyl or glycolyl groups in the sialic acid molecules, but may also depend on the presence of characteristic components of each animal. Especially, the presence of appreciable amounts of unusual gangliosides found individually in chicken and frog gave rise to large differences in ganglioside patterns among mammals, birds and amphibians.

Another possibility is that the variation of ganglioside patterns reported above is also due to different distribution sites of fat tissue. In this respect the intraspecies variability was checked by separately analyzing three fat tissues obtained from different sites in the rat; that is, epididymal fat, abdominal adipose and back adipose tissues. Figure 8 shows thin layer chromatograms of the ganglioside distribution from these rat tissues together with that of the rat sample used in Figure 4. The chromatography in this case was carried out with solvent system (A) to the first direction followed by (C) to the second direction, by developing twice each. The ganglioside patterns in the three fat tissues (Fig. 8, a, b and c) as well as in the previous sample (Fig. 8, d) were found to be almost similar, although some of the small spots were delicately different in regard to the position and relative intensity. It was now concluded that in the adipose gangliosides there is not so much variability within one species as among different species.

This investigation shows that two solvent pairs of two-dimensional TLC are useful for the separation and preliminary identification of gangliosides on a microscale (4-5 μg of sialic acid/spot), and such pairs of chromatograms provide much information about the possible structures of the carbohydrate moieties of the gangliosides, although the complete structure cannot be predicted.

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Effect of Marine Oil and Rapeseed Oil on Composition of Fatty Acids in Lipoprotein Triacylglycerols from Rat Blood Plasma and Liver Perfusate

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ABSTRACT

The fatty acid patterns of triacylglycerols (TG) from very low density lipoprotein (VLDL) in blood plasma and liver-perfusate from rats fed partially hydrogenated marine oil or rapeseed oil were determined. In the plasma from rats fed rapeseed oil for three days and three weeks, there was a small but significant decrease in the percentage of 22:1 fatty acid from 17.2 to 11.2% with length of feeding. In liver-perfusate, the comparable decrease with dietary rapeseed oil was from 18.5 to 5.2%, and with dietary marine oil from 13.4 to 8.0%. In contrast to the liver-perfusate, the remaining liver had only a very low 22:1 composition (ca 2%) independent of feeding period or diet. The results indicated that the liver exported the very long chain fatty acids and that an adaptation took place after three days feeding with rapeseed oil or marine oil. This adaptation in the liver could possibly explain why TG accumulation in hearts, which appears after three days' feeding with rapeseed oil or marine oil, disappears after an extended feeding period.

INTRODUCTION

Rats and several other animals when fed diets containing high levels of rapeseed oil rich in erucic acid *cis*-22:1(ω9), develop an acute accumulation of triacylglycerols (TG) in the heart and skeletal muscles (1-4). Similar effects are also found with partially hydrogenated marine oils (5,6). In the young rat, the fat content reaches a peak after three to seven days of feeding, after which it falls during the next four weeks of feeding to an almost normal value (7).

The TG accumulation in the heart has been ascribed to an inhibitory effect of 22:1 on the mitochondrial oxidation of other fatty acids and to the low rate of oxidation of 22:1 in the mitochondria (8-10). However, as oxidation of palmitoyl-carnitine by heart mitochondria from species less susceptible to lipidosis than the rat appear to be similarly inhibited by 22:1 acyl-carnitine (11), it seems unlikely that a decreased rate of β-oxidation in the heart is the sole determining factor in the accumulation.

The mechanism of the adaptation process is even more enigmatic. Studies with isolated heart mitochondria from rats fed rapeseed oil for several weeks have not shown any improved ability above control rats to oxidize erucic acid (12-14); neither is there any clue from available data on triacylglycerol lipase activity in heart to explain the adaptation (12,14,15).

Changes in the composition of the lipids reaching the heart by the blood-stream, especially with regard to 22:1 fatty acids, may be of importance to the lipid metabolism in this organ. Such changes would most likely be due to modifications in the liver 22:1 fatty acid metabolism and export. In the present work, therefore, the interests were mainly focused on the relative amounts of different fatty acids in the TG of very low density lipoproteins (VLDL–TG) of plasma and perfusate from liver as a function of dietary fat composition and feeding period.

MATERIALS AND METHODS

Animals and Diets

Male weanling rats of the Wistar strain were purchased from Møllegaard Laboratory, Denmark. After three to five days on a standard pellet diet, the animals were fed semisynethetic diets for three days or three weeks. The composition of the semisynthetic diet was, in weight % of total: sucrose, 20.0; corn starch, 37.5; casein (with 2% methionine), 20.0; cellulose, 1.0; vitamin mixture, 1.5; salt mixture, 5.0; diet oil, 15.0. The composition of the vitamin mixture was essentially after Methods of Analysis (16), and salt mixture after United States Pharmacopeia (17). The vitamins and salts were purchased from E. Merck AG, Darmstadt, GFR; Nutritional Biochemicals, ICN Pharmaceuticals, Inc., Cleveland, OH and Sigma Chemical Co., St. Louis, MO. The diet contained 30% of the calories as dietary oils. These were either partially hydrogenated marine oil (mostly from capelin; *Mallotus villosus*), rapeseed oil (from *Brassica varians*) or groundnut oil (i.e., peanut oil from *Arachis hypogaea*). The fatty acid compositions of these oils are described in Table I. The oils, including their analytical data, were obtained from DeNoFa and Lilleborg Co., Norway.

The animals were housed two and two in

metal cages, and were fed diet and water ad libitum. The climatic conditions were 23 C, 60% relative humidity, and 12 hr light period. Before the experiments, the animals were fasted for ca. 3 hr (from 0700 to 1000).

Chemicals

Chemicals of highest purity were used, mainly from E. Merck AG, Darmstadt, and Sigma Chemical Co., St. Louis, MO.

Blood Sampling

Samples (3-10 ml) were collected by aortic puncture from rats given a light ether anaesthesia. After 3 hr clotting at 0 C, the serum was separated at 800 g for 30 min.

Liver Perfusion

The liver was perfused essentially as described by Seglen (18) for an experimental period of 30 min. The animals were given a light ether anaesthesia before perfusion. The perfusion fluid consisted of 50-60 ml Krebs-Ringer bicarbonate buffer, pH 7.45, 0.1% glucose and 0.028% $CaCl_2$ and was continuosly gassed with 95% O_2 – 5% CO_2.

The perfusate was recirculated through the liver at a flow rate of 3 ml/min/g liver. Samples (0.5 ml) were taken (at intervals) during the perfusion for protein determination.

Isolation of VLDL and TG

VLDL fractions from serum or liver perfusate were separated by centrifugation of 5 ml samples for 18 hr at 4 C using 115,000 g. The layer of VLDL (d.= 1.006) floated on top of a separation layer of saline (d.= 1.006). Lipids were extracted with chloroform-methanol (2:1) after the method of Folch et al. (19).

The hearts were removed immediately after operation, chilled, minced, washed and extracted for lipids. The perfused liver was chilled immediately after perfusion, and ca. 1 g was used for lipid extraction. The lipids from heart and liver were extracted essentially as above after the modified method described by Christie (20).

Lipid extracts were separated by thin layer chromatography using 0.4 mm Silica Gel H. The solvent system was petroleum ether (60 C-70 C) – diethylether - acetic acid (113:20:1). Zones corresponding to free fatty acid (FFA) and TG fractions were scraped into glass-stoppered tubes and prepared for gas chromatography.

Gas chromatographic Analysis

Methylation of samples with FFA was performed with 2 ml methanolic HCl at 37 C for 10 min (21), while samples with TG were methylated with dimethoxypropane in addition and left at room temperature over night (22). The mixture was neutralized with 2% $NaHCO_3$ and extracted into hexane.

The gas liquid chromatography of the methyl esters was performed isothermally at 180 C in a Perkin-Elmer gas chromatograph model 3920, equipped with a six-foot stainless steel column packed with diethanolamine-stabilized DEGS 10%, on anachrom ABS 80-100 mesh, AW-DMCS, and a flame ionization detector. Nitrogen flow was 36 ml/min at the outlet. Injection port, column, and detector temperatures were 195, 185 and 215 C, respectively.

Identification of major peaks was made by comparison of retention times with standards from Supelco, Inc., Bellefonte, PA. The percentage of each methylated fatty acid was determined by means of triangulation.

Analytical Methods

Protein was determined by the method of Lowry et al. (22), total lipids according to Merckotest Gesamtlipide (24). Lactic dehydrogenase, to test the viability of perfused liver, was determined by Sigma diagnostic kit no. 500.

RESULTS

Serum TG and Free Fatty Acids (FFA)

The fatty acid composition of VLDL–TG from rats fed partially hydrogenated marine oil, rapeseed oil, or groundnut oil is shown in Table I. The fatty acid composition of the respective dietary oils is also presented. The acyl pattern of VLDL–TG from the control group (groundnut oil), both after three days and three weeks, was close to that of the dietary oil. Feeding with rapeseed oil or marine oil yielded a different picture. The 22:1 percentage of total fatty acids, which was high in the dietary rapeseed oil, was less than half that value in the serum VLDL–TG after three days' feeding. In the marine oil fed group, this difference was even greater. A compensation for this decrease was found as an increase in percentage of 16:0 and 18:1.

Throughout this investigation, a comparison was carried out of the fatty acid composition of serum, liver perfusate, liver and heart in rats fed for three days or three weeks on the various dietary oils. These feeding periods were chosen to reflect the time of maximal TG accumulation in the heart and the time for adaptation during which the elevated TG content of the heart is reduced.

TABLE I

Fatty Acid Composition of Serum Lipoprotein-Triacylglycerols (VLDL-TG) and Dietary Oils
% of Total Methylated Fatty Acids

	Rapeseed oil VLDL – TG			Partially hydr. marine oil VLDL – TG			Groundnut oil VLDL – TG		
Fatty acid[a]	3 days feeding (7)[b]	3 weeks feeding (9)	Dietary oil	3 days feeding (8)	3 weeks feeding (13)	Dietary oil[f]	3 days feeding (7)	3 weeks feeding (5)	Dietary oil
14:0	trace[c]	trace	0.7	3.6 ± 2.3	1.8 ± 1.6[x]	6.5	trace	trace	0.7
16:0	15.2 ± 5.6[d]	21.3 ± 3.8[xx e]	2.8	24.1 ± 4.3	26.4 ± 4.7	16.1	16.5 ± 4.2	21.3 ± 3.2[xx]	17.6
16:1	2.8 ± 0.7	1.6 ± 0.5[xxx]	0	10.6 ± 1.1	10.7 ± 2.9	6.9	1.1 ± 0.6	1.5 ± 0.7	0
18:0	2.0 ± 0.5	3.6 ± 1.1[xxx]	1.0	4.8 ± 1.1	4.4 ± 1.2	7.7	2.6 ± 0.9	3.8 ± 1.0[x]	2.8
18:1	35.4 ± 4.9	33.2 ± 2.2	16.3	42.2 ± 6.0	42.9 ± 5.1	12.8	37.4 ± 3.4	29.9 ± 4.7[xxx]	36.2
18:2	16.9 ± 1.7	16.7 ± 3.5	17.9	2.3 ± 3.0	3.0 ± 3.3	2.1	35.4 ± 3.6	32.7 ± 7.1	40.8
18:3, 20:0	4.3 ± 1.2	2.9 ± 1.3[xx]	9.2	1.6 ± 1.2	1.4 ± 0.9	4.9	0.5 ± 0.6	trace	0.7
20:1	5.5 ± 1.0	5.7 ± 2.9	0.5	6.0 ± 2.3	4.7 ± 2.6	11.5	0.9 ± 0.7	0.8 ± 1.2	1.1
20:4,22:0	trace	2.6 ± 2.0	0	trace	trace	5.0	4.2 ± 1.7	7.1 ± 2.1[xx]	0
22:1	17.2 ± 6.9	11.2 ± 4.8[x]	42.7	2.4 ± 1.8	3.0 ± 2.2	11.5	0	0	0

[a]Indicates number of carbon atoms: number of double bonds.
[b]Figures in parantheses are the numbers of animals per group.
[c]trace < 0.5%.
[d]Mean ± standard deviation.
[e]Significantly different from 3 days group; x=0.05> p> 0.025, xx=0.025> p> 0.01, xxx=0.01> p (t distribution).
[f]Partially hydr. marine oil contain as well; 5.1% 20:2 and 20:3 and 5.9% 22:2 and 22:3. 54.3% of the total fatty acid double bonds were *trans* isomers.

The 22:1 percentage of total fatty acids in serum VLDL–TG from the rapeseed oil fed group was significantly decreased from three days' to three weeks' feeding, and compensated by an increase of 16:0. Only minor differences with length of feeding time were found in the marine oil fed group. The fatty acid composition of the groundnut oil fed group indicated a decrease of 18:1 and 18:2, and an increase in composition of 16:0 and 20:4.

The composition of FFA in serum from rats fed different dietary oils was also measured (not shown). The distribution reflected to a great extent the same tendencies as described for fatty acids from VLDL–TG (Table I), but the difference in fatty acid composition between three days' and three weeks' feeding with rapeseed oil or marine oil was insignificant. The composition of 22:1 fatty acid in FFA after feeding with these diets was even lower than in serum VLDL–TG.

Triacylglycerols in Liver Perfusate

The data above suggested an adaptation to utilize 22:1 after three weeks' feeding with rapeseed oil in the diet. The decrease seen in serum TG was small, however, and since the composition of plasma TG reflects metabolic processes in the whole animal, i.e., secretion of TG from liver and intestine, uptake of TG by most organs and also catabolism in the plasma itself, it is evident that this system is complex. Since an important part of TG in plasma is secreted from the liver, we decided to study a simpler experimental model; i.e., the secretion of VLDL–TG into perfusate from the perfused rat liver.

We investigated the pattern: was the composition of fatty acids from VLDL–TG secreted after three days' feeding with various dietary oils different from those secreted after three weeks' feeding? The results are presented in Table II. The total amount of lipids secreted from the liver during 30 min perfusion was 0.17 mg/g liver (average number for all the dietary groups and feeding periods). The composition of fatty acids from the three days' fed group was surprisingly similar to the fatty acid composition of serum VLDL–TG after three days' feeding on the respective oil diets. An important exception may be noted after three days' marine oil feeding where the 22:1 content was even higher than the 22:1 content of the dietary oil; however, there was no 20:1 present.

After three weeks' feeding with rapeseed oil, there was a significant decrease in 22:1, and compensatory increase in lower fatty acids (18:1, 16:0). Thus, the data from serum VLDL–TG indicating an adaptation in fatty

TABLE II

Fatty Acid Composition of Liver Perfusate VLDL – TG
% of Total Methylated Fatty Acids[1]

Fatty acid[a]	Rapeseed oil		Partially hydr. marine oil		Groundnut oil	
	3 days (8)[b]	3 weeks (6)	3 days (5)	3 weeks (5)	3 days (5)	3 weeks (6)
14:0	trace[c]	trace	1.1 ± 2.5	4.0 ± 2.0[x]	trace	0.9 ± 1.3
16:0	14.5 ± 5.7[d]	24.0 ± 2.7[xxx e]	25.3 ± 2.0	29.3 ± 4.9	14.5 ± 3.7	26.8 ± 4.9[xxx]
16:1	2.3 ± 1.1	3.7 ± 1.1[xx]	7.9 ± 3.5	9.8 ± 2.7	1.2 ± 1.0	3.3 ± 1.4[xxx]
18:0	5.2 ± 2.7	4.4 ± 1.6	4.8 ± 2.5	5.7 ± 2.3	5.0 ± 2.3	5.9 ± 5.4
18:1	36.6 ± 7.9	45.2 ± 3.2[xxx]	38.9 ± 8.4	36.3 ± 6.6	36.4 ± 8.1	34.3 ± 8.5
18:2	13.4 ± 3.4	14.3 ± 1.3	6.4 ± 2.8	5.0 ± 2.4	28.5 ± 3.4	21.4 ± 5.6[xx]
18:3,20:0	2.4 ± 1.3	1.1 ± 1.3[x]	0	0	trace	trace
20:1	4.0 ± 2.9	0.6 ± 1.2[xxx]	0	1.9 ± 1.8	1.0 ± 2.1	0.5 ± 0.9
20:4,22:0	trace	trace	trace	0	6.2 ± 0.6	5.0 ± 4.0
22:1	18.5 ± 8.4	5.2 ± 4.3[xxx]	13.4 ± 7.5	8.0 ± 5.6	0	0

[1]Footnotes and Symbols as indicated in Table I.

TABLE III

Fatty Acid Composition of Liver Triacylglycerols
% of Total Methylated Fatty Acids[1]

Fatty acid[a]	Rapeseed oil		Partially hydr. marine oil		Groundnut oil	
	3 days (8)[b]	3 weeks (6)	3 days (5)	3 weeks (5)	3 days (6)	3 weeks (6)
14:0	trace[c]	0.6 ± 0.5	trace	0.8 ± 1.2	0	trace
16:0	22.2 ± 6.4[d]	23.2 ± 3.8	23.5 ± 8.1	30.8 ± 3.2[x e]	19.4 ± 4.7	20.0 ± 6.9
16:1	3.9 ± 1.1	3.7 ± 0.7	6.5 ± 3.2	7.3 ± 4.1	1.9 ± 2.2	1.5 ± 0.5
18:0	2.0 ± 0.7	2.7 ± 1.5	4.7 ± 2.7	5.1 ± 0.9	3.6 ± 0.7	3.1 ± 1.1
18:1	51.6 ± 4.1	51.9 ± 5.3	58.3 ± 7.2	51.1 ± 9.9	38.1 ± 2.4	43.5 ± 2.8[xxx]
18:2	11.5 ± 4.5	11.9 ± 2.5	4.3 ± 1.7	3.4 ± 2.8	30.8 ± 3.9	26.9 ± 6.2
18:3,20:0	2.1 ± 0.7	2.1 ± 0.9	1.4 ± 1.2	trace	trace	trace
20:1	2.3 ± 1.3	2.1 ± 1.0	trace	1.3 ± 2.0	trace	trace
20:4,22:0	trace	trace	0	trace	5.6 ± 2.7	3.7 ± 2.0
22:1	2.5 ± 1.7	1.4 ± 1.3	0.6 ± 1.4	1.8 ± 3.0	0	0

[1] Footnotes and Symbols as indicated in Table I.

acid metabolism were supported by the data from VLDL–TG in the liver perfusate. In the animals fed three weeks' on marine oil, there was a tendency to a lower percentage of 22:1, which was, however, not statistically significant.

Triacylglycerols in Liver and Heart

The fatty acid composition of TG in the liver remaining after perfusion is presented in Table III. The most striking point in these data is the small content of C_{20} and C_{22} fatty acids. This is very different from what was found in the liver perfusate (Table II). No statistically significant differences in fatty acid composition were found between the three days' and three weeks' fed dietary groups. The amounts of total lipids in liver are presented in Table IV. There was a statistically significant difference between the three days and three weeks rapeseed oil fed groups but no significant differences within the other dietary groups.

The composition of TG from hearts is presented in Table V. It may be noted that the accumulation of 22:1 after three days' feeding with rapeseed oil was extensive, and after three weeks' feeding there was a small but significant decrease. If it is also taken into account that the total lipid accumulation was considerable after three days on rapeseed oil (Table IV) followed by a decrease to about 1/5 after three weeks' feeding, the total difference in accumulation of 22:1 between three days and three weeks fed groups was even more striking. In the marine oil-fed group, there was a small decrease in 22:1 percentage in heart TG after three weeks' feeding, which was, however, not statistically significant (Table V). Likewise, there was a tendency to lipid accumulation in the heart after three days' feeding, followed by a decrease after three weeks' feeding (Table IV).

DISCUSSION

Heart receives its main fuel from serum TG and FFA. During the absorptive and early postabsorptive phases, the heart derives most of its energy from chylomicrons and VLDL.

Rat heart accumulates TG on a high fat diet rich in 22:1, perhaps due to an impaired oxidative capacity. After three to seven days, the amount of accumulated TG decreases, an indication that an adaptation may have taken place. The adaptation could either have taken place in the heart proper or in an organ which delivers fatty acids or TG to the heart. In this report, we have tested the latter hypothesis and have chosen liver as the most likely organ. We have tested rats in an early postabsorptive

TABLE IV

Content of Total Lipids in Heart and Liver
mg Lipids/g Tissue[1]

	Rapeseed oil		Partially hydr. marine oil		Groundnut oil	
	3 days (6)[b]	3 weeks (6)	3 days (6)	3 weeks (6)	3 days (7)	3 weeks (6)
Heart	148.5 ± 53.6[d]	31.9 ± 5.1[xxx e]	44.2 ± 15.8	29.6 ± 3.9[x]	36.0 ± 16.5	23.2 ± 7.6[x]
Liver	65.6 ± 32.2	34.5 ± 15.3[x]	40.3 ± 13.1	34.5 ± 12.0	46.6 ± 21.6	34.3 ± 16.7

[1]Footnotes and Symbols as indicated in Table I.

TABLE V

Fatty Acid Composition of Heart Triacylglycerols
% of Total Methylated Fatty Acids[1]

Fatty acid[a]	Rapeseed oil		Partially hydr. marine oil		Groundnut oil	
	3 days (8)[b]	3 weeks (6)	3 days (5)	3 weeks (5)	3 days (6)	3 weeks (6)
14:0	trace[c]	0.6 ± 0.4	1.2 ± 2.7	2.2 ± 3.0	trace	0.8 ± 0.9
16:0	7.2 ± 1.7[d]	10.3 ± 1.5[xxx e]	20.5 ± 5.5	23.0 ± 9.8	17.6 ± 4.0	21.8 ± 3.2[x]
16:1	0.6 ± 0.3	1.6 ± 0.7[xxx]	3.5 ± 1.6	5.7 ± 3.3	1.4 ± 0.9	2.4 ± 0.9[x]
18:0	3.2 ± 0.7	3.3 ± 0.6	11.6 ± 4.9	7.8 ± 2.2	7.4 ± 1.2	6.2 ± 1.4
18:1	31.4 ± 5.2	31.9 ± 3.8	33.7 ± 4.2	41.5 ± 7.8[x]	39.4 ± 5.5	41.3 ± 2.2
18:2	10.7 ± 1.4	13.0 ± 0.9[xxx]	8.0 ± 2.3	2.8 ± 1.4[xxx]	26.8 ± 3.2	24.8 ± 3.0
18:3,20:0	3.0 ± 0.7	3.1 ± 1.0	1.9 ± 1.7	2.0 ± 1.1	1.2 ± 1.0	trace
20:1	12.6 ± 1.6	11.0 ± 1.8	9.8 ± 5.5	9.6 ± 5.7	1.1 ± 1.0	trace
20:4,22:0	trace	trace	0.7 ± 0.9	trace	2.3 ± 0.5	2.0 ± 1.9
22:1	30.6 ± 4.1	24.8 ± 3.8[xxx]	7.3 ± 2.9	4.4 ± 3.2	0	0

[1]Footnotes and symbols as indicated in Table I.

phase. In this phase the chylomicron concentration in blood is probably low enough not to mask changes in the VLDL acyl pattern, and the liver VLDL production is high.

We explored the possibility of differences occuring in the acyl pattern of circulating TG (and FFA) when animals were given the same diet for three days and three weeks.

The plasma VLDL–TG contained substantially lower 22:1 than the diet, both after three days and three weeks on either rapeseed oil or partially hydrogenated marine oil, indicating differences in metabolism or handling of the different fatty acids of the dietary lipids.

In rats fed rapeseed oil, there was a decrease in 22:1 in the plasma VLDL–TG with the longer feeding time, indicating that possibly an adaptation to the excessive 22:1 had taken place. However, the change was barely significant, and in the animals fed marine oil, the 22:1 concentration after three days was so low that we could not expect much further decrease. No changes were seen in the marine oil-treated rats. In a similar study by Roquelin et al. (25), there was also a decrease in the 22:1 content of plasma-TG from that of dietary rapeseed oil; however, they could not demonstrate any adaptation.

As the acyl pattern of plasma-TG is a result of several processes, i.e., secretion from liver and intestine, and uptake in most organs of the animal, we decided to study the VLDL in a more simple experimental model. Perfusion of liver revealed that the composition of TG which were secreted varied much from three days' to three weeks' feeding on dietary oil with a decrease in 22:1 from 18.5 to 5.2%. Perfusate from rats fed marine oil showed the same tendency, although not statistically significant.

A possible explanation for the data is that increased chain shortening had occurred in the liver. Indications of a chain shortening of 22:1 taking place in various organs were first obtained by Craig and Beare (26). Ong et al. (27) used radioactively labelled erucic acid and showed that the liver took the most active part in the chain shortening of erucic acid, which was mainly converted to oleic acid. Recently, it has been demonstrated that isolated liver cells can metabolize erucic acid in a chain shortening process (28-30), especially after clofibrate feeding of the rats, a procedure which appeared to stimulate extramitochondrial conversion of erucic acid to shorter monoenic fatty acids (28, 29).

Although the pattern of fatty acids in TG exported from the liver changed from three days' to three weeks' feeding on dietary oils, the remaining liver tissue after perfusion indicated no differences. But a very low level of 22:1 indicated that the long chain fatty acids in the liver were either exported, chain shortened, or oxidized. Calculations from our data revealed that 0.25-0.5% of total lipids from the liver were secreted into the perfusate during the perfusion period, while more selectively ca. 2% of total 22:1 was secreted. This handling of long chain fatty acids in the liver may also explain why there was no significant increase in TG of liver after feeding with rapeseed oil (1,2).

The adaptation of liver metabolism after long term feeding with rapeseed oil or marine oil may offer an explanation of the observed changes in the heart. Our data indicated that the 22:1 percentage of total fatty acids in heart-TG after long term feeding with rapeseed oil was significantly decreased. This decrease was even higher when the level of total lipids in heart was taken into consideration.

Alterations in the ability of the heart to oxidize fatty acids after long term feeding with rapeseed oil or marine oil may have taken place. However, recent data by Clandenin (31) demonstrated a lowered capacity of isolated heart mitochondria from rats fed three weeks on rapeseed oil to oxidize substrates from the citric acid cycle, compared to the three days fed group. Forsyth et al. (6) found a slightly increased capacity to oxidize palmitoyl-CoA in heart mitochondria after feeding rats with herring oil for long periods of time. The effect of rapeseed oil feeding on the rat heart lipoprotein lipase activity was investigated by Jansen et al. (15). After three days the activity was stimulated but did not indicate adaptation after prolonged feeding of rapeseed oil. Hsu and Kummerow (14), however, demonstrated a slight adaptation after prolonged rapeseed oil feeding using trierucate or dietary corn oil as substract.

Thus, the adaptation taking place in the liver with respect to the metabolism of long chain fatty acids due to feeding with rapeseed oil or marine oil is the most striking observation which may offer an explanation to the adaptation taking place in the heart. We are presently pursuing this point and are studying the adaptation by incorporation of label from ^{14}C-erucic acid into shorter fatty acids in perfused liver from rats fed marine oil or rapeseed oil. The data reveal a significant increase in chain shortening capacity with partially hydrogenated marine oil as well as rapeseed oil (32).

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COMMUNICATIONS

Isolated Brain Capillary Endothelia: Influence of Various Levels of Essential Fatty Acids on the Acyl Group Composition of Glycerophospholipids

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ABSTRACT

On day seven of gestation, Wistar rats were assigned to a high essential fatty acid (EFA), low EFA, or a fat free diet. The same diets were continued during lactation. On weaning, the offspring were fed the same diets as their mother. Rats were killed at 222 days, brain capillary endothelia isolated, and total lipids extracted from the purified capillaries. The composition of the constituent fatty acids of ethanolamine glycerophospholipid (EGP), choline glycerophospholipid (CGP), and the alk-1-enyl EGP composition from each diet is reported. A decrease in dietary EFA led to reduced proportions of total saturated acyl groups in EGP with no change observed in the total saturated acyl groups from CGP, and an increase in monoenoic fatty acids, particularly 18:1n-9 for each phospholipid class. The proportions of 20:4n-6 in alk-1-enyl EGP were reduced in fat-free fed animals. In addition, the relationship between 20:3n-9 and 20:4n-6 fatty acids in brain capillary endothelia were markedly increased with a reduction in dietary fat. Low EFA and fat deficient animals showed a tendency to sequester 22:6n-3.

INTRODUCTION

The importance of the endothelial cells of cerebral capillaries to the transport properties of the blood brain barrier (BBB) has been well established (1-3). As a result of tight junctions between the plasma membranes of adjacent cells, a continuous membranous barrier between blood and brain interstitium is formed. The flux of circulating substances through this barrier occurs either through the lipid matrix of the plasma membrane bilayer or by membrane-associated mediated transport (4). Independent membrane-bound transport systems for hexoses, monocarboxylic acids, amino acids, amines, purines, and nucleosides have been identified in brain (3). It has been shown that the activity of membrane transport enzymes can be altered by changes in the acyl group composition of phospholipids (5). The importance of membrane lipids, specifically the fatty acyl groups of membrane phospholipids, to the optimal functioning of BBB transport systems has not been determined.

In the present study, changes in the acyl group composition of the major phospholipids in brain endothelial cell membranes were correlated to changes in dietary lipid containing various levels of essential fatty acids (EFA).

EXPERIMENTAL PROCEDURES

Pregnant rats of the Wistar strain in the 7th day of gestation were fed a semisynthetic diet containing one of the following levels of essential fatty acids (EFA): high EFA, (10% corn oil, 12.0 cal % as linoleate); low EFA (1% corn oil, 1.2 cal % as linoleate); or a fat-free diet. The semisynthetic diets contained 20% casein, 4% α cellulose, and 4% Jones-Foster salt mix. Dextrin was adjusted from 62% at 10% corn oil, 71% at 1% corn oil, to 72% in the fat-free diet. The vitamin mix was the same as described previously (6). All animals were fed ad libitum and food consumption measured every other day. The average daily calorie intake was essentially the same for each group. Animals were housed individually in polypropylene "shoe box" cages. Room temperature was maintained at 75 C and humidity at 50%. A diurnal light cycle of 12 hr was used throughout this experiment. Each litter was adjusted to nine pups within 36 hr of parturition, and the

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TABLE I

Composition of the Constituent Fatty Acids (WT%) of Ethanolamine Glycerophospholipids (EGP) and Choline Glycerophospholipids (CGP) in Brain Capillary Endothelia of Rats Fed Various Levels of Essential Fatty Acids.[a]

	Alk-1-enyl EGP			Diacyl EGP[b]			Diacyl CGP[c]		
	diet[d]			diet			diet		
Acyl groups	High EFA	Low EFA	Fat free	High EFA	Low EFA	Fat free	High EFA	Low EFA	Fat free
14:0[c]	0.7	1.1	---	3.6	3.2	1.4	0.5	0.6	0.4
16:0	7.7	8.1	4.1	29.4	21.3	15.4	33.7	37.5	33.4
16:1	0.4	tr[e]	0.5	3.3	3.3	3.0	0.8	1.5	1.6
18:0	9.8	4.1	3.5	20.9	26.8	24.6	15.5	15.9	15.2
18:1n-9	13.6	15.1	19.3	11.2	16.9	17.8	21.7	26.9	28.5
18:2n-6	5.0	3.3	1.0	5.2	3.3	1.8	10.4	2.9	1.5
20:1n-9	1.7	3.9	3.1	0.3	1.4	0.5	1.1	1.6	1.3
20:3n-9	0.8	2.8	7.5	0.5	0.3	2.5	tr	0.3	1.5
20:4n-6	47.7	49.5	38.2	18.4	16.4	18.2	13.2	9.7	10.9
22:4n-6	4.6	4.2	4.0	1.9	tr	2.9	1.5	1.4	1.4
22:6n-3	6.2	6.2	14.5	5.2	4.7	11.0	tr	tr	3.2
Total saturated:	18.2	13.3	7.6	53.9	51.3	41.4	49.7	54.0	49.0
Total monounsaturated:	15.7	19.0	22.9	14.8	21.6	21.3	23.6	30.0	31.4
Total polyunsaturated:	64.6	67.0	67.9	31.2	25.2	36.4	25.5	15.0	19.3
Ratio saturated/unsaturated:	0.23	0.16	0.08	1.17	1.09	0.72	1.00	1.20	0.96
Unsaturation index[f]	278	300	320	139	126	183	105	83	111
20:3n-9 |20:4n-6	0.02	0.06	0.20	0.03	0.02	0.14	---	0.03	0.14
n-9 |n-6	0.28	0.38	0.69	0.47	0.92	0.91	0.89	1.96	2.14

[a]Acyl group analysis in duplicate on capillaries isolated from seven brains pooled on killing of animals (see text).

[b]Includes a small amount of alkyl acyl.

[c]Traces of 20:2, 20:3n-6, 20:5n-3, 22:5n-6, and 22:5n-3 were also present in all lipid classes listed.

[d]Diet, calorie % as essential fatty acid.

[e]tr = trace.

[f]Unsaturation index is defined as Σ (number of double bonds in each fatty acid) x (mol % of each fatty acid).

dams continued on the diets fed during gestation. Pups were weaned at 21 days, housed individually, and placed on the same diet as their mother.

Brain capillary endothelia were isolated from rats of both sexes at 222 days of age as described previously (7). Total lipids were extracted from purified capillaries (8) and the individual phospholipids separated by separation-reaction-separation thin layer chromatography by the procedure of Horrocks (9). Methanolysis of ethanolamine glycerophospholipids (EGP) and choline glycerophospholipids (CGP), purification of the methyl esters, and analysis by gas liquid chromatography (GLC) were done as previously described (10). Peak areas were determined with a Hewlett-Packard 3380A integrator. The percent relative deviation between duplicate samples for all analyses ranged from 0-5.6% for major components (>5% of sample) and 0-8.0% for minor components (<5% of sample). Peaks were identified by comparison of relative retention times with standards PUFA No. 1 and PUFA No. 2 (Supelco Inc., Bellefonte, PA), standard methyl esters prepared from beef testes (11), and by determination of equivalent chain length according to Hofstetter et al. (12).

RESULTS AND DISCUSSION

The acyl group composition of the major phospholipid classes (EGP and CGP) for the capillary endothelia preparations from high EFA, low EFA, and fat-free animals are presented in Table I. In general a decrease in dietary fat led to decreased proportions of total saturated acyl groups in EGP with essentially no change observed in the proportions of total saturated acyl groups of CGP. A decrease in dietary fat led to an increase in levels of monoenoic fatty acids, particularly the levels of 18:1n-9 for each phospholipid class. The total polyunsaturated fatty acids from EGP generally increased with decreasing levels of dietary fat. This higher degree of unsaturation is caused mainly by increases in 22:6n-3 and is reflected in the higher unsaturation index (U.I.) and lower ratio of saturated to unsaturated fatty acids (SFA/UFA). Choline glycerophospholipids, on the other hand, showed little differences in polyunsaturated fatty acids in response to reduced levels of dietary fat. There was no change in either the U.I. or the ratio of SFA:UFA. However, there was a tendency for the fat deficient animals to sequester 22:6n-3 in diacyl CGP. It has been shown that the level of 22:6n-3 in brain is maintained during nutritional studies in which little or no linolenic acid occurs in the diet or during subsequent supplementation (13-15). That a mechanism exists in order to maintain a constant percent of highly unsaturated pentaenes and hexaenes has been suggested (15). A biological requirement may exist for a minimal percent of fatty acids with double bonds in the Δ 4, 7, 10, 13, 16 positions (or any combination of these), as these double bonds are common to both 22:5n-6 and 22:6n-3. Trapp and Bernsohn (15) have pointed out that the position of the double bonds appear to play a role in the replacement of 20:4n-6 by 20:3n-9 in EFA deficiency with both acids having Δ 5, 8, 11 bonds.

The acyl group composition of EGP from the present study differs from that reported previously (7). Notably, EGP from high and low EFA endothelia are much higher in 16:0 and 18:0 and lower in 18:1, 20:1, and 22:6. This discrepancy can be explained on the basis of differences in dietary fatty acids. In agreement with the results of the present study, a high percentage of corn oil in the diet has been shown to significantly increase the concentration of saturated fatty acids in tissue lipids (16). Furthermore, decreased amounts of monoenoic fatty acids in livers of rats fed high levels of linoleic acid has been reported (17,18). The results of our previous study (7) were obtained from animals fed a commercial laboratory chow containing beef tallow as the primary fat source (personal communication with H.L. Schilt, Ralston Purina Co.). The major fatty acids in beef tallow are 16:0 (26%), 18:0 (17%), and 18:1 (43%). The interrelationship of dietary fatty acids and endothelia lipids observed in our findings agrees with the suggestion of competitive inhibition of Rahm and Holman (18).

That acyl groups in EGP underwent greater changes than those in CGP, especially those in alk-1-enyl EGP is possibly an indication that alk-1-enyl EGP is more metabolically active than diacyl EGP or diacyl CGP, a response that has been noted previously (10). In a study of brain fatty acids during EFA deficiency in the mouse, Sun et al. (19) concluded that alk-1-enyl acyl EGP was more metabolically active than previously supposed. Moreover, Trewhella and Collins (20) reported that 1-stearoyl-2-arachidonyl CGP had the slowest rates of turnover in rat liver during EFA deficiency.

Exposure of brain capillary endothelia to a diet deficient in fat resulted in a marked reduction of 20:4n-6 in alk-1-enyl EGP. Furthermore, a ten-fold increase in the 20:3n-9:20:4n-6 ratio was observed in alk-1-enyl EGP, with a five-fold increase observed in

diacyl EGP and CGP (Table I). The ratio of trienoic to tetraenoic acids in tissue lipids, which is an indication of the degree of replacement of polyunsaturated derivatives of linoleic acid, increases during EFA deficiency (21). The effects of EFA deficiency on the central nervous system indicate that brain is affected by the levels of dietary EFA, but that the effects are much less pronounced than those observed in other tissues, for example, liver (13). Several investigators have also shown a wide range of values for triene/tetraene ratios between brain subcellular fractions from rats subjected to a deficiency in EFA (19,22,23).

The ratio of n-9/n-6 was more reflective of a response to dietary EFA. This was due largely to the aforementioned increases in 18:1n-9 fatty acids. Although the physical properties of phospholipids and their manner of interaction within the microenvironment of the lipid bilayer are influenced by all constituent acyl groups, the exchange of monounsaturated fatty acids may be of greater significance to membrane properties (24). Studies with model systems have shown that the introduction of the first double bond, as opposed to the addition of subsequent double bonds, resulted in the most significant changes in bilayer properties (25).

It seems that either dietary or toxicant (26,27) induced alterations in EFA could have some effect on the permeability of brain capillaries affecting vital transport processes of BBB. The functional consequences on this regulatory system remain unknown.

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Correlation in the Proportion of Oleic to Vaccenic Acid of Plasma Phospholipids with the Early Stages of Hepatoma 7288CTC Growth

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ABSTRACT

The major octadecenoate isomers, oleate (Δ9) and vaccenate (Δ11), were measured in the plasma phospholipids of rats bearing hepatoma 7288CTC as the tumor developed. The percentage of vaccenate decreased from 45% of the octadecenoate fraction at day zero to 25% by the 15th day. A significant decrease (45% to 35%) in the percentage of vaccenate occurred by the sixth day, well in advance of detectable tumor growth. The percentage of vaccenate continued to decrease as a function of time until day 15, after which it remained constant. Detection of alterations in plasma phospholipids at an early stage of tumor development in rats suggests that experiments should be carried out to determine if the same effects occur in humans.

INTRODUCTION

Previous studies from this laboratory have shown that the percentage of vaccenate (Δ11) and oleate (Δ9) isomers of octadecenoate is characteristic of the lipid class from which they are isolated (1-3). Neutral lipid octadecenoate from liver contains small amounts of vaccenate (25%) and high amounts of oleate (75%). The phospholipids from liver, on the other hand, contain at least 50% vaccenate. Thirty days after implantation of hepatoma 7288CTC, the percentage of vaccenate in the phospholipid fraction of the host liver drops to 25% with a corresponding increase in oleate. The percentages of vaccenate and oleate in the neutral lipid of the host liver are not altered by the hepatoma. These results suggest that the hepatoma induces the change in the percentage of vaccenate in the host liver phospholipids. The present investigation was prompted by these results to determine if the octadecenoate composition of phospholipids changes as a result of hepatoma growth. A time course after transplantation showed that the percentage of vaccenate in the plasma phospholipids significantly decreases early in the growth of the hepatoma.

METHODS

Animals

Groups of four male Buffalo rats (175-225 g) were anesthesized with ether and implanted bilaterally with Morris hepatoma 7288CTC (0.4 ml of homogenate in sucrose, 0.25 M; 1:1, w/v). A second group of rats was injected bilaterally with 0.4 ml of sucrose to serve as sham-operated control rats. The blood from one group of the transplanted animals was drawn immediately after implantation to serve as the zero time controls. The remainder were returned to their cages and fed a chow diet. Groups of animals were killed at three day intervals until

TABLE I

Effect of Implantation of Hepatoma 7288CTC on the Double Bond Position of Octadecenoates from Phospholipids of Blood Plasma[a]

Days after implantation	Number of animals	Percentage Δ9	Percentage Δ11	Ratio Δ11/Δ9
0	6	55 ± 3	45 ± 3	0.82 ± 0.09
3	4	59 ± 12	41 ± 12	0.76 ± 0.39
6	4	65 ± 2[b]	35 ± 2[b]	0.53 ± 0.04[b]
6-sham	3	54 ± 5	46 ± 5	0.86 ± 0.18
9	2	66	34	0.50
12	3	72 ± 4[b]	28 ± 4[b]	0.39 ± 0.09[b]
15	3	74 ± 4[b]	26 ± 4[b]	0.36 ± 0.07[b]

[a]At three day intervals following hepatoma implantation or sham operations, the percentages of Δ9 and Δ11 isomers of octadecenoates were determined on the plasma from these animals. The values are means ± S.D. when the number of animals is larger than two. At day nine the value is the mean of the two animals.

[b]$P < 0.001$ compared to zero day control.

30 days had elapsed. Blood was drawn into a heparinized syringe from the inferior vena cava of anesthesized animals, centrifuged, and the plasma stored at -76 C until analyzed. Both the sham-operated and hepatoma-injected rats had the same growth rates for at least the first 15 days after injection.

Lipid Analysis

The plasma was thawed and 1 ml was pipetted into a glass tube. The tube was heated in a steam bath until the protein was denatured. Total lipids were extracted twice by the Bligh and Dyer procedure (4) and fractionated into neutral and phospholipids (5). Fatty acid methyl esters were prepared from the total phospholipid fraction by transesterification in 2% H_2SO_4 in CH_3OH as previously described (6). The methyl esters of the phospholipids were chromatographed on a 33M stainless steel capillary column (0.25 mm I.D.) coated with diethylene glycol succinate for determination of the position of the double bond in the octadecenoate fraction. It was established by ozonolysis that oleate and vaccenate were the major octadecenoate isomers in the plasma phospholipids.

RESULTS AND DISCUSSION

At the initial or zero time, plasma phospholipid octadecenoates contain 55% oleate and 45% vaccenate (Table I). The earliest time period (three days) showed a change toward a decrease in the percentage of vaccenate which became highly significant ($P < 0.001$) by the sixth day. The sham controls showed that the change at day six was due to the hepatoma and not to transplantation procedures, different weight gains or variation in the lipid analysis. The percentage decrease in plasma phospholipid vaccenate reached a low by the 15th day and remained at this level through the 30th day. Therefore, early phases of hepatoma growth can be correlated with the decrease in the percentage of vaccenate. This change might have a potential diagnostic value because it can be measured on a small blood sample and because it occurs very early in the growth of the hepatoma, even before the hepatoma can be palpated and before it can be easily found by dissection. It is unlikely that the observed alterations in octadecenoate isomers could be due to the nutritional state of the animals. Wood et al. (3) showed that the quantity of octadecenoate could be changed by extremes in dietary fats, but the ratio of vaccenate to oleate retained the characteristic ratio of the lipid class from which they were isolated (3). Thus, the diet or the nutritional state of the animal would have little effect on the ratio of octadecenoate isomers and could not explain the alteration that we have documented.

The significance of the mechanism maintaining the ratio of vaccenate to oleate in normal tissue and the change induced by the hepatoma are unclear. The early alteration in the vaccenate to oleate ratios which precedes significant tumor growth may represent a condition that precedes tumor cell proliferation. Carcinogens may also induce a precancerous condition which decreases the percentage of vaccenate in phospholipids. This hypothesis is supported by the fact that the characteristic vaccenate to oleate ratios of liver phospholipids are altered to resemble hepatoma tissue by feeding *Sterculia foetida* oil (0.5% of the diet) (7), a reported carcinogen and cocarcinogen (8-10).

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Lactobacillic and Methyl-Branched Olefinic Acids in *Byrsocarpus coccineus* Seed Oil

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ABSTRACT

A detailed investigation of the seed oil of *Byrsocarpus coccineus* Schum. and Thonn. has disclosed *cis*-11,12-methyleneoctadecanoic (lactobacillic) (13%) and two branched octadecenoic acids (0.1%). Other fatty acids in the oil are those normally associated with seed lipids except for an unusually high proportion (12%) of *cis*-11-octadecenoic acid. Lactobacillic acid has long been known as a constituent of certain bacterial lipids, but this is the first report of its presence in a seed oil. The branched olefinic acids have not heretofore been found to occur in plants.

INTRODUCTION

Byrsocarpus is a genus of tropical shrubs and small trees in the family Connaraceae. *B. coccineus* is widely distributed throughout central Africa; the sample investigated here was collected in Ghana. It is a straggling or scandent shrub attaining a height to 3 m and bearing oblong seeds about 1.5 cm long and 0.8 cm in diameter. Isolation and characterization of the fatty acids in the seed oil were undertaken when preliminary data suggested that a cyclopropane fatty acid might be present. This type of acid (unknown in the Connaraceae) occurs so infrequently in seeds that further studies of the oil were warranted. As a result, lactobacillic and two seemingly related branched, olefinic fatty acids were disclosed for the first time in plants.

EXPERIMENTAL PROCEDURES

Oil was extracted from the ground seed with petroleum ether, and the preliminary analytical tests were conducted as previously described (1,2). Methyl esters were prepared by acid-catalyzed transesterification procedures with 10% BF_3 in MeOH used for small scale (<200 mg) preparations and 5% HCl in MeOH for the large scale one. Equivalent chain length (ECL) values and quantitation of the methyl esters were obtained by gas chromatography (GC) on two columns (Apiezon L and LAC-2-R 446) (3,4).

The gas chromatography-mass spectrometry (GC-MS) and computerized data acquisition system (5) utilized a 122 x 0.2 cm glass column packed with 3% OV-1 on Gas Chrom Q (Applied Science Laboratories, State College, PA). Temperature programming was used to optimize GC separations for some samples (e.g., ozonolysis products). Infrared (IR) spectra were obtained from CCl_4 solutions in 1 mm NaCl cells, and proton magnetic resonance (PMR) spectra from $CDCl_3$ solutions with a Varian HA-100 or a Brucker WH-90 spectrometer.

Microozonolysis and GC were used for double bond localization (6); GC-MS facilitated identification of certain ozonolysis products. Microhydrogenation was carried out in EtOH with 10% Pd-on-charcoal as the catalyst. The cyclopropane ring was located by GC-MS of the pyrrolidide (7,8) and methoxy (9) derivatives.

Esters were isolated by column chromatography on an $AgNO_3$-impregnated support followed by high pressure liquid chromatography (HPLC) in the reverse-phase mode. For the first $AgNO_3$ chromatography, 2.2 g of esters were applied to a 50 x 1.5 cm dry column packed with 30 g of 20% $AgNO_3$ on Hi-Flosil (Applied Science Labs.). Elution was with 320 ml of hexane followed by 140 ml of hexane-chloroform (50:50) and then 80 ml of hexane-ether (90:10). Fractions (20 ml) were collected and the progress of the chromatography was monitored by GC. After the hexane-ether (90:10) elution, the column was washed with 100 ml of ether. The column was equilibrated with 750 ml of isooctane; fractions 1 and 2 from the first chromatography were combined, reapplied to the column, and eluted with 200 ml of isooctane (3-ml fractions collected) followed by 240 ml of hexane-ether (50:50). Again, progress was monitored by GC. Separation was now complete enough that four fractions composed primarily of saturated, monoenoic, dienoic, and trienoic esters were ob-

[1]Mention of firm names or trade products does not imply endorsement or recommendation by the Department of Agriculture over other firms or similar products not mentioned.

tained.

A Waters ALC-201 HPLC instrument equipped with a 30 x 0.78 cm μ-Bondapak C_{18} column (Waters Associates, Milford, MA) and a differential refractometer was used to complete the isolation of individual esters. The solvent system was CH_3CN-H_2O (90:10).

RESULTS AND DISCUSSION

B. coccineus seeds weighed about 0.2 g each and contained 19% oil (dry basis). GC analysis of the methyl esters prepared from *B. coccineus* oil showed a peak with ECL values of 18.8 on Apiezon L and 19.5 on LAC-2-R 446, which are indicative of a 19-carbon fatty acid containing either a cyclopropane ring or a double bond (3,10). GC-MS confirmed this assumption giving an apparent molecular ion (M^+) at 310, together with the ions generally associated with long chain methyl esters (74,87,etc).

Isolation of Unknown Esters

The first chromatography on $AgNO_3$-impregnated support gave good separation between dienes and trienes but did not adequately resolve the monoenes from the saturated esters. Accordingly, the first two fractions, which contained the saturated plus a large portion of the monoeneoic esters, were combined, reapplied, and eluted with a less polar solvent (isooctane). This chromatography gave saturated fractions (fractions 14-50), a mixture of branched and normal monoenes (fractions 51-61) and the remaining normal monoenes (fractions 62-68). The cyclopropane esters (ECL = 18.8, 19.5) was isolated from the saturated fractions by HPLC, eluting between palmitate and stearate. The branched monoenes, which had eluted between the saturated esters and "normal" monoenes on the $AgNO_3$ column, had ECL values of 17.7 on Apiezon L and 18.2 on LAC-2-R 446. These compounds eluted after the 18:1 during HPLC. Detection of branched monoenes was made possible by fortuitous choices of solvent and fraction size for the second $AgNO_3$-chromatography, since they were present in very small amounts and had ECL values similar to the normal 18:1. They would have undoubtedly remained unnoticed in most other chromatographic systems.

These chromatographic techniques afforded highly purified (by GC) fractions of the following esters: 16:0, 18:0, 16:1, 18:1, 18:2, 18:3, cyclopropane (ECL = 18.8, 19.5) and branched monoenes (ECL = 17.7, 18.2).

Identification of Esters

The 16:0 and 18:0 were identified by GC retention and by GC-MS. The 16:1 was shown to be palmitoleate by ozonolysis. Ozonolysis of the 18:1 gave fragments arising from n-9 (9-carbon aldehyde [9A] and 9-carbon aldehyde-ester [9-AE]) and n-7 (7A and 11AE) monoenes in a ratio of 60:40. The 18:2 proved to be linoleate and the 18:3 linolenate by ozonolysis. The lack of absorption in the 900 to 1000 cm^{-1} region in any of the IR spectra of the normal esters showed that the unsaturation had *cis* geometry.

The cyclopropane ester gave a medium intensity band at 1025 cm^{-1} (IR) and broad multiplets at 0.6 ppm and -0.3 ppm relative to tetramethylsilane (PMR), which are consistent with a *cis*-cyclopropane structure (10). The IR and PMR spectra were otherwise normal for fatty esters, with no indications of olefinic protons (PMR). GC-MS of the pyrrolidide derivative gave diminished intensities for the C_{10}, C_{11}, and C_{12} cleavages, which indicate a functional group at the 11,12 positions (8). Treatment of cyclopropane esters with 50% BF_3 /MeOH gives six isomeric methoxy adducts that can be used to locate the cyclopropane group via GC-MS (9). In this present study, abundant ions at 129, 143, 157, 229, 243, and 257 as well as small amounts of M^+, $M\text{-}15^+$, and $M\text{-}31^+$ located the ring at the 11,12 positions. Therefore, the cyclopropanoic acid is *cis*-11,12-methyleneoctadecanoic or lactobaccilic acid.

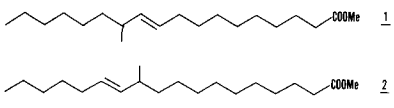
The branched monoenes gave a medium intensity band at 975 cm^{-1} (IR), a doublet centered at 0.93 ppm (J = 9Hz) with the normal terminal methyl multiplet at 0.88 ppm superimposed (PMR), and a multiplet centered at 5.3 ppm (PMR). Coupling constants at the latter resonance were not definitive, since coupling occurred not only between the olefinic protons but also with the methine proton on the adjacent branched carbon atom. Not enough sample was available for multiple resonance studies. Nonetheless, the IR and PMR spectra indicated branched, *trans*-olefinic esters. Ozonolysis yielded four fragments, two of which were easily identified as 6A and 10AE by GC retention and GC-MS. The other two fragments did not give ECL values of saturated, n-aldehydes or n-aldehyde-esters (6), but were identifiable by GC-MS as a 9-carbon, methyl-branched aldehyde and a 13-carbon, methyl-branched aldehyde-ester. The spectrum of the 9A had a base peak at 58 with the ion at 44 representing only 0.2%, a behavior known for α-methyl aldehydes (11). The 13AE gave ions at 214 (20%) and 211 (5%) representing $m\text{-}28^+$

TABLE I

Fatty Acid Profile of *Byrsocarpus coccineus* Seed Oil

Fatty acid	Area % by GC
12:0	0.2
14:0	0.7
14:1	0.2
15:0	trace
16:0	42
$16:1^{9}$	5.5
17:0	trace
17:1	0.2
18:0	3.0
$18:1^{9}$	17
$18:1^{11}$	12
$18:2^{9,12}$	2.4
$18:3^{9,12,15}$	1.0
20:0	0.2
20:1	0.3
22:0	0.3
Lactobacillic	13
11-me-$18:1^{12}$ / 12-me-$18:1^{10}$	0.1

and $M-31^+$, respectively, (12) but no ion at 199 for $M-43^+$ was evident. Instead, a major ion occurred at 185 (31%) which would result from β-cleavage ($M-57^+$) if the carbon α to the aldehyde group was methyl-branched (11). GC-MS of a hydrogenated sample gave spectra virtually identical to those reported by McCloskey and Law for a mixture of 11- and 12-methyl octadecanoates (13). Ozonolysis data (6) indicated that the two isomers, *1* and *2* below,



were present in equal amounts. Due to the extreme difficulty in separation of these compounds from stearate and oleate by GC of the mixed esters, their proportion of the fatty acid mixture must be approximated. Their combined total represents not more than 0.1% of the total.

The overall fatty acid composition of *B. coccineus* oil is given in Table I. To our knowledge, this is the first report of lactobacillic acid in a seed oil (14). The accompanying, branched, olefinic acids are an interesting biosynthetic anomaly. One can easily postulate pathways in which the branched olefins are precursors to, are products from, or are formed in conjunction with lactobacillic acid, since an unusually high proportion of *cis*-11-octadecenoic acid is available as a substrate.

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1,25-Dihydroxyvitamin D_3 Increases the Activity of the Intestinal Phosphatidylcholine Deacylation-Reacylation Cycle

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ABSTRACT

The activity of the intestinal phosphatidylcholine deacylation-reacylation cycle has been found to be stimulated by 1,25-dihydroxy-vitamin D_3. The stimulation of this cycle thus provides a possible mechanism for the reported retailoring of the fatty acid composition of phosphatidylcholine in intestinal cell membranes by 1,25-dihydroxy-vitamin D_3 and its analogue, 1α-hydroxyvitamin D_3.

INTRODUCTION

Since the demonstration that 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2$ D_3) is the principal metabolically active form of vitamin D_3 that is involved in calcium homeostasis (1), much interest has been focused on the effects and mechanism(s) of action of this hormonal form of vitamin D and its analogues at the target tissues. It has been demonstrated repeatedly that a major physiological effect of $1,25(OH)_2$ D_3 is to increase the active transport of calcium in the duodenum and upper jejunum of nearly all mammalian and avian species (1). In spite of these demonstrations and studies, the biochemical basis of the action of $1,25(OH)_2$ D_3 is unknown. Walling (2) observed a biphasic response of rat duodenal active calcium absorption following $1,25(OH)_2$ D_3 administration and has suggested that this can be explained by an acute response at 6 hr occurring in the villus cell and a later (24-48 hr) elevation of transport resulting from crypt cells that have subsequently migrated up the villi. Although it has recently been reported (3) that the administration of 1α-hydroxyvitamin D_3, an analogue of $1,25(OH)_2$ D_3, to chicks caused a change in the fatty acid composition of the phosphatidylcholine fraction of the intestinal microvillus membrane, no mechanism was suggested by which this could be achieved.

One established mechanism by which the fatty acid composition of membranes could be retailored is by the phosphoglyceride deacylation-reacylation cycle (4,5). The present report provides the first demonstration that $1,25(OH)_2$ D_3 can stimulate the activities of the enzymes involved in the phosphatidyl choline deacylaton-reacylation cycle in duodenal villus cells of rat intestine.

MATERIALS AND METHODS

Mixed acid 1-acyl-*sn*-1-[1-^{3}H] glycero-3-phosphocholine was prepared as described previously (6) and 1-palmitoyl-2-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine was prepared according to Lands and Merkl (7). 1,25-Dihydroxyvitamin D_3 was a gift from Dr. M. Uskokovic, Hoffman La-Roche, Nutley, NJ. Weanling male Holtzmann rats were fed the Vitamin D-deficient, low calcium diet of Suda et al. (8) for 3 weeks as previously described (9). They exhibited low serum calcium concentration and averaged 80±10 g of body weight at the time of the experiment. To determine the effect of the vitamin D metabolite on the deacylation-reacylation cycle, the animals received 650 pmol of $1,25(OH)_2$ D_3 intrajugularly in 0.05 ml ethanol. The control group received the vehicle alone. At 3 and 5 hr after dosing, the animals were sacrificed and isolated duodenal villus cells prepared by collagenase dispersion (10). Phospholipase A_2 [E.C. 3.1.1.4] was assayed in duodenal cell homogenates as described by Subbaiah and Ganguly (11), using 6 μmol of 1-palmitoyl-2-[1-^{14}C] oleoyl-*sn*-3-phosphocholine as substrate. Each incubation contained 1.5 mg of cell protein. The reactions were carried out for 60 min at 37 C. Lysophosphatidylcholine acyltransferase [E.C. 2.3.1.23] was assayed in cell homogenates as previously described (12). The incubations were terminated by addition of chloroform-methanol, 2:1, and total lipid extracts were prepared according to the method of Folch et al. (13). The phospholipids were resolved into their various classes by thin layer chromatography using chloroform/methanol/glacial acetic acid/water (25:15:4:4, v/v/v/v) as the developing solvent, and the various lipid fractions were eluted from the silica gel as previously described (6). As radioactive acyl-CoAs were used in the acyltransferase assay, the radioactivity at the *sn*-2 position of the synthesized phosphatidylcholine was determined after phospholipase A_2 hydrolysis (14). Liquid scintillation spectrometry was carried out as described (6). The recovery of the tritium isotope was 92% and the recovery of the ^{14}C isotope was 90%. Protein was determined by

TABLE I

Effect of 1,25(OH)$_2$ D$_3$ on Phospholipase A$_2$ Activity In Rat Intestinal Cells

Experimental condition	Time (hr)	Lysophosphatidyl choline formed[a]	Phosphatidyl choline recovered[a]	Disappearance of total ester bonds[a]
Control	3	252 ± 28	5227 ± 548	228 ± 27
	5	263 ± 27	5348 ± 556	231 ± 24
1,25(OH)$_2$ D$_3$-Treated	3	646 ± 76	4483 ± 463	695 ± 81
	5	921 ± 88	3887 ± 379	993 ± 126

[a]pmol/hr/mg protein. There were five animals in each group. At each time point, the enzyme was assayed in isolated duodenal cells from each animal as described in Materials and Methods using 6 μmol of 1-palmitoyl-2-[1-^{14}C] oleoyl-*sn*-glycero-3-phosphocholine as substrate. Results are the mean ± SEM of five animals.

TABLE II

Lysophosphatidylcholine Acyltransferase Activity In Intestinal Cells from Control and 1,25-Dihydroxyvitamin D$_3$-Treated Rats.[a]

Fatty acyl incubated with 1-acyl-*sn*-[2-^{3}H] glycero-3-phosphocholine	Control		1,25-Dihydroxyvitamin D$_3$-treated	
	3 hr	5 hr	3 hr	5 hr
[1-^{14}C] Oleoyl CoA	882 ± 114	912 ± 102	2238 ± 216	3798 ± 426
[1-^{14}C] Linoleoyl CoA	1098 ± 96	1134 ± 116	3516 ± 384	4764 ± 576
[1-^{14}C] Arachidonyl CoA	1350 ± 144	1278 ± 174	4176 ± 438	5244 ± 546

[a]nmol/hr/mg protein. There were five animals in each group. At each time point, the enzyme was assayed in isolated duodenal cells from each animal as described in Materials and methods. Results are the mean ± SEM of five animals.

the method of Lowry et al. (15) using bovine serum albumin as standard.

RESULTS

The effect of 1,25(OH)$_2$ D$_3$ on phospholipase A$_2$ activity in homogenates of isolated intestinal duodenal cells is shown in Table I. Cell homogenates were chosen for the assay because it has been demonstrated (11) that the enzyme activity is distributed between the brush border, microsomal and cytosolic fractions. The results show that phospholipase A$_2$ activity was increased 2 to 3-fold at 3 hr after administration of 1,25(OH)$_2$ D$_3$, and this was increased to 4-fold at 5 hr. The second enzyme involved in phosphatidylcholine deacylation-reacylation is lysophosphatidylcholine acyltransferase (4,5). Table II shows the effect of 1,25(OH)$_2$ D$_3$ treatment on the activity of this enzyme. In the control experiments, the yield of phosphatidylcholine varied with the acyl CoA used in the assay with arachidonyl CoA being more effective than oleoyl CoA or linoleoyl CoA. 1,25(OH)$_2$ D$_3$ treatment resulted in a 3 to 4-fold stimulation of the enzyme activity without significant alteration of the acyl CoA specificity. These results indicate that 1,25(OH)$_2$ D$_3$ can stimulate the activity of the phosphatidylcholine deacylation-reacylation cycle in intestinal duodenal cells.

DISCUSSION

1,25(OH)$_2$ D$_3$ acts on intestine to stimulate calcium transport (1). Although its structure, biological synthesis (16) and certain features of its mechanism of action (1,17,18) closely resemble other steroid hormones, little is known about the biochemical basis of its mechanism(s) of action in intestinal cells. It had been demonstrated earlier (19,20) that actinomycin D did not prevent the stimulation of intestinal calcium transport by 1,25(OH)$_2$ D$_3$ in rat duodenum. Bikle et al. (21) recently reported similar findings with chick duodenal loops using both actinomycin D and cycloheximide. These authors suggested that the inability of either inhibitor to block 1,25(OH)$_2$ D$_3$-mediated calcium transport despite inhibition of calcium-binding protein production and alkaline phosphatase activity by cycloheximide, indicates that *de novo* RNA and protein synthesis, and, in particular, calcium binding protein and alkaline phosphatase are not required for the 1,25(OH)$_2$ D$_3$ stimulation of calcium transport.

One attractive alternative is a mechanism by which the fatty acid composition of the villus cell membranes could be retailored, thus modifying general properties of the membranes such as fluidity and permeability. This could be affected by increased activity of the phospho-

glyceride deacylation-reacylation cycle (4,5). The results presented above demonstrate that administration of $1,25(OH)_2$ D_3 can increase the activity of the intestinal phospholipase A_2, thus generating lysophosphatidylcholine which can then be reacylated by the acyltransferase reaction, which is also shown to be stimulated by $1,25(OH)_2$ D_3, to form a retailored molecular species of phosphatidylcholine. It is pertinent to point out that we have recently also suggested (22) that the detergent properties of lysophosphatidylcholine may provide the physical basis for a role by this monoacylphospholipid as a metabolic modulator in the intestinal cell.

Yorio and Bentley have recently shown (23) that aldosterone can increase phospholipase A activity in toad bladder and suggested that this was a mechanism by which transepithelial sodium transport was increased. They did not, however, investigate the activity of the acyltransferase reaction. Our present findings provide the first demonstration that $1,25(OH)_2$ D_3 can increase the activities of the enzymes involved in the phosphatidylcholine deacylation-reacylation cycle in intestinal cells. This retailoring of the fatty acid composition of membrane phospholipids by increasing the activity of the deacylation-reacylation cycle could help explain how calcium transport can occur without *de novo* RNA and protein synthesis (21), as well as the change in the fatty acid composition of the phosphatidylcholine fraction of the intestinal microvillus membrane mediated by both 1α-hydroxyvitamin D_3 (3), and $1,25(OH)_2$ D_3 (24). The fact that the enzymes of this cycle can be stimulated 4-fold by 5 hr, could also explain the acute duodenal calcium absorption observed by Walling (2) 6 hr after $1,25(OH)_2$ D_3 administration.

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The Effect of Essential Fatty Acid-Deficient Diet on the Levels of Prostaglandins and Their Fatty Acid Precursors in the Rabbit Brain

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ABSTRACT

Rabbits were maintained on an EFA-deficient diet. After eight weeks on this diet, lipid analysis showed no major alterations in the levels of brain dihomo-gamma-linolenic and arachidonic acids when compared with animals maintained on the standard laboratory diet. However, there were substantial reductions in the brain prostaglandin contents. It is suggested that the dihomo-gamma-linolenic acid and arachidonic acid utilized for prostaglandin production may be more directly related to the dietary essential fatty acid input rather than to the size of the precursor pool in the principal phospholipids.

INTRODUCTION

Brain phosphoglycerides are rich in the long chain polyunsaturated fatty acids derived from linoleic and α-linolenic acids. These fatty acids play a vital role in the development and normal functioning of the brain. In the laboratory rat, it has been demonstrated that feeding of essential fatty acid (EFA)-deficient diets to pregnant animals effects brain development in the foetus and offspring (1,2). In young growing rats, EFA deficiency increases the susceptibility of myelin to attack by encephalitogenic proteins (3) and influences the activity of the Na^+,K^+-ATPase in the synaptosomes (4). It is commonly believed that the role of these long chain essential fatty acids in the brain is to maintain the membrane structure and fluidity which, in turn, influences membrane functions.

However, two of these long chain polyunsaturated fatty acids (dihomo-gamma-linolenic [DHLA] and arachidonic [AA] acids) present in the brain structural lipids are the biosynthetic precursors for prostaglandins (PG) of the PG_1 and PG_2 series, respectively (5). Furthermore, it has been shown that prostaglandins are synthesized locally in the brain where they may modify neuronal function (6), cyclic nucleotide metabolism (7), and also produce behavioral changes (8). In the kidney of EFA-deficient rabbits (9) and in thrombin-stimulated platelet from EFA-deficient rats (10), the prostaglandin formation is markedly reduced, although there was no evidence for any lack of correlation between amounts of prostaglandins and their precursors.

In the present study in rabbits, we have examined the relationship between dietary input of essential fatty acids, membrane phosphoglyceride fatty acids and prostaglandin production. For the first time, we report evidence to suggest that brain prostaglandin production does not appear to be related to the amounts of precursors present in the principal membrane phosphoglycerides.

MATERIALS AND METHODS

Weanling Dutch male rabbits (Hyeline) were maintained for up to eight weeks on either the normal laboratory diet (Diet RAF, The Christopher Hill Group Ltd., Poole, Dorset) or a semisynthetic diet deficient in essential fatty acids. The semisynthetic diet was otherwise complete in respect of minerals, vitamins and energy (11).

The animals were killed and the brains removed. One-half of the brain of each animal was immediately frozen in liquid nitrogen, stored at -20 C until powdered and extracted for prostaglandin (PG) determination (12). Prostaglandin values are expressed in terms of authentic standards and were assayed on superfused rat stomach strips after isolation by thin-layer chromatography (TLC). Details of these methods have been published (13). All values are corrected for recovery of ^3H-tracer. The other half of each brain was homogenized in an ice-cold mixture of chloroform-methanol (2:1, v/v), containing an antioxidant (14). The lipids were extracted, separated into phosphoglyceride classes on TLC and transmethylated in concentrated sulphuric acid in methanol. The fatty acid methyl esters were estimated by gas liquid chromatography. The detailed procedures of lipid extraction and fatty acid analyses have been described elsewhere (14).

RESULTS AND DISCUSSION

There were no significant differences in the DHLA content in brain choline phosphogly-

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TABLE I

Dihomo-gamma-linolenic Acid (DHLA) and Arachidonic Acid (AA) Contents in the Phosphoglyceride Fractions of the Brains from Rabbits Fed a Standard Laboratory Diet or an Essential Fatty Acid-deficient Diet[a]

Brain phosphoglyceride fraction	Normal diet (n=4)	EFA-deficient diet (n=3)	P
Ethanolamine phosphoglycerides			
DHLA	0.8 ± 0.1[b]	0.5 ± 0.1	0.05
AA	10 ± 0.3	10 ± 0.5	NS
Choline phosphoglycerides			
DHLA	0.3 ± 0.1	0.2 ± 0.1	NS
AA	5 ± 0.7	6 ± 0.3	NS
Serine phosphoglycerides			
DHLA	0.5 ± 0.1	0.4 ± 0.1	NS
AA	7 ± 0.8	5 ± 0.4	0.05

[a]Results expressed as wt. %.
[b]Mean ± 1 SEM.

TABLE II

Prostaglandin Content of the Brains from Rabbits Fed a Standard Laboratory Diet or an Essential Fatty Acid-deficient Diet[a]

Prostaglandins	Normal diet	EFA-deficient diet	P
E_1	76 ± 14[b] (9)[c]	15 ± 4 (8)	0.001
E_2	290 ± 80 (7)	80 ± 13 (8)	0.025
$F_{2\alpha}$	400 ± 68 (5)	76 ± 16 (8)	0.001

[a]Results expressed as ng/g brain wet weight. Prostaglandin values are expressed in terms of authentic standards and were assayed on superfused rat stomach strips after isolation by thin layer chromatography. All values are corrected for recovery of ^{3}H-tracer.
[b]Mean ± 1 SEM
[c]No. of animals.

cerides (PC) and serine phosphoglycerides (PS) between the animals fed the normal diet compared with those fed an essential fatty acid-deficient diet (Table I). However, in the ethanolamine phosphoglyceride (PE) fraction, there were marginally lower levels of DHLA in animals fed the EFA-deficient diet. There were also no systematic differences in the AA levels of the brain PE, PC and PS fractions; in the PC fraction, the animals on EFA-deficient diet had slightly higher levels of AA compared with controls, but lower levels in the PS fraction. This general lack of response in the brain lipids to the EFA-deficient diet is consistent with what is known about the resistance of the brain lipids to alter under different short term dietary conditions (15,16). By contrast, the brain prostaglandin content (E_1 derived from DHLA and E_2 and $F_{2\alpha}$ derived from AA) were consistently reduced by over 70% in the brains from the animals that were maintained on the EFA-deficient diet (Table II). Although Galli et al. (17) have shown that much prostaglandin synthesis in rat brain may occur between killing the animal and removal of the organ, the differences we report here are significant since both groups of rabbits were treated identically.

These substantial reductions in the brain prostaglandin contents in rabbits fed EFA-deficient diets, despite little comparable changes in the DHLA and AA levels in the membrane phosphoglyceride, do not appear to be consistent with the widely held assumption that the rate limiting step in prostaglandin production is the liberation of their precursors (DHLA and AA) from membrane phospholipids by phospholipase A (5).

There are a number of possible explanations for the dissociation between levels of phospholipid PG precursors and their prostaglandin metabolites. What is clear is that the DHLA and AA contents of the principal phospholipids in the brain and other tissues we examined (18) were unchanged, while prostaglandin production was reduced. Weston and Johnston (19) have recently shown a reduction in brain PGF synthesis in EFA-deficient rats despite no significant differences in its precursor, arachi-

donic acid, in the brain ethanolamine phosphoglyceride fraction when compared with the nondeficient animals. It has also been recently shown that although there were no differences in the prostaglandin precursors, dihomo-gamma-linolenic and arachidonic acids, in the plasma cholesterol and phosphoglyceride fractions of infants with respiratory distress syndrome and age-matched controls, the levels of prostaglandins were markedly different in the two groups (20). It would, therefore, seem that the levels of DHLA and AA in these phospholipids are not controlling prostaglandin production. Either PG production is dependent for its substrates on a small amount of the measured phospholipids in a specific location or on some other minor lipid fractions.

The DHLA and AA content of total brain lipids was also no different in the EFA-deficient animals and controls. Since the principal variable was dietary EFA intake, we conclude that PG production is more dependent on dietary factors than on the total amount of PG precursors in the tissues.

This conclusion suggests a hypothesis that the DHLA and AA utilized for PG production is derived from a metabolic pool which is more directly related to dietary EFA input rather than the principal membrane structural lipids.

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^{13}C Nuclear Magnetic Resonance of Mono- and Dihydroxy Saturated and Unsaturated Fatty Methyl Esters

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ABSTRACT

^{13}C nuclear magnetic resonance spectra were obtained for methyl esters of *erythro-* and *threo-*9,10-dihydroxystearates, for 12-hydroxy-*cis-* and *trans-*9-octadecenoates, and for *threo-*12,13-dihydroxy-*cis-* and *trans-*9-octadecenoates. *Erythro* and *threo* compounds may be distinguished easily by the difference in the chemical shifts of the carbons alpha to the hydroxy-bearing carbons. Mono-hydroxy compounds are easily distinguished from *vicinal* dihydroxy compounds by differences in chemical shifts of both the hydroxy-bearing carbons and of the carbons alpha to them. The presence of a hydroxy-bearing carbon beta to a double bond results in the two carbons of the double bond having different chemical shifts, with the numerical values being different for the *cis* and *trans* isomers. The chemical shift of a carbon alpha to both a doubly bonded carbon and a hydroxy-bearing carbon is influenced both by the geometry of the double bond and the number of hydroxy-bearing carbons.

INTRODUCTION

In a previous publication (1), we reported on the behavior of several mono- and dihydroxy saturated and unsaturated fatty methyl esters on a chromatographic column packed with a silver-ion saturated Amberlyst XN1010 cation exchange resin. In this communication we wish to report the ^{13}C nuclear magnetic resonance (NMR) spectra of these compounds.

EXPERIMENTAL PROCEDURES

Materials

Methyl *erythro-* and *threo-*9,10-dihydroxystearates and methyl 12-hydroxy-*cis-* and *trans-*9-octadecenoates were available from other workers in this Center. Methyl *threo-*12,13-dihydroxy-*cis-* and *trans-*9-octadecenoates were derived from *Vernonia anthelmintica* seed oil as previously described (1). The saturated compounds were racemates while the unsaturated compounds, which were natural products or derived from them, were enantiomers.

Methods

The ^{13}C NMR spectra were recorded in 10-mm tubes at ambient probe temperature with a Bruker WH-90 Fourier Transform spectrometer operating at 22.6 MHz. The spectra were obtained from solutions in $CDCl_3$, which also served as the internal deuterium lock. Chemical shifts are reported as δ values (ppm downfield from TMS). Sweep widths of 6024 Hz with 4096 plot data points were used to give chemical shift values to within ± 1.5 Hz, i.e., ± 0.1 ppm. A pulse width of 5 μsec (ca. 30°) was used, and the computer data memory size (8192 addresses) limited the data acquisition to 0.68 sec. No pulse delays were used.

DISCUSSION

The chemical shifts determined for C-1 through C-6 and C-13 through C-18 for the saturated compounds, methyl *erythro-* and *threo-*9,10-dihydroxystearates (I and II), and for C-1 through C-7 and C-16 through C-18 for the unsaturated compounds, methyl 12-hydroxy-*cis-* and *trans-*9-octadecenoates and methyl *threo-*12,13-dihydroxy-*cis-* and *trans-*9-octadecenoates (III through VI), agreed with published values (2-6) for similar carbons in other long chain esters. The values obtained by us for the other positions in these molecules are presented in Table I. Assigments for some of these positions are based on values reported by Tulloch and Mazurek (7).

In the saturated dihydroxy esters, I and II, the carbon shifts for the carbons (C-9 and C-10) bearing the hydroxy groups are essentially the same (δ74.6, 74.8) in the two compounds. There is a substantial difference, however, between the shifts of the methylene carbons (C-8 and C-11) adjacent to C-9 and C-10 in the two compounds. In the *erythro* compound, I, this shift is δ31.3, while in the *threo* compound, II, it is δ33.7. Thus, *erythro* and *threo* dihydroxy compounds may be easily identified or distinguished in this manner. The influence of the geometry of the dihydroxy compounds

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

TABLE I

^{13}C NMR Chemical Shifts

Number	Compound name	Carbon number									
		15	14	13	12	11	10	9	8	7	
I	Methyl *erythro*-9,10-dihydroxystearate				26.0	31.3	74.8	74.8	31.3	26.0	
II	Methyl *threo*-9,10-dihydroxystearate				25.6	33.7	74.6	74.6	33.7	25.6	
III	Methyl 12-hydroxy-*cis*-9-octadecenoate		25.8	37.0	71.6	35.5	133.0	125.5	27.4		
IV	Methyl 12-hydroxy-*trans*-9-octadecenoate		25.7	36.9	71.2	40.9	134.4	126.2	32.7		
V	Methyl *threo*-12,13-dihydroxy-*cis*-9-octadecenoate	25.4	33.7	73.9	74.0	31.8	133.0	125.1	27.4		
VI	Methyl *threo*-12,13-dihydroxy-*trans*-9-octadecenoate	25.5	33.7	73.8	74.0	37.2	133.9	126.1	32.7		
	Methyl oleate				29.9	27.3	130.1	129.8	27.3	29.8	
	Methyl elaidate				29.8	32.7	130.5	130.3	32.6	29.7	

is also apparent, although not as strikingly so, for the beta carbon. Thus, the shift is δ25.4-25.6 for the *threo* compounds (II, V, and VI) and δ26.0 for the *erythro* compound.

Note that C-14 of the *threo*-dihydroxy esters (V and VI) also appears at δ33.7 in both the *cis* and *trans* isomers. In these compounds C-11 does not demonstrate this same shift because it is influenced by the presence of the double bond.

Introduction of a double bond produces a small but significant chemical shift in the hydroxy-bearing carbons of the diol from δ74.6, 74.8 in I and II to δ73.8, 74.0 in V and VI, respectively. In the monohydroxy compounds III and IV, however, this shift is very different, δ71.2, 71.6. Thus, it is easy to distinguish *vicinal* dihydroxy compounds from monohydroxy compounds by this difference in chemical shift. One further striking difference between mono- and dihydroxy compounds is the shift of the carbon alpha to the carbon bearing the hydroxy group. The shifts of C-13 of III and IV appear at δ37.0, some 3-6 ppm downfield from the shifts of C-8 and C-11 of I and II or C-14 of V and VI. This downfield shift results from the absence of a beta shielding effect caused by the second hydroxy group (7).

The olefinic carbons of the *cis* unsaturated compounds are characterized by large differences in their chemical shifts caused by the presence of the hydroxy groups. Thus, C-9 appears at δ125.5 and δ125.1 in III and V while C-10 is at δ133.0 in both III and V. A similar large difference is observed for the *trans* compounds IV and VI. By contrast, the shifts for C-9 and C-10 are δ129.8 and δ130.1, respectively, in methyl oleate and δ130.3 and δ130.5, respectively, in methyl elaidate (Table I). The assignments of C-9 and C-10 in IV were verified by a shift reagent experiment with $Eu(fod)_3d_{27}$ and are the reverse of those listed by Wenkert et al. (8) for ricinelaidic acid.

As has already been reported (2), C-8 in the *cis* compounds resonates at δ27.4 while C-8 in the *trans* compounds appears at δ32.7. The shift of a carbon between a double bond and a hydroxy group is dependent upon the geometry of the double bond and on the number of hydroxy groups. In III and IV, C-11 has a shift of δ35.5 for the *cis* compound and δ40.9 for the *trans* compound. With the dihydroxy compounds V and VI, the shifts for C-11 are δ31.8 for *cis* and δ37.2 for *trans*. Thus, double bond geometry and number of hydroxy groups are immediately apparent from this absorption.

In summary, ^{13}C NMR spectroscopy may be used to identify or distinguish *erythro* and

threo dihydroxy compounds, geometrically isomeric unsaturated monohydroxy compounds and geometrically isomeric unsaturated dihydroxy compounds, as well as distinguishing monohydroxy from dihydroxy compounds.

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The Importance of the Steric Configuration of Lysophosphatidylcholine in the Lymphatic Transport of Fat

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ABSTRACT

The importance of the steric configuration of lysophosphatidylcholine in the lymphatic transport of fat was investigated in bile fistula rats. It was found that the feeding of 1-palmitoyl-*sn*-glycero-3-phosphocholine increased the lymphatic output of phosphatidyl choline and triacylglycerol, while the feeding of 3-palmitoyl-*sn*-glycero-1-phosphocholine had no effect. In intestinal microsomes of the bile fistula rats, it was found that the lysophosphatidylcholine acyltransferase was stereospecific in acylating the 1-acyl-*sn*-glycero-3-phosphocholine enantiomer. The significance of these findings is briefly discussed.

INTRODUCTION

In previous studies we provided in vivo (1) and in vitro (2-5) evidence that the synthesis of phosphatidyl choline is necessary for the formation and release of chylomicrons in rat intestinal mucosa. It was demonstrated (1) by electron microscopy and by measurement of the uptake of radioactivity into liver and adipose tissue, that in one-day bile fistula rats fed a micellar solution of bile salt, monoacylglycerol and labelled free fatty acids, there was a significant impairment of fat release from the intestinal mucosa. Fat clearance was effected by the feeding of phosphatidyl choline, lysophosphatidylcholine or choline. The role of luminal lysophosphatidylcholine in this process was rationalized (1,4,5) on the basis of its ability to supply a precursor of phosphatidyl choline. These findings have been recently confirmed by Tso et al. (6) by direct measurements of the fat in the lymph following large doses of dietary fats with or without lysophosphatidylcholine.

The question arises whether or not the effect of lysophosphatidylcholine in the lymphatic transport of fat is stereospecific, or is it due to the detergent properties of this monoacylphospholipid? The present study was undertaken to answer this question.

MATERIALS AND METHODS

Materials

1-Palmitoyl-*sn*-glycero-3-phosphocholine and 3-palmitoyl-*sn*-glycero-1-phosphocholine were prepared as previously described (7). [1-^{14}C] Oleoyl-CoA and [1-^{14}C]linoleoyl-CoA were prepared as described (9).

Methods

Male Wistar rats (250-260 g) were fasted overnight before operation. Under ether anaesthesia, the thoracic duct and common bile duct were cannulated as described by Tso et al. (6). Postoperatively, the animals were infused via the duodenal tube at a rate of 2.9 ml/hr with saline (145 mM/4 mM KCl). The operated animals were allowed to recover for at least 36 hr in restraint cages before lipid infusions were given. On the day of the experiment, lipid was infused in the same volume of fluid as postoperatively. There were three groups of rats: (1) bile fistula, group A; (2) bile fistula with 1-palmitoyl-*sn*-glycero-1-phosphocholine supplemented, group B; and (3) bile fistula with 3-palmitoyl-*sn*-glycero-1-phosphocholine supplemented, group C. The bile fistula group received a mixture of oleic acid and monoolein in the molar ratio 2:1 (6). The total fatty acid infused per hour was 170 μmol. In group B the lipid was the same as the rats in group A, except that 1-palmitoyl-*sn*-glycero-3-phosphocholine, 10 μmol/hr, was added as a supplement. In group C the lipid dose was the same as in group A, except that 3-palmitoyl-*sn*-glycero-1-phosphocholine, 10 μmol/hr, was added as a supplement. All groups received sodium taurocholate, 55 μmol/hr, to ensure an adequate, continuous luminal supply of bile salts. In all experiments the lipid was labelled with [1-^{14}C]oleic acid (ca. 5X10^{-4} Ci/mol of fatty acid. On the day of experiment, stock lipid solutions were mixed and the solvent evaporated under a stream of nitrogen. Sodium taurocholate (19 μmol/ml, dissolved in phosphate-buffered saline) (6) was then added to make up the required lipid concentration and the mixture sonicated. Both infusates formed a stable, slightly milky mixture; the pH of the infusate was adjusted to 6.4. Lymph was collected into precooled tubes contained 2 ml methanol for 2 hr before lipid infusion (6). The lymph sample was analyzed as control. Lymph samples were collected as above at various time intervals (see Results) during the 8 hr infusion.

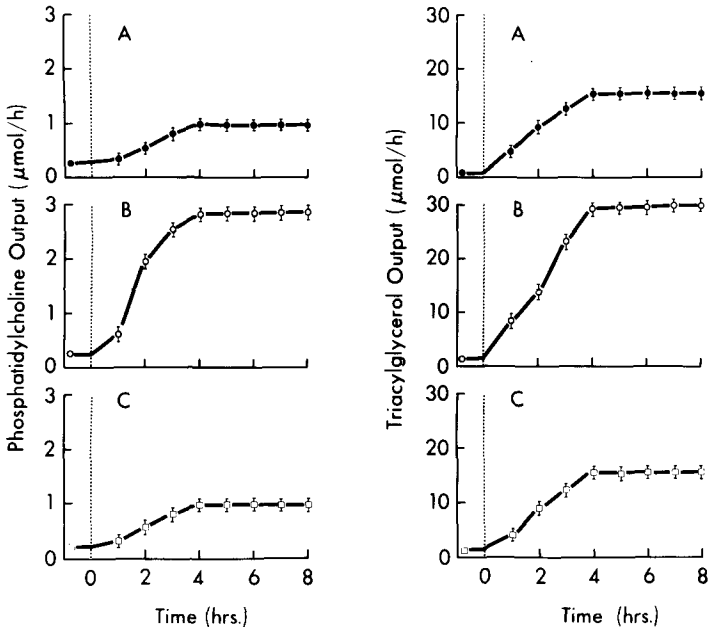

FIG. 1. Phosphatidyl choline and triacylglycerol output before and during lipid infusion. The lipid test meal was infused into bile fistula rats, using three experimental groups. Group A was bile fistula without supplement; group B, bile fistula with 1-palmitoyl-*sn*-glycero-3-phosphocholine supplement; and group C, bile fistula with 3-palmitoyl-*sn*-glycero-1-phosphocholine supplement. Both outputs were measured 2 hr preinfusion and also during the 8 hr infusion period. All values are expressed as the mean ± S.E. for 5 rats.

Lipid Analyses

Lymph lipid was extracted by the method of Bligh and Dyer (8) and dissolved in chloroform. The triacylglycerol and phosphatidyl choline fractions were resolved by thin layer chromatography, and the lipid masses quantitated by gas chromatography as previously described (4). Lipid scintillation spectrometry was carried our as described (4).

Preparation of Microsomes

Microsomes were prepared from the intestinal mucosa of normal and bile fistula rats as previously described (3).

Assay of Lysophosphatidylcholine Acyltransferase [EC 2.3.1.23]

Lysophosphatidylcholine acyltransferase was assayed as previously described (9). A typical reaction mixture contained 20 nmol of acyl-CoA, 150 nmol of 1-palmitoyl-*sn*-glycero-3-phosphocholine, or, where appropriate, 150 nmol of 3-palmitoyl-*sn*-glycerol-1-phosphocholine, 1 μmol of 5,5-dithiobis(2-nitrobenzoic acid), 80 μmol of Tris-HCl (pH 7.5), and 0.2 mg of microsomal protein in a final volume of 1 ml. The reaction was followed spectrophotometrically at 412 nm. Control values without acceptor were subtracted to give net acyl transfer rates. As radioactive acyl-CoAs were used, the reaction products were extracted and purified by thin layer chromatography, and the radioactivity at the *sn*-2 position of the phospholipid was determined after phospholipase A_2 hydrolysis (10). Protein was determined by the method of Lowry et al. (11), using bovine serum albumin as standard.

RESULTS

The fasting output of phosphatidyl choline in lymph was low in all three groups of rats, being about 0.3 μmol/hr as shown in Figure 1. During lipid infusion the lymph phosphatidyl choline increased and reached a steady output after 5 hr. Taking the 7- and 8-hr values as the steady-state output, the corresponding values in μmol/hr (mean ± S.E.) were group A (bile fistula) 0.98 ± 0.1; group B (bile fistula with 1-palmitoyl-*sn*-glycero-3-phosphocholine supplement) 2.8 ± 0.3; and group C (bile fistula with 3-palmitoyl-*sn*-glycero-1-phosphocholine supplement) 0.96 ± 0.1. The difference in phosphatidyl choline output was significant with $p < 0.001$ for both group B vs. group A, and group B vs. group C.

Prior to lipid infusion, the output of triacylglycerol was low in all three groups of rats.

TABLE I

Lysophosphatidylcholine Acyltransferase Activity[a] in Mucosal Microsomes of Normal and Bile Fistula Rats

Lysophosphatide acceptor	Fatty Acyl-CoA used in assay	Source of microsomes	
		Control	Bile fistula
1-Palmitoyl-*sn*-glycero-3-phosphocholine	[1-^{14}C] Oleoyl CoA	14.7 ± 1.3	14.5 ± 1.4
	[1-^{14}C] Linoleoyl CoA	18.3 ± 1.8	18.4 ± 1.8
3-Palmitoyl-*sn*-glycero-1-phosphocholine	[1-^{14}C] Oleoyl CoA	n.d.	n.d.
	[1-^{14}C] Linoleoyl CoA	n.d.	n.d.
1-Palmitoyl-*sn*-glycero-3-phosphocholine plus 3-Palmitoyl-*sn*-glycero-1-phosphocholine (1:1 mixture)	[1-^{14}C] Oleoyl CoA	7.6 ± 0.8	7.4 ± 0.6
	[1-^{14}C] Linoleoyl CoA	9.4 ± 0.7	9.8 ± 1.1

[a]nmol/min/mg protein. The results represent the mean ± SE of five experiments. n.d. = not detectable.

The output increased after lipid infusion and reached a steady level at 6 hr. The steady-state triacylglycerol output followed a trend similar to phosphatidyl choline output in the three groups. The rats in group B transported significantly more triacylglycerol than the rats in group A and group C ($p<0.02$.). The steady-state triacylglycerol outputs in μmol/hr (mean ± S.E.) were group A, 15.6 ± 1.2, group B, 28.1 ± 1.5 and group C, 15.5 ± 1.1.

The inability of the bile fistula group supplemented with 3-palmitoyl-*sn*-glycero-1-phosphocholine to increase the level of both phosphatidyl choline and triacylglycerol in lymph above the level of the unsupplemented bile fistula group could have been due to the inability of the intestinal mucosa to acylate this compound. It was, therefore, decided to compare the rate of acylation of 1-palmitoyl-*sn*-glycero-3-phosphocholine and its enantiomer by the intestinal lysophosphatidylcholine acyltransferase.

As shown in Table I, when the 3-palmitoyl-*sn*-glycero-1-phosphocholine was used as the lysophosphatide acceptor, no acylation was observed. With the racemic substrate, the amount acylated was never more than 50%, indicating that only one enantiomer was being enzymatically acylated. As mentioned under Methods, the reaction product was also hydrolyzed by phospholipase A_2, which has been shown to be specific only for the 1,2 diacyl-*sn*-glycero-3-phosphocholine enantiomer (12). These results indicate that the intestinal lysophophatidylcholine acyltransferase accepts only the 1-acyl-*sn*-glycero-3-phosphocholine enantiomer as its substrate.

DISCUSSION

In previous studies we (1,5) and others (6) have shown that the clearance of dietary fat in the intestinal mucosa of the bile fistula rat was affected by the feeding of lysophosphatidylcholine. The question arose whether or not the effect of lysophosphatidylcholine in the lymphatic transport of fat was stereospecific, or was it due to its detergent properties. In other studies we have demonstrated that 1-palmitoyl-*sn*-glycero-3-phosphocholine and its enantiomer were equipotent in stimulating the phosphocholine cytidylyltransferase activity in the cytosol fraction of intestinal villus cells (7), in stimulating the glycosyltransferase activity in villus cell microsomes (13), in stimulating the guanylate cyclase activity of the microvillus membrane, and in inhibiting the adenylate cyclase activity of the basal lateral membrane of the villus cell (14). These findings led us to propose (13) that the stimulatory or inhibitory effects of lysophosphatidylcholine may be related to a specific detergent property dependent upon the peculiar balance of hydrophilic and hydrophobic components in the molecule, and that the detergent properties of lysophosphatidylcholine may provide the physical basis for a role by this monoacylphospholipid as a metabolic modulator in the intestinal villus cell.

The results from the present study provide the first demonstration for a stereospecific effect of lysophosphatidylcholine in the intestinal mucosa. The data documented above shows that the feeding of 1-palmitoyl-*sn*-glycero-3-phosphocholine to the bile fistula rat results in increased levels of phosphatidyl choline and triacylglycerol in the lymph, whereas the feeding of the enantiomer 3-palmitoyl-*sn*-glycero-1-phosphocholine did not increase the lymph levels of these lipids. The inability of this enantiomer to icrease the lymphatic transport of fat may have been due to its unsuit-

ability as a substrate for the lysophosphatidyl choline acyltransferase which we have shown (Table I) to be stereospecific for the 1-acyl-*sn*-glycero-3-phosphocholine enantiomer. It is of interest to note here that Fleming and Hajra (15) have recently shown that the 1-alkyl-*sn*-glycero-3-phosphate:acyl-CoA acyltransferase in rat brain microsomes was stereospecific in acylating the alkylglycerophosphate substrate. The other optical enantiomer was inactive with the brain acyltransferase.

Taken overall, the results from both the present and our previous studies suggest that lysophosphatidylcholine as a substrate for the acyltransferase reaction to form phosphatidyl choline exhibits stereospecificity, whereas its action as an enzyme modulator is not stereospecific, but due to its detergent properties.

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METHODS

Improved Synthesis of Choline Phospholipids

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ABSTRACT

Choline phospholipids (diether and dialkyl analogs of phosphatidyl choline, cholesteryl phosphocholine) were prepared, in yields of 72-83%, by condensation of the diglyceride analogs (or cholesterol) with phosphorusoxychloride and choline toluene-sulfonate.

INTRODUCTION

While phosphatidycholines are most easily accessible through acylation of glycerophosphocholine or lysophosphatidylcholine (1), their ether or alkyl analogs must be synthesized by addition of the phosphocholine moiety to the corresponding 1,2-diglyceride analog. This can be done by condensation with 2-bromoethyldichlorophosphate, isolation of the phosphatidylbromoethanol and replacement of the bromine by trimethylamine (2,3). The method produced dipalmitoyl phosphatidylcholine in 57% yield (3); starting from unsaturated diether and dialkyl glycerols, we have not been able to obtain yields higher than 35%, and we found the necessary chromatographic purification of intermediate and product difficult because of the presence of many by-products. A much simpler method, condensation of phosphorusoxychloride first with a diglyceride, then with choline (as a salt), without isolation of the intermediate product, is reported to give 40% (with choline chloride) or 50% (with the iodide) yield (4). We find that with slight modifications, mainly by use of a soluble choline salt, high yields of choline phospholipids can be obtained.

EXPERIMENTAL METHODS

Choline toluenesulfonate was prepared by neutralizing commercial aqueous 40% choline hydroxyde with toluenesulfonic acid, removing the water by repeated evaporation with toluene, and crystallyzing the salt from acetone (for an alternate method, see ref. 5.) The salt was dried in vacuum over P_2O_5 and stored in air-tight bottles.

To 1.87 ml (20 mmole) of $POCl_3$ in a flame-dried 500 ml three-neck flask equipped with magnetic stirrer, drying tube, reflux condenser and dropping funnel, under N_2, we added (within 5 min, room-temperature) 9.1 g (16 mmole) 1-palmityl-2-oleyl-*sn*-glycerol (6) dissolved in 100 ml ethanol-free chloroform containing 2.37 ml (20 mmole) of dry quinoline (distilled, kept over molecular sieve 4A). The temperature was then raised to 45 C for 30 min. After cooling, 10 ml of dry pyridine and 9.6 g (35 mmole) of choline toluenesulfonate were added, and stirring was continued for 5 hr at room temperature. Then, 3 ml water was added, and stirring continued for 30 min. Chloroform, 150 ml, was then added, and the mixture was extracted successively with 50 ml portions, and two washings each, of water, 3% aqueous Na_2CO_3, water, 5% HCl, and water. At each extraction methanol was added in an amount just sufficient to break any emulsion formed. After drying (Na_2SO_4) and evaporation of the chloroform, the crude product, 11.0 g, contained almost no by-products, as judged by thin layer chromatography. The chromatographic purification was carried out as follows: silicic acid (Mallinckrodt), 100 g in chloroform-methanol (5:1, VN) in a column of 2.5 cm diameter; loaded with 2.2 g lipid in 20 ml chloroform; linear gradient, from chloroform-methanol 3:1 (600 ml in mixing chamber) to methanol (995 ml in the reservoir chamber). Five runs yielded 9.95 g (83% of theory) of pure diether phosphatidyl choline, with thin layer chromatographic behavior identical to previously synthesized (6) and commercial material. (Serdary Research Laboratories, Inc., London, Ont., Canada). Phosphorus, calculated, 4.1%; found, 4.1%.

A dialkyl analog of phosphatidyl choline (6)

was synthesized in an identical manner from 2-tetradecyl-2-hexadecenyl-ethanol, except that the quinoline was replaced by 20 mmoles triethylamine, with a yield of 74%. The chromatographic purification was carried out as follows aluminum oxide, neutral, Woelm (ICN Pharmaceuticals, Inc., Cleveland, Ohio); column diameter, 4 cm; 330 g Al_2O_3 in chloroform-methanol 5:1; replace solvent with chloroform; load lipid, 8g in 30 ml chloroform; elute with solvents A) chloroform, 250 ml; B) chloroform-methanol 95:5, 500 ml; C) chloroform-methanol 90:10, 500 ml; and D) chloroform-methanol 75:25, 1000 ml. The phospholipid appeared in the last 150 ml of C and in D, and was identical with previously prepared lipid (6); P, 4.2%, found 4.2%.

Cholesterylphosphocholine was prepared in the same manner and purified by column chromatography on Unisil[R] (Clarkson Chemical Co., Williamsport, PA) with a gradient starting from chloroform-methanol-water (40:55:5) to methanol-water (60:40), with a yield of 72%. The preparation thus obtained contained 1.73 moles of water according to elementary analysis: $C_{32}H_{58}O_4NP \cdot 2H_2O$[587.83]; C, 65.39; H, 10.62; N, 2.38; P, 5.27. Found, C, 65.95; H, 10.72; N, 2.32; P, 5.29.

It would seem probable that phosphatidyl cholines could be synthesized from diglycerides with improved yield by use of choline toluenesulfonate, but we have not tried this out.

ACKNOWLEDGMENT

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Serum High Density Lipoprotein and Its Relationship to Cardiovascular Disease Risk Factor Variables in Children — The Bogalusa Heart Study

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ABSTRACT

Serum high density lipoprotein is increasingly recognized as a negative risk for cardiovascular disease. The distribution and interrelationship of serum lipids, lipoproteins, anthropometric measurements and blood pressures were determined in some 5,000 children. Children had mean ± S.D. α-lipoprotein cholesterol levels (mg/100 ml) of 36 ± 15 at birth, 51 ± 22 at 6 mo, 53 ± 18 at 1 yr, 60 ± 19 at preschool age (2½-5½ yr) and 68 ± 22 at school age (5-14 yr), reflecting a sharp increase in α-lipoprotein between birth and school-age years, when these levels remained relatively stable through age 14. Although white children tended to have higher levels of total cholesterol and α-lipoprotein at birth than black children, during childhood this trend was reversed and the differences were pronounced in school-age children ($p<0.0001$). Unlike in adulthood, boys had slightly higher levels of α-lipoprotein than girls. The α-lipoprotein was negatively correlated with pre-β-lipoprotein and to a lesser extent with β-lipoprotein classes. There was an inverse relationship between α-lipoprotein and obesity with a consistently significant relationship ($p<0.01$) in older children (10-14 yr). Children with higher levels of α-lipoprotein have lower levels of blood pressure, β-lipoprotein and a lower obesity index.

INTRODUCTION

A number of epidemiologic studies indicate that cardiovascular risk factors are associated with an increased probability for coronary heart disease. High serum total cholesterol levels represent one of the major risks. While a major portion of the serum cholesterol exists as a component of low density lipoproteins in adults, several epidemiologic studies have recently suggested that low levels of serum high density lipoproteins are associated with higher rates of coronary heart disease (1-3). Even in the presence of normal (acceptable) levels of serum total cholesterol, low levels of HDL may relate to more severe coronary atherosclerosis. Further, relationships of HDL with body weight, weight changes, and exercise have also been shown (4-5).

Most of the information on HDL has been obtained on adults. Little is known about lipoproteins and their changes in childhood, yet it is important to begin to learn about HDL in early life. In 1972, we reported preliminary observations on 333 children and noted high concentration of α-lipoproteins relative to levels found in adults (6,7). The development of laboratory techniques (8,9) for quantitating serum lipoproteins on small amounts of serum and on large numbers of samples (9) has now made studies possible on large populations.

Regarding lipoproteins, and HDL in particular, the following questions can be posed: 1) What are the levels and distributions of serum HDL in children? 2) How do HDL change over time, especially in early life? 3) What are the interrelationships of HDL with other risk factor variables, particularly with physical parameters such as body weight?

The observations reported here are primarily from a population study of children in a biracial community — The Bogalusa Heart Study.

MATERIAL AND METHODS

Population Sample

Some 5,000 black and white children in Bogalusa, LA, are being observed for risk factor variables in the Bogalusa Heart Study as part of a Specialized Center of Research-Arteriosclerosis (SCOR-A) Three major cohorts are being examined in cross-sectional and longitudinal studies: school-aged children, preschool-aged children, and a newborn infant cohort. Detailed observations on these children have been presented earlier (10-14) on selected anthropometric measurements, blood pressure recordings, estimation of maturation by secondary sex characteristics and, of particular interest, analyses of serum lipids and lipoproteins.

Lipid and Lipoprotein Analyses

Serum total cholesterol and triglycerides were analyzed simultaneously with the use of the Technicon AutoAnalyzer II according to methods designed by the Lipid Research Clinics in collaboration with the Center for Disease

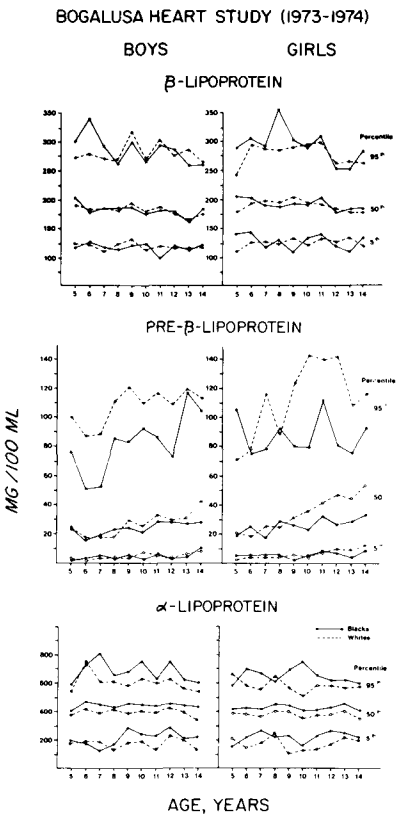

FIG. 1. Selected percentiles for β-, pre-β- and α-lipoproteins for children, ages 5-14 yr. Alpha-lipoprotein levels are essentially the same for the median of this age span; β-lipoproteins tend to decrease around age 11 yr; and pre-β-lipoproteins show a progressive increase (With permission from Circulation.)

Control, Atlanta, GA (15). The method used in these studies for quantitating serum lipoproteins is essentially a heparin-Ca^{++} precipitation coupled with agar agarose gel electrophoresis (8,9). (Since our method involves an electrophoretic separation of lipoproteins, in this presentation, we have used electrophoretic nomenclature of α-, β-, and pre-β-lipoproteins, which are equivalent to high (HDL), low (LDL), and very low (VLDL) density lipoproteins, respectively. Briefly, heparin and Ca^{++} are added to serum to precipitate selectively β- and pre-β-lipoproteins for quantitation of cholesterol in these two lipoproteins. α-Lipoprotein cholesterol is quantitated by subtraction of β- and pre-β-lipoproteincholesterol from the serum total cholesterol. The electrophoreticratio of β- to pre-β-lipoproteins was used to calculate β- and pre-β-lipoproteins from the precipitate. Although ideally suited for determination of β- and pre-β-lipoproteins, when the method was evaluated by blind analyses and compared with a preparative ultracentrifuge method, the analyses of α-lipoprotein showed good agreement (16). This method is now being used to study 40 to 50 children each day of screening in Bogalusa.

RESULTS

Levels and Distributions of α-Lipoproteins in Children

The first cross-sectional study of children ages 5-14 years was conducted in 1973-1974 on 3,524 children, with 93% participation. Since the population is 67% white and 33% black, the study provides an opportunity to observe racial differences in the children.

A unimodal distribution of both serum total cholesterol and triglycerides was observed, with black children having higher serum cholesterol levels and lower triglyceride levels than white children (11). Higher serum triglycerides were noted in girls than boys, especially white girls, and serum triglycerides tended to increase with age. The studies of serum lipoproteins indicated β-lipoprotein levels to be identical in both races, but white children showed somewhat higher pre-β-lipoproteins than their black counterparts (10). Interestingly, high levels of α-lipoproteins explained the relatively high serum total cholesterol noted in the black children.

Figure 1 provides selected percentiles for the serum lipoproteins by race and sex over the age span of the school-aged children. As an example, studies of the pre-β-lipoproteins show a consistent increase with age while the α-lipoproteins for this age span remain essentially horizontal. Consistently, higher levels of α-lipoproteins are shown in the black children, although no statistically significant sex differences were observed in this group. Of particular interest is a significant ($p<0.05$) decrease of the β-lipoproteins, particularly in boys, that occurs between ages 11 and 14 (10). Subsequent observations on those children show this decreasing trend to continue through age 16 with an unusual decrease of α-lipoproteins in white boys.

Table I shows the interrelationship among serum lipoproteins and serum lipids. As might be expected, a high correlation between serum total cholesterol and the β-lipoproteins is noted; triglycerides have a high correlation with pre-β-lipoproteins. In contrast, α-lipoproteins correlated negatively with β-lipoprotein and to a greater extent with triglycerides and pre-β-lipoproteins, especially in white children, suggesting an inverse relationship among the lipoprotein classes.

TABLE I

Correlation Coefficients (r) for Serum Lipoprotein and Lipid Variables by Race in Children, Ages 5-14 Years

	β-lipoprotein	Pre-β-lipoprotein	α-lipoprotein	Total cholesterol
Pre-β-lipoprotein				
Black, N = 1173	0.231[b]			
White, N = 2008	0.393[b,e]			
α-lipoprotein				
Black, N = 1173	-0.093[a]	-0.359[b]		
White, N = 2009	-0.231[b,d]	-0.505[b,e]		
Total Cholesterol				
Black, N = 1174	0.727[b]	0.097[a]	0.595[b,e]	
White, N = 2009	0.745[b]	0.206[b,c]	0.441[b]	
Triglyceride				
Black, N = 1174	0.282[b]	0.667[d]	-0.286[b]	0.127[b]
White, N = 2009	0.442[b,e]	0.798[d,e]	-0.422[b,e]	0.253[b,d]

[a,b]Level of significance for (r) $p<0.01$; $p<0.0001$.
[c,d,e]Race differences at $p<0.01$; $p<0.001$; $p<0.0001$.

TABLE II

Serum Lipoprotein Cholesterol and Triglycerides by Age
Bogalusa Heart Study, 1973-1974. Mean (95th Percentile)

Age	N[a]	Cholesterol				Triglycerides[b]
		Total	α-[b]	β-[b]	Pre-β[b]	
Birth	419	70 (103)	36 (60)	30 (50)	4 (12)	40 (54)
6 Months	312	135 (185)	51 (88)	74 (111)	10 (25)	92 (169)
1 Year	291	145 (193)	53 (81)	83 (121)	9 (25)	82 (158)
2½-5½ Years	694	157 (198)	60 (90)	91 (129)	6 (18)	63 (113)
5-14 Years	3446	165 (215)	68 (104)	89 (130)	8 (22)	69 (126)

[a]Nonfasting included in total numbers; numbers vary slightly for each variable.
[b]Fasting samples only.

Comment

Lipoprotein levels of children from a total community are now available for comparison with other studies (17). Interestingly, α-lipoprotein levels in school-aged children show race and sex differences, with black children having higher levels than white children, and girls, especially white girls ($p<0.05$), tending to have lower α-lipoproteins than boys. Observations of these differences in α-lipoproteins in childhood and their changes with age should contribute to a better understanding of the early natural history of atherosclerosis.

The Time Course Changes of α-Lipoproteins

One of our major objectives has been to monitor the changes in risk factor variables over time, particularly the evolution of serum lipoproteins from early life onward. Our data have shown remarkable changes of serum total cholesterol in the first few years of life from longitudinal studies of a newborn infant cohort. We reported earlier that levels of serum total cholesterol of 3-year-old children were as high as we observed in medical students (6,7). Consequently, we began a study on infants, focusing on lipoprotein cholesterol fractions (18,19). Table II shows the levels of serum total cholesterol, triglycerides, and serum lipoprotein cholesterol from cross-sectional studies on the Bogalusa children. Figure 2 compares the serum lipoprotein cholesterol levels of Bogalusa children to those of adults reported by Fredrickson and coworkers (20). As in adults, the β-lipoprotein cholesterol accounts for the greatest percent of the total cholesterol, but α-lipoprotein cholesterol accounts for relatively greater amounts in children than in adults.

Lipoprotein cholesterol values change dramatically in the first two years of life, with β-lipoproteins accounting for the major increase of serum cholesterol during the first year. A slower but progressive rise of the α-lipoproteins is also noted during this period. Although an interesting elevation of serum triglycerides occurs at six months of age compared to the first and second year's pre-β-lipoprotein cholesterol increases much less than α- and β-lipoprotein cholesterol. In infants and children, the ratio of

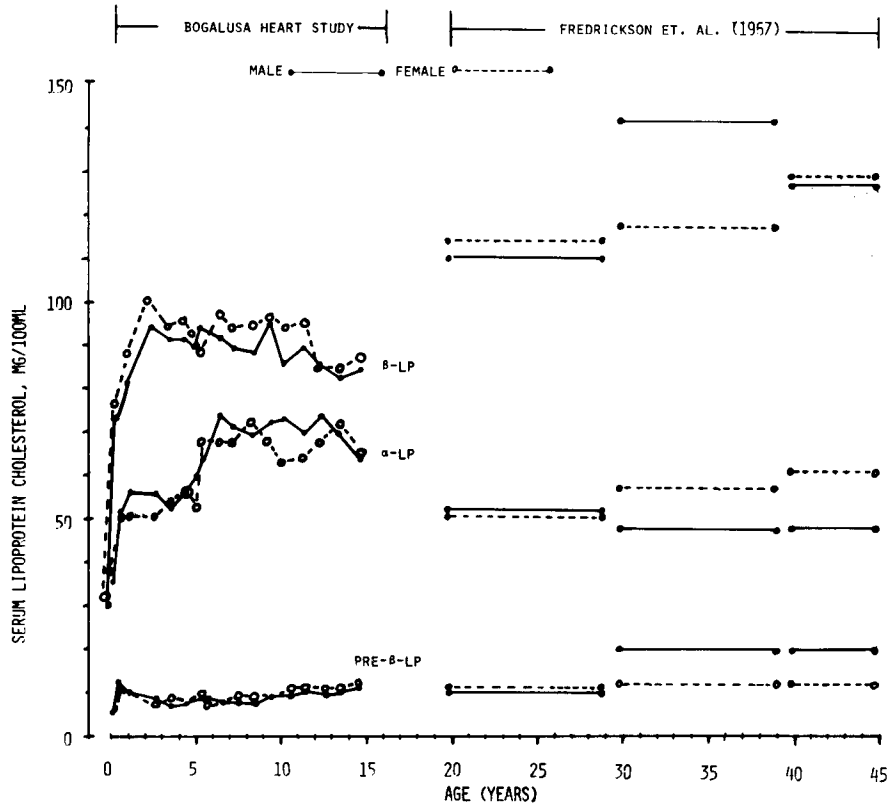

FIG. 2. Mean lipoprotein cholesterol levels by sex for infants and children in the Bogalusa Heart Study and those from Fredrickson et al. (20) for adults are shown for comparison. Analysis of the data on children indicate both race and sex differences (Please refer to text).

β- to α-lipoprotein cholesterol related to sex differences is less than observed for adults, and is also the reverse of the adult ratio. These data suggest that a transition from an infantile to an adult pattern begins to occur between ages 15-20. At what age the higher mean α-lipoprotein level in females first becomes apparent is not known.

Our data now provide observations on α-lipoprotein cholesterol by age, race and sex from birth to 14 yr in ca. 5,000 children. It is important to document how lipids change over time in a given individual; unfortunately, observations obtained from cross-sectional studies represent only one point in time. In the Bogalusa Heart Study, we are making longitudinal observations on four cohorts within the school age, at 5, 8, 11 and 14 yr. At present, analyses of only the first and second years of study are available (21).

Observations of serum total cholesterol levels at two successive years show high correlation coefficients, ca. 0.7 for each of the several age groups (Table III). From multiple regression analyses (see Table III for independent variables), we observed that the cholesterol level of the first year was the parameter most predictive for subsequent cholesterol levels. We also noted that 45% of the children at the top 90th or the bottom 10th percentile in the first year persisted at those respective levels during the second year. The persistence within those ranks seems remarkably high when regression toward the mean, measurement error, and individual biologic variations are taken into account. Somewhat more consistent levels over time were observed with β-lipoproteins, whose persistence within ranks is greater than that observed for pre-β and α-lipoproteins. In fact, the multiple R^2 for the β-lipoprotein analysis was highest, accounting for some 43% to 69% of the variability in the second year.

Analyses of serum α-lipoprotein data over two consecutive years show some tendency to persist within ranks, but considerable variation occurs from one year to the next. Correlations from year 2 to year 1 indicate somewhat lower r values, ca. 0.45. Multiple linear regression

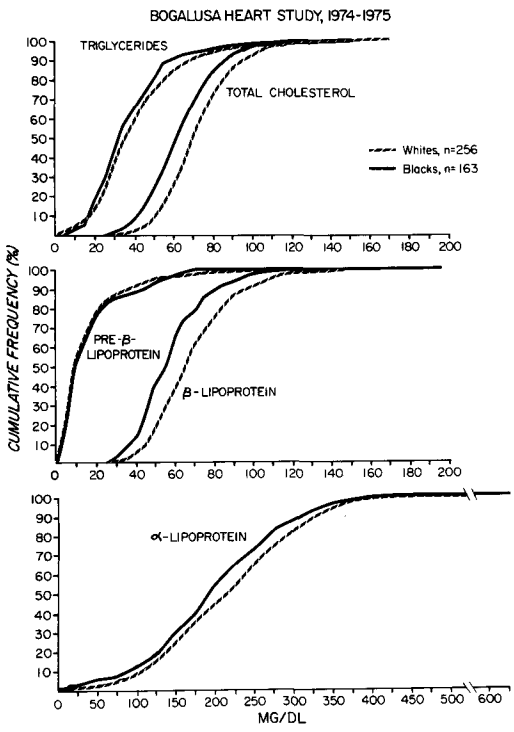

FIG. 3. Cumulative frequency distributions for lipids and lipoproteins at birth of black and white Bogalusa newborns. (With permission from Pediatr. Res.)

analyses account for ca. 25% of the variability or about one half of that observed for β-lipoproteins. More variability was observed for α- and pre-β-lipoprotein, which either may be related to a greater biologic change reflecting both metabolic and environmental influences than noted for β-lipoproteins or due to somewhat higher measurement errors for these two variables.

Newborn Infant Cohort

In the Bogalusa Heart Study, we are observing a newborn cohort through infancy. Cord blood was obtained from 477 infants born in Bogalusa over an 18-month period, 97% of all births in the community. Figure 3 illustrates the distribution of levels of lipids and lipoproteins in cord blood as cumulative frequencies in the black and white neonates. White infants at birth tended to have higher serum levels of total cholesterol, triglycerides, β-lipoprotein, and α-lipoprotein than black infants. The observed black-white differences in serum total cholesterol and β-lipoprotein at birth persisted even when comparing newborns of the same weight and socioeconomic status. During infancy (6

TABLE III

Relationship in Two Successive Years (Bivariate and Multiple Correlation Coefficients) of Serum Lipids and Lipoproteins in Four Cohorts by Age at Initial Examination[a]

Variables	Age (years) 5 (N)	5 Bivariate r	5 Bivariate r^2	5 Multiple R^b	8 (N)	8 Bivariate r	8 Bivariate r^2	8 Multiple R^b	11 (N)	11 Bivariate r	11 Bivariate r^2	11 Multiple R^b	14 (N)	14 Bivariate r	14 Bivariate r^2	14 Multiple R^b
Total cholesterol	(212)	0.64	0.41	0.42	(281)	0.75	0.56	0.58	(323)	0.69	0.47	0.49	(241)	0.69	0.48	0.52
Triglycerides[c]	(154)	0.40	0.16	0.21	(247)	0.50	0.25	0.31	(270)	0.54	0.29	0.35	(200)	0.37	0.14	0.22
β-Lipoprotein cholesterol[c]	(154)	0.65	0.42	0.43	(245)	0.82	0.67	0.69	(270)	0.79	0.62	0.64	(200)	0.75	0.56	0.58
Pre-β-lipoprotein cholesterol[c]	(154)	0.51	0.26	0.29	(245)	0.46	0.21	0.26	(270)	0.51	0.26	0.32	(200)	0.51	0.26	0.29
α-Lipoprotein cholesterol[c]	(154)	0.34	0.11	0.14	(247)	0.42	0.17	0.23	(270)	0.51	0.26	0.30	(200)	0.56	0.31	0.41

[a]All coefficients are statistically significant, $p < .0.0001$.
[b]Dependent variable: Variable (Year 2). Independent variables: Variable (Year 1), Race, Sex, height (Year 1), $W/H^{2.77}$ (Year 1), Δheight (Year 2-Year 1), $\Delta W/H^{2.77}$ (Year 2 - Year 1).
[c]Fasting children only.

TABLE IV

Correlation between Serum Lipids and $W/H^{2.77}$ in Bogalusa Boys by Age and Race

	White males		Black males	
	Ages 5-9 (N= 456)	Ages 10-14 (N= 572)	Ages 5-9 (N- 262)	Ages 10-14 (N= 342)
Total cholesterol	0.10[a]	0.21[b]	0.10	-0.05
Triglycerides	0.18[b]	0.35[b]	0.09	0.17[c]
β-Lipoprotein	0.20[b]	0.27[b]	0.16[a]	0.08
Pre-β-lipoprotein	0.19[b]	0.31[b]	0.09[a]	0.21[b]
α-Lipoprotein	-0.12[c]	-0.14[c]	-0.03	-0.19[b]

[a] $p < 0.05$
[b] $p < 0.001$.
[c] $p < 0.01$.

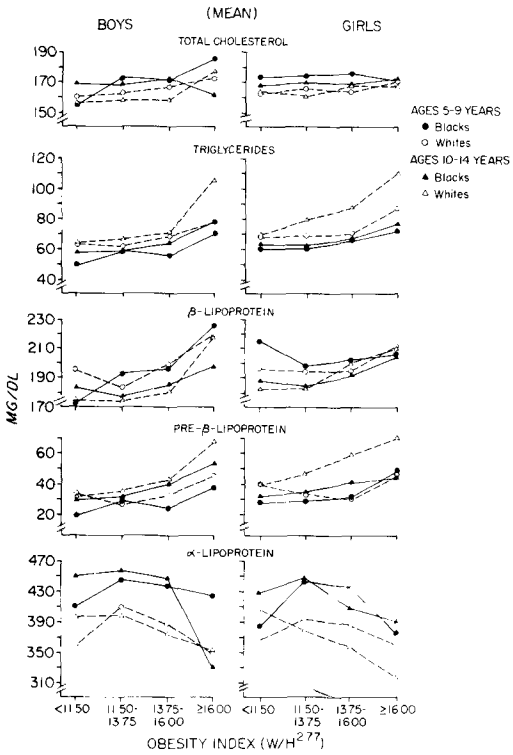

FIG. 4. Relationship of serum lipids and lipoproteins with the obesity index, $W/H^{2.77}$, in children by age, race and sex. The mean levels of total cholesterol, triglycerides, and lipoproteins (β, pre-β, α) are plotted for each of four $W/H^{2.77}$ categories for black and white children ages 5-9 and 10-14 yr. Multiplying by the following factors converted lipoproteins into corresponding lipoprotein cholesterol: β-lipoprotein x 0.469; pre-β-lipoprotein x 0.222; α-lipoprotein x 0.17. (With permission from Am. J. Epidemiol.)

mo and 1 yr) the race-related differences persisted only in triglycerides and pre-β-lipoproteins, with white infants having relatively higher levels than the black infants. Surprisingly, α-lipoprotein levels tended to be higher in preschool-aged black children than in white children; however, consistently high levels of α-lipoprotein in black children became more evident only from the school age onward. The triglyceride and pre-β-lipoprotein levels remained lower in the blacks into and throughout the school age.

The infant cohort has now been observed through the first year of life (22). We note that lipid levels of cord blood are poor predictors of subsequent levels at six months of age or one year, even for the β-lipoprotein concentrations. At 6 mo of age, however, the ranks of lipid levels become consistent, and the levels begin to correlate with values at one year of age. As described for successive samples for the school-age children, correlations are highest for β-lipoproteins and less for α- and pre-β-lipoproteins.

Comment

Almost no longitudinal observations on serum lipoproteins from free-living children are yet available. Such observations are obviously needed since changes for a given individual may not be predicted from cross-sectional studies alone. It becomes important to learn with what consistency children will tend to persist in their respective ranks over time – the "tracking" of serum lipoproteins. The greater the degree of tracking, the greater the predictive value of blood studies in early life for hyperlipoproteinemia in adulthood. Although genetic disorders are diagnosed by detecting extremely high values, from the standpoint of the general adult population at risk for coronary artery disease, we need to learn much more about the transition of serum lipoproteins from childhood through adulthood.

Interrelationship of α-Lipoproteins with Body Weight

Since this study involves observations on several risk factor variables, such as blood pres-

sure and height (ht)-weight (wt) indices, we can look at the interrelationship of serum α-lipoproteins with other factors. We noted no correlation with blood pressure but a significant relationship with body weight. An obesity index, $wt/ht^{2.77}$, was selected using the Benn formula (23) to derive an index for this population which correlates least with height and more closely with skinfold thickness, a measure of body fatness. Bivariate analysis of lipids and $wt/ht^{2.77}$ showed a closer relationship of triglycerides and pre-β-lipoproteins to weight than other variables (Table IV). This might be expected from studies in adults (5,24). An inverse relationship was observed between α-lipoproteins and the obesity index in white boys and girls of both races. Figure 4 shows the relationship of the obesity index to the concentration of the various lipids. With an increasing $wt/ht^{2.77}$, a closer relationship to lipoproteins was observed.

Table V shows multiple regression analysis using all observations: age, race, sex, skinfold, obesity index, and maturation. These analyses show an overall low relationship; that is, ca. 10% of the variability of the children's lipid values can be explained. This is considerably less, for example, than was observed for weight and blood pressure in the same population of children. Further, dietary studies indicated that inclusion of dietary factors as a variable cannot account for the majority of the variability (25).

Further analysis of the data by dividing the children into obese or lean groups based on the obesity index and triceps skinfold thickness showed a consistently higher level of α-lipoproteins in thin white boys, somewhat higher levels in girls, but no difference in black boys. Weight and perhaps body fatness seemed to relate inversely more to α-lipoprotein levels in the white children than in the blacks (26).

Comment

Although these studies indicate an interrelationship of serum lipoproteins with measurements of weight or fatness, only a small percentage of the variability in serum lipids and lipoproteins of young individals can be accounted for by multiple characteristics, including the diet of that person. These observations suggest that the marked variability is largely due to the general metabolic makeup or genetic background of the individual rather than environmental factors.

DISCUSSION

One of the major considerations in the study of serum lipoproteins in early life is the clinical implication of α-lipoprotein levels in childhood. In adults it is suggested that high serum α-lipoproteins may have a greater protective role than adverse role as exists for high serum total cholesterol or β-lipoproteins. Consequently, α-lipoproteins should be evaluated in children for their potential in the development of cardiovascular disease. The accumulation of such information will take time, but the study of lipoproteins in children is a distinct advance.

TABLE V

Multiple Linear Regression for Serum Lipids, Bogalusa Children, Ages 5-14 Years (N=3, 151)[a]

Dependent Variables	Multiple R^2
Total cholesterol	0.04
Triglycerides	0.11
β-Lipoprotein	0.06
Pre-β-lipoprotein	0.11
α-Lipoprotein	0.06

[a]Independent variables: age, race, sex, triceps skinfold, $W/H^{2.77}$, maturation scale (pubic hair, Tanner grades 1-5)

Technical advances in the analysis of serum lipoproteins have simplified the study of the cholesterol distribution in the lipoprotein fractions. Our population studies now provide the distribution of levels or normative values from birth to adolescence from a total and well defined bi-racial group of free-living children. A better appreciation of serum lipoprotein levels can limit errors in characterizing types of hyperlipoproteinemia, as are currently made by analyses of serum cholesterol, triglycerides and qualitative electrophoresis. Since many children and selected groups of adults have high α-lipoprotein levels, it has become even more important to quantitate serum lipoprotein fractions as we learn more of their function. Although nothing is yet known about the composition of HDL or subfractions in children, ultracentrifuge studies are defining this more clearly in adults (27). Similar studies will be needed to study the heterogeneity of HDL (27,28) in children in the near future.

From observations of children, it is now possible to study a combination of risk factor variables, i.e., blood pressure, weight indices, and serum lipids and lipoproteins. An analysis of multiple risk factor variables from the Bogalusa children indicate that an aggregation or clustering of risk factors occurs greater than would be expected if no association existed. This aggregation is particularly apparent in white boys and has a tendency to increase with age. Little or no relationship among the various risk parameters was observed in children younger than school age. Although individual genetic makeup may be the cause of the

marked interindividual variability, this observation implies that environmental factors increasingly influence the risk factor variables over time. Focusing on the protective effect of α-lipoprotein levels on cardiovascular disease, it is now possible to study children for low risk rather than high risk, by selecting high concentrations of α-lipoproteins to cluster with low levels of blood pressure, β-lipoproteins or total cholesterol, and an obesity index. Again, when studies were extended into the preschool age, less clear associations were observed. It remains to be seen how the combinations of risk factors, whether for high or low risks, persist through adulthood and ultimately relate to cardiovascular disease. These observations in early life are important to establish, since they provide a means of identifying susceptibility for coronary artery disease at a time optimum for prevention.

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High and Low Density Lipoprotein Cholesterol Levels in Hypercholesterolemic School Children

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ABSTRACT

To most fully explicate risk to coronary heart disease (CHD) in adults and children with elevated plasma total cholesterol, the levels of high and low density lipoprotein cholesterol (C-HDL, C-LDL) must be quantitated. This report focuses upon C-HDL and C-LDL levels in children identified in a lipid and lipoprotein sampling survey of 6,775 Princeton School children, by either total plasma cholesterol $\geq$ 205 mg/dl, the approximate 95th percentile for children 6-17 years of age, or age-, sex-, and race-specific 95th percentiles for cholesterol. Using the sex-, race-specific local 95th percentiles for C-HDL and C-LDL, the hypercholesterolemic children were separated into four categories according to whether they had elevations of both C-HDL and C-LDL, C-HDL only, C-LDL only, or neither. When selection for hypercholesterolemia was based on the overall 95th percentile (205 mg/dl), black children were more likely than white to have elevations of C-HDL only, which accounted for their hypercholesterolemia, $p<.05$, whereas white children were much more likely to have elevations of C-LDL only, than were black children, $p<.005$. However, when selection for hypercholesterolemia was based on age-, sex-, and race-specific 95th percentile cholesterol levels, there were no differences in the proportion of black and white children having elevations of C-HDL and C-LDL, accounting for their hypercholesterolemia. Elevated levels of C-HDL can explain apparent hypercholesterolemia in at least 16% of children, ages 6-17, who may putatively be at reduced, rather than increased CHD risk.

INTRODUCTION

Population surveys and studies of kindreds with familial aggregations of elevated high density lipoprotein cholesterol (C-HDL) have indicated that C-HDL is inversely correlated with the risk of developing coronary heart disease (CHD) (1-5). Conversely, population surveys and studies of kindreds with familial hypercholesterolemia have shown that elevated low density lipoprotein cholesterol (C-LDL) is positively associated with subsequent risk of CHD (3,6-10). Consequently, to most fully explicate CHD risk in adults and children with elevated plasma total cholesterol, C-HDL and C-LDL must be accurately measured. Elevated levels of C-HDL can explain apparent hypercholesterolemia in some children (11,12) who may putatively be at reduced (1-5), rather than increased CHD risk (3,6-10).

The purpose of this report is to assess the relative frequency that an elevated cholesterol measurement in children is due to elevated C-LDL and/or C-HDL, with special focus on the association of C-HDL and C-LDL with sex and race.

MATERIALS AND METHODS

Study Population

The Cincinnati Lipid Research Clinic's Princeton Family Lipid Program, a lipid survey of schoolchildren and their parents in a racially integrated suburban school district, has been previously described in detail (13,14). At an initial screening (Visit 1), plasma cholesterol and triglyceride were measured in 6,775 children fasting 12 or more hours and not using lipid altering medications and 522 children admittedly not fasting at least 12 hr, and/or using exogenous hormones or antidiabetic drugs. At follow-up (Visit 2, ca. 6 weeks later), lipoproteins and lipids were measured in 948 students randomly selected from the entire participating pediatric population and in 724 students selected for retesting because of elevated cholesterol, elevated triglyceride, or use of antidiabetic medication.

This report focuses on the C-HDL and C-LDL levels in 240 children re-evaluated at Visit 2 and found (at Visit 2) to have total plasma cholesterol $\geq$ 205 mg/dl, the approximate 95th percentile for children age 6-17 years (13-15).

The 95th percentile cholesterol level of 205 mg/dl was arbitrarily used throughout this report to define severe hypercholesterolemia, despite the continuous nature of risk to CHD associated with plasma cholesterol throughout its entire distribution. We used this 205 mg/dl as a "cut point" for the following reasons. One,

TABLE I

Plasma Total Cholesterol (TC), Triglyceride (TG), High and Low Density Lipoprotein Cholesterol (C-HDL, C-LDL), mg/dl: Percentile Distribution[a] for 927 Fasting Schoolchildren, Ages 6-17

	Males						Females					
	White (n=358)			Black (n=119)			White (n=326)			Black (n=124)		
Percentiles	TC	TG	C-HDL	C-LDL	TC	TG	C-HDL	C-LDL	TC	TG	C-HDL	C-LDL	TC	TG	C-HDL	C-LDL
5th	121	33	36	64	122	33	38	61	126	38	37	67	129	31	37	70
10th	128	38	39	71	132	35	43	70	130	44	39	71	139	40	41	74
50th	156	57	52	93	156	51	56	94	159	65	51	95	165	56	57	101
90th	185	99	67	123	203	86	74	127	189	108	65	122	193	92	72	128
95th	203	116	71	131	212	93	76	151	201	124	72	133	204	113	75	139

[a]Percentile distribution for 927 schoolchildren, randomly selected from Princeton City School population study.

in large scale, multicenter epidemiologic studies, some common decision point is required for the consistent classification and selection for more intensive follow-up of subjects. In the Lipid Research Clinics, this cut point was 205 mg/dl for children up to age 19 years (13-15). Two, in clinical practice some kind of decision point for intervention is pragmatically used whether it be based on the clinician's experience or on percentiles obtained from a well defined population. Three, the selection of the 95th percentile in epidemologic studies allows one to focus on a population with statistically extreme values and, presumably, greatly increased risk.

If, however arbitrary, the overall 95th percentile for choelsterol is utilized as a tool to identify children for further study, then the use of age-, race-, and sex-specific values from the same cohort provides optimal specificity. In this study we compared the diagnostic ramifications of the use of either a broad population 95th percentile or age-, race-, and sex-specific values.

Blood Sampling Techniques

Blood sampling techniques at Visit 2 were similar to those previously described for Visit 1 (13,14). Fasting plasma cholesterol, C-HDL, C-LDL, and Triglyceride levels were quantitated following the methods of the Lipid Research Clinic's Laboratory Methods Manual (16). Inter- and intraday coefficients of variation for determination of plasma cholesterol and triglyceride have been previously described (13,14). For plasma pools containing 50.3 and 43.8 mg/dl C-HDL, interday coefficients of variation were 3.7% and 4.2%

Study Design

For each lipoprotein studied (C-HDL and C-LDL), children with (Visit 2) total plasma cholesterol $\geqslant$ 205 mg/dl were arbitrarily classified "elevated" or "normal" using the sex-race specific empirical 95th percentile of C-HDL and C-LDL distributions in the randomly selected retest population (Table I).

Additionally, for comparison purposes, age-, sex-, and race-specific cholesterol 95th percentile levels of 203 (white males), 212 (black males), 201 (white females), and 204 (black females) (Table I) were used to select "hypercholesterolemic" children for classification by lipoprotein levels, as above. In establishing these distributions, only those randomly selected children (n = 927) who fasted 12 hr or more at Visit 2 and were not using lipid-altering drugs were included. Hence, the distributions are based on 358 white males, 119 black males,

TABLE II

Lipoprotein Classification of 240 Hypercholesterolemic Children,[a] Ages 6-17, by Sex and Race

	Elevated C-HDL, C-LDL		Elevated C-HDL only		Elevated C-LDL only		Neither elevated		
	n	%	n	%	n	%	n	%	
White males	8	10.4%	9	11.7%	54	70.1%	6	7.8%	77
Black males	4	7.8%	12	23.5%	20	39.2%	15	29.4%	51
White females	6	11.8%	5	9.8%	36	70.6%	4	7.8%	51
Black females	4	6.6%	13	21.3%	38	62.3%	6	9.8%	61
Total	22	9.2%	39	16.2%	148	61.7%	31	12.9%	240
White males and whte females compared to black males and females, X^2, p									
	.63, >.1		4.88, <.05		7.91, <.005		5.42, <.02		

[a]Selected by overall population 95th percentile for total plasma cholesterol (205 mg/dl).

TABLE III

Lipoprotein Classification of 245 Hypercholesterolemic Children,[a] Ages 6-17, by Sex and Race

	Elevated C-HDL, C-LDL		Elevated C-HDL only		Elevated C-LDL only		Neither elevated		
	n	%	n	%	n	%	n	%	Total
White males	8	10%	11	13%	57	70%	6	7%	82
Black males	4	12%	6	18%	20	59%	4	12%	34
White females	6	9%	7	10%	46	68%	9	13%	68
Black females	4	7%	13	21%	38	62%	6	10%	61
Total	22	9%	37	15%	161	66%	25	10%	245
White males and white females compared to black males and females, X^2, p									
	.007, NS		2.31, NS		1.18 NS		0 NS		

[a]Selected by age-, sex-, and race-specific 95th percentiles for total plasma cholesterol (203 mg/dl, WM) (212 mg/dl, BM), (201 mg/dl, WF), (204 mg/dl, BF).

326 white females, and 124 black females, (Table I). The children were then separated into four categories according to whether they had elevations of both C-HDL and C-LDL, or C-HDL only, of C-LDL only, or of neither, (Tables II, III).

Statistical Analysis

The relationship between sex-race groups and their respective distributions in the four lipoprotein categories was tested for significance using the chi-square statistic (17). The proportion of observations of each sex-race group which occurred in the four lipoprotein categories was viewed as a vector, and the hypothesis tested was: are the respective elements of these four vectors equal? Linear model techniques for the analysis of categorical data (18) were also used to obtain estimates of the effects of race and sex on lipoprotein categorization.

RESULTS

Lipid and Lipoprotein Distributions

In Table I, the 5th, 10th, 50th, 90th, and 95th percentile levels for total plasma cholesterol, triglyceride, C-HDL, and C-LDL for the random recall group of 927 school children (19) are summarized.

C-HDL and C-LDL in Hypercholesterolemic School Children

As summarized in Table II, 9.2% of children with total plasma cholesterol $\geqslant$ 205 mg/dl had both elevated C-HDL and C-LDL, 16.2% had elevated C-HDL alone, 61.7% had elevated C-LDL alone, and 12.9% had neither elevated C-HDL nor C-LDL. Thus, in 29.1% of the hypercholesterolemic children, the C-LDL fraction was not elevated above the sex-race specific 95th percentile level.

As summarized in Table III, when children

were selected by the age-sex-race specific cholesterol 95th percentiles, they were distributed among the four lipoprotein categories in a fashion similar to those children (above) selected by the broad population 95th percentile for cholesterol. Nine percent of the children had both elevated C-HDL and C-LDL, 15% had elevated C-HDL alone, 66% had elevated C-LDL alone, and 10% had neither elevated C-HDL or C-LDL. Thus, following this classification scheme, the C-LDL fraction was not elevated above the sex-race specific 95th percentile level in 25% of the "hypercholesterolemic" children.

Comparison of All Black to All White Children Selected by the Overall 95th Percentile for Cholesterol: Classification by C-HDL and C-LDL

Overall, the association between sex-race groups and lipoprotein classification was significant (X^2 = 25.5, 9 df, $p<.01$) when the overall 95th percentile was used to define hypercholesterolemia. The distribution of hypercholesterolemic black and white children over the four lipoprotein categories differed significantly (X^2 = 14.6, 3 df, $p<.005$). As summarized in Table II, hypercholesterolemic black children were more likely than white to have elevations of C-HDL only, accounting for their hypercholesterolemia, $p<.05$. Hypercholesterolemic white children were much more likely to have elevations of C-LDL only, than were black children, $p<.005$, Table II. Hypercholesterolemic black children were also more likely than whites to have neither C-LDL nor C-HDL elevated, $p<.02$, Table II.

Using the linear models technique, assignment of a numeric code to the four lipoprotein categories (e.g., 1,2,3,4) allows one to express these response variables as functions of a model which contains a term for the overall mean, the race effect, and the sex effect. The error term is thus the race-by-sex interaction term, (18). The model provided an adequate fit to the data. The sex effect was nonsignificant ($p = 0.15$). The race effect, however, was highly significant, $p<0.001$.

Comparisons Within Sex, by Race, Hypercholesterolemic Children Classified by C-HDL and C-LDL

The distribution of hypercholesterolemic white males over the four lipoprotein categories differed from that of black males (X^2 = 16.6, 3 df, $p<.01$).

Hypercholesterolemic white males were more likely to have elevated C-LDL alone than were black males (X^2 = 12.9, $p<.01$), while black males were more likely to have both C-HDL and C-LDL below the 95th percentile (X^2 = 10.2, $p<.01$).

The difference in the proportions of white and black males with elevated C-HDL alone was at the borderline of significance (X^2 = 3.2, $p=.07$) with the proportion of black males being greater.

The distribution of hypercholesterolemic white females over the four lipoprotein categories did not differ significantly from that of black females (X^2 = 3.5, 3 df, $p>.1$). Black females tended to have a larger proportion with elevated C-HDL than white females, but the difference did not attain statistical significance (X^2 = 2.72, $p=.1$.).

Comparison within Race, by Sex, Hypercholesterolemic Children Classified by C-HDL and C-LDL

Differences between the proportions of white males and females in the lipoprotein categories were not statistically significant (X^2 = 0.15, 3 df, $p>.1$). Differences in the distribution of black males and females in the lipoprotein categories were significant (X^2 = 8.7, 3 df, $p<.05$). Black males had a larger proportion than did black females with neither C-HDL nor C-LDL elevated (X^2 = 5.8, $p<.01$), while black females had a larger proportion with C-LDL alone elevated (X^2 = 6.8, $p<.01$). There was no difference in the proportions of black males and females with elevated C-HDL only (23.5% vs 21.3%).

Sex-Race Comparisons of Children Selected by Age-, Sex-, Race-Specific Cholesterol 95th Percentiles, Classification by C-HDL and C-LDL

When hypercholesterolemia was defined using the age-, sex-, and race-specific 95th percentiles, the association between sex-race groups and lipoprotein classification was not statistically significant (X^2 = 5.59, 9 df, NS), Table III.

DISCUSSION

Pediatricians and family physicians, by virtue of early recognition of childhood hyperlipoproteinemia and other risk factors for CHD, play a central role in the collective approach to primary prevention of atherosclerosis (13-15,20-29). Progressive increments in pediatric population lipid sampling will inevitably provide increasing numbers of children diagnosed as "hypercholesterolemic" children. As emphasized by this study, and by earlier reports by Neill et al. (11) and Glueck et al. (12), elevated levels of C-HDL can explain apparent hypercholesterolemia in many children, who may represent a subgroup putatively at reduced (1-5) rather than increased (3,6-10) CHD risk by virtue of the antiatherogenic

aspects of C-HDL.

Since elevated C-HDL levels may aggregate in families (12), identification of a hypercholesterolemic child whose elevated C-HDL accounts for the hypercholesterolemia, may lead to identification of similarly affected siblings and parents (12). In a recent study (12), 17 kindreds were identified by virtue of hypercholesterolemic school children whose hypercholesterolemia was accounted for by elevated C-HDL. Family lipid and lipoprotein sampling revealed three-generation vertical appearance of elevated C-HDL in two kindreds, and two-generation vertical appearance in eight additional kindreds. High C-LDL levels also aggregate in families, and are closely associated with increased CHD risk (7-11,27). Moreover, in children with familial hypercholesterolemia, C-HDL is lower than in normal children (8), suggesting incremental CHD risk from both low C-HDL and high C-LDL.

This study focused upon C-HDL and C-LDL relationships in hypercholesterolemic children identified during population lipid sampling from a probability sample in a biracial school district. Although the Princeton School District is not a simple microcosm of U.S. School districts, the data should be usefully generalizable to pediatric practice and to subsequent pediatric population lipid surveys.

Although a majority of the hypercholesterolemic children (61.7%) had elevations of C-LDL alone which accounted for their elevated total plasma cholesterol, and 9.2% had elevations of C-LDL plus C-HDL, 30% did not have C-LDL above the 95th percentile. Of this 30% ca. one-half (16.2%) had elevated C-HDL which accounted for their elevated total plasma cholesterol, and the rest (12.9%) had neither elevated C-HDL nor C-LDL. Since black children and adults have higher C-HDL levels than whites (19-20), hypercholesterolemic black girls and boys were more likely to have C-HDL, and not C-LDL, accounting for their hypercholesterolemia, than were their white counterparts.

For the 9.2% of hypercholesterolemic children having conjoint elevations of C-HDL and C-LDL, we spectulate that the opposing CHD risk influences of elevated C-HDL and C-LDL might produce an aggregate CHD risk not dissimilar to that observed in normocholesterolemic subjects with "normal" C-HDL and C-LDL. Detailed family studies in kindreds with pediatric probands having elevated C-HDL and C-LDL would be important. Longitudinal evaluation of nutritional modification in hypercholesterolemic children with elevated C-HDL and C-LDL also needs to be done to determine if C-LDL, but not C-HDL, can be lowered by dietary intervention.

When age-, sex-, and race-specific 95th percentiles for cholesterol were used for further evaluation of children, no racial differences in lipoprotein classes accounting for hypercholesterolemia were observed. However, for any given plasma cholesterol level, black children have higher C-HDL and slightly lower C-LDL (30). It is important to note that the 95th percentile selection process is arbitrary and does not imply that the remaining 95% of the the pediatric population would be categorized as "normo-cholesterolemic." The entire distribution for plasma cholesterol and the lipoprotein cholesterols (as in Table I) should be used in the placement of individual children within a distribution of continuous risk for CHD, positive for C-LDL and negative for C-HDL. However, it should also be noted that the differential risk to CHD between blacks and whites with high cholesterol is more clearly appreciated when a common population plasma cholesterol "cut point" is used to define hypercholesterolemia.

The findings of this, and earlier studies (11,12), suggest that C-HDL and C-LDL should be routinely quantitated in all children found to have hypercholesterolemia on initial sampling, whether in clinical practice or in epidemiologic studies. C-HDL and C-LDL measurement is particularly important in black children, who have higher C-HDL levels and are more likely to have their hypercholesterolemia solely accounted for by elevated C-HDL. C-HDL and C-LDL measurement in hypercholesterolemic children will also provide cohorts of children to evaluate "tracking" of C-HDL and C-LDL in upper deciles, and to allow longitudinal assessment of the relationship of C-HDL to eventual development of CHD.

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Distribution of High Density and Other Lipoproteins in Selected LRC Prevalence Study Populations: A Brief Survey

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ABSTRACT

Selected lipid and lipoprotein data from the Lipid Research Clinics (LRC) Prevalence Study are presented, with particular emphasis given to high-density lipoprotein (HDL) values. Cross-sectional age- and sex-specific mean values are shown for 7007 white participants in the ten North American LRCs. Comparisons are drawn for males and females (including the pediatric group) and for females using or not using sex hormones. The US-USSR Collaborative Program is summarized, and selected comparisons are noted for the Soviet and United States samples.

INTRODUCTION

Case-control and prospective epidemiological studies indicate that high-density lipoprotein (HDL) concentrations are strongly, inversely and independently related to coronary heart disease (CHD) risk (1). While the need continues for additional information about this lipoprotein (e.g., its effect on younger subjects), little doubt remains that HDL levels should be added to the list of major risk factors for CHD.

Within this context it is important, for various clinical and epidemiological purposes, to obtain information on the distribution of HDL in the population, its characteristics and its determinants. Valuable insights into the interrelationship of diet, serum cholesterol and CHD have been obtained through comparative studies of cholesterol levels in different populations and subpopulations. Similar information for HDL, together with the other plasma lipoproteins, is clearly needed. Although some data of this nature have been obtained from several studies outside and within the United States, few were population-based. Hence, the small numbers in these studies often precluded age-, sex- or race-specific analysis. Recently, however, the Lipid Research Clinics (LRC) Program has collected data from a sufficiently large population base to permit detailed analyses of lipid and lipoprotein distributions.

Details of the two major collaborative studies of the LRC Program, the Population Studies and the Coronary Primary Prevention Trial are described elsewhere (2,3). The Population Studies encompass three studies that share the same general population base: the Prevalence Study, designed to obtain data on the distribution of lipids and lipoproteins and on the prevalence of dyslipoproteinemia, in diverse populations; the Family Study, established to measure the familial and genetic attributes of plasma lipids and lipoproteins; and the Mortality Follow-up Study, initiated to complement the earlier cross-sectional observations by determining the subsequent mortality experience of approximately 9,000 Prevalence Study participants. Here we report data from the Prevalence Study.

POPULATIONS AND METHODS

Study Populations

The Prevalence Study embraces a series of 14 individual population studies (Table I) in four countries, all of which have used a common protocol and highly standardized methodology. The use of a common protocol permits pooling of the data, but each study also has the potential, based on the size and definition of its population sample, to be analyzed independently of the other studies. The 14 populations were selected to provide considerable diversity and to permit various intra- and international comparisons. The aggregated population base covers a broad range of socioeconomic, geographic, sex and ethnic groups, and spans an age range of 0 to 100 years.

North American Populations

Each of the ten participating LRCs screened participants selected according to standardized procedures from a well defined target population. The sampling strategies utilized for the different populations included the following broad categories: school children and their parents, households in specific geographical

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TABLE I

Lipid Research Clinics Prevalence Study Populations

Sampling frame	Clinic name
Adults in occupational and industrial groups	Seattle Stanford Toronto-McMaster, Canada
Households or family units	Iowa Johns Hopkins (two separate populations) La Jolla Minnesota Oklahoma
Children and their parents	Baylor Cincinnati Jerusalem, Israel
Adult males in defined residential areas	Moscow, USSR Leningrad, USSR

locations, and occupational-industrial groups. The number of individuals eligible for participation totaled 81,970.

Collaborative US-USSR Study Populations

As a result of an agreement signed between the governments of the United States and the Union of Soviet Socialist Republics, LRCs were established in Moscow and Leningrad. Each Soviet clinic conducted a Prevalence Studyu using a protocol essentially identical to that of the North American surveys (4,5).

The Moscow LRC sampled men ages 40-59 residing in the Oktyabrskii District, as identified by the 1974 voting list. A random sample of 5,000 was selected from the target population of 25,000 individuals. A similar strategy was adopted by the Leningrad LRC; of the 10,000 men ages 40-59 identified as residents of the Petrogradskii District, 5,000 were randomly selected for the survey.

For US-USSR comparisons, the LRC Program used lipid data from 9,435 50 to 59-year-old white male participants in the four US clinics that had sampled individuals based on residence in towns or cities. Data for lipoprotein levels were taken from a 15% randomly selected sample of these participants.

Visit 1 and Visit 2 Screens

The Prevalence Study involved two sequential examinations. Visit 1 was a brief screen to collect information on some sociodemographic variables and on utilization of five types of lipid-altering medications, and to measure plasma cholesterol and triglyceride levels in fasting participants ($\geq$ 12 hr). a 15% random sample of the Visit 1 population, as well as all participants who were found to have hyperlipidemia (i.e., lipid values exceeding the age-, race-, sex-specific 90th percentile) or were taking lipid-altering medication, were then asked to return for Visit 2. This more extensive examination involved medical and family histories (including a detailed drug history), blood pressure measurements, lipid and lipoprotein determinations, nutrient intake evaluation by means of a 24-hr dietary recall, resting and exercise electrocardiogrphy, clinical chemistries, and anthropometric measurements.

Lipid and Lipoprotein Measurements

Standardization: An important feature of the LRC Program has been its emphasis on standardization of the lipid and lipoprotein determinations, in order to achieve high precision and accuracy despite the processing of large numbers of samples over a long period of time. All blood specimens were drawn and processed according to carefully specified instructions in the LRC Manual of Laboratory Operations (6). Extensive internal and external quality control systems were employed.

Techniques for lipid and lipoprotein measurements at the Soviet clinics were developed to be directly comparable to those made by the North American clinics. Standardization was reinforced by the regular exchange of personnel and frequent monitoring of data quality.

Lipids: Cholesterol and triglyceride levels were determined in plasma in each LRC core lipid laboratory by use of either the Technicon AutoAnalyzer I (AA-I) or II (AA-II) analytical system, as modified for the LRC Program.

In general, precision and accuracy were extremely high for cholesterol and triglyceride measurements, regardless of which AutoAnalyzer was used (7,8). Most of the variability in lipid measurements arose from differences from sample to sample within a given laboratory rather than from differences between instruments.

Lipoproteins: Concentrations of plasma LDL-, VLDL-, and HDL-cholesterol (LDL-C, VLDL-C, HDL-C) were determined by use of the so-called beta quantification procedure, which involved a combination of preparative ultracentrifugation and heparin-manganese precipitation (6).

HDL-C estimation: Evaluation of the LRC precipitation procedure for determining HDL-C in unfractionated plasma has shown that HDL is not precipitated by heparin-manganese treatment. Although many treated plasma samples do contain some spo-B-associated cholesterol in the supernatant, the resulting

TABLE II

Mean Plasma Total Cholesterol, VLDL-C, LDL-C and HDL-C in White Females in 11 North American Populations[a]

AGE	N	Total cholesterol[b] mg/dl	VLDL-C[b] mg/dl	LDL-C[b] mg/dl	HDL-C[b] mg/dl
5-9	126	164.0 ± 1.8	9.7 ± 0.7	100.4 ± 2.1	53.2 ± 1.0
10-14	248	160.1 ± 1.5	10.9 ± 0.4	97.4 ± 1.3	52.2 ± 0.7
15-19	297	159.5 ± 1.6	11.8 ± 0.5	95.7 ± 1.5	52.3 ± 0.7
20-24	199	170.3 ± 2.5	13.5 ± 0.6	103.7 ± 2.2	53.3 ± 1.0
25-29	314	179.5 ± 1.7	13.4 ± 0.5	110.2 ± 1.6	56.0 ± 0.8
30-34	337	179.2 ± 1.7	12.2 ± 0.5	111.3 ± 1.5	56.0 ± 0.7
35-39	300	189.6 ± 2.1	15.4 ± 0.7	119.7 ± 2.0	55.0 ± 0.8
40-44	319	197.5 ± 1.9	14.7 ± 0.5	125.1 ± 1.8	57.8 ± 0.9
45-49	329	206.2 ± 2.0	17.4 ± 0.7	129.4 ± 1.9	59.4 ± 1.0
50-54	257	217.3 ± 2.4	17.2 ± 0.7	138.1 ± 2.3	62.0 ± 1.0
55-59	251	228.7 ± 2.4	20.7 ± 1.0	146.1 ± 2.4	62.2 ± 1.1
60-64	145	232.3 ± 3.7	16.7 ± 1.8	152.0 ± 3.6	63.8 ± 1.4
65-69	130	234.1 ± 4.0	17.0 ± 1.3	153.8 ± 4.1	63.3 ± 1.8
70+	143	224.5 ± 2.8	15.6 ± 1.2	148.6 ± 2.7	60.7 ± 1.4

[a]Source: LRC Prevalence Study, Visit 2 Random Sample.
[b]Mean ± SEM.

overestimation of HDL-C is slight (ca. 2 mg/dl) (9).

RESULTS AND DISCUSSION

This report highlights three aspects of the data gathered by the LRCs: lipid and lipoprotein distributions in the white North American populations (10), effects of sex hormone use on lipid and lipoprotein levels in females, and comparisons of HDL-C levels in US and USSR populations.

Response Rates

For the North American Prevalence Study, 81,970 individuals were eligible for the Visit 1 screen at the ten LRCs; 60,502 were screened, for an overall response rate of 73.9%.

Approximately 25% (16,335) of the Visit 1 participants were eligible for Visit 2, of whom 13,852 were screened (85% response rate).

In the Union of Soviet Socialist Republics, both clinics achieved a response rate of ca. 80% (3,908 men in Moscow, 3,907 men in Leningrad).

Lipid and Lipoprotein Distributions (North American)

The data described here are from white male (n = 3,581) and female (n = 3,426) Visit 2 participants, ages 5 to 100 years. Data from these 7,007 participants correspond to the 15% random sample of Visit 1 white participants. Cross-sectional age- and sex-specific distributions of mean plasma total cholesterol and VLDL-C, LDL-C and HDL-C are presented in Tables II and III and Figure 1.

In the females a mean HDL-C level of about 53 mg/dl is maintained between the ages 5 to 24 years, after which it rises to 56-58 mg/dl between 25-44 and subsequently to a peak of 64 mg/dl in the 60-65 age band. It then drops to 61 mg/dl by age 70 and beyond.

In males between the ages 5-14 years, mean HDL-C is about 55 mg/dl. After age 14 the HDL-C level decreases sharply to 46 mg/dl, and remains at 44-46 mg/dl through the 50-54 age band, after which it rises to about 51 mg/dl.

The cross-sectional changes with age in the pediatric and adolescent age group are shown in Table IV and Figure 2. Between the ages of 6 and 13 years, males have slightly higher HDL-C concentrations than females, but then a crossover occurs, and after age 13 females have substantially higher HDL-C. This difference increases with age (Tables II,III). In the 45-54 year age groups, the female HDL-C level is 30% higher than the male level. The greatest difference (> 17 mg/dl) is seen at ages 50-54.

Similar male-female differences were described in the Cooperative Lipoprotein Phenotyping Study which was restricted to age groups 40 years and older (11). Higher concentrations of HDL were found in white females in all the age groups studied.

Male-female differences in a postadolescent and adult (16-49 for females and 17-65 for males) population were also observed by Nichols who measured HDL subfractions using analytical ultracentrifugation (12). Markedly higher HDL_2 and slightly higher HDL_3 levels were found in females aged 16 to 49 than in the

TABLE III

Mean Plasma Total Cholesterol, VLDL-C, LDL-C and HDL-C in White Males in 11 North American Populations[a]

Age	N	Total cholesterol[b] mg/dl	VLDL-C[b] mg/dl	LDL-C[b] mg/dl	HDL-C[b] mg/dl
5-9	148	155.3 ± 1.8	8.2 ± 0.5	92.5 ± 1.8	55.8 ± 1.0
10-14	299	160.9 ± 1.5	9.9 ± 0.4	96.8 ± 1.4	54.9 ± 0.7
15-19	299	153.1 ± 1.4	12.8 ± 0.5	94.4 ± 1.3	46.1 ± 0.6
20-24	118	162.2 ± 2.5	13.7 ± 0.8	103.3 ± 2.4	45.4 ± 1.0
25-29	253	178.7 ± 2.1	17.4 ± 0.9	116.7 ± 1.9	44.7 ± 0.7
30-34	403	193.1 ± 1.8	21.3 ± 0.9	126.4 ± 1.6	45.5 ± 0.6
35-39	372	200.6 ± 1.9	24.1 ± 1.0	133.2 ± 1.7	43.5 ± 0.6
40-44	385	205.2 ± 1.9	25.5 ± 1.2	135.6 ± 1.6	44.2 ± 0.6
45-49	327	213.4 ± 1.9	24.4 ± 1.1	143.7 ± 1.8	45.5 ± 0.6
50-54	340	213.2 ± 1.9	26.8 ± 1.1	142.3 ± 1.7	44.1 ± 0.6
55-59	261	215.0 ± 2.2	21.6 ± 1.1	145.8 ± 2.1	47.6 ± 0.9
60-64	131	216.6 ± 3.3	18.9 ± 1.3	146.3 ± 3.1	51.5 ± 1.3
65-69	105	221.0 ± 3.8	19.7 ± 2.0	150.4 ± 3.5	51.1 ± 1.5
70 +	119	210.3 ± 3.4	17.0 ± 1.2	142.9 ± 2.9	50.5 ± 1.7

[a]Source: LRC Prevalence Study, Visit 2 Random Sample.
[b]Mean ± SEM.

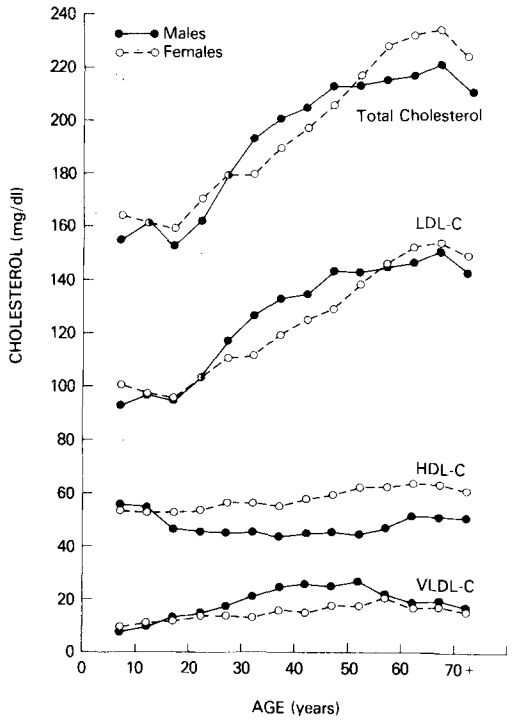

FIG. 1. Mean plasma total cholesterol, LDL-C, HDL-C and VLDL-C by 10-year age groups for 3581 males and 3426 females. LRC Prevalence Study, Visit 2 random sample.

males of the same age.

The cross-sectional changes with age in HDL-C are different from those observed for total cholesterol, VLDL-C and LDL-C (Fig. 1,2). In childhood and adolescence, females have slightly higher total cholesterol, LDL-C and VLDL-C than males (Fig. 2). In the females total cholesterol and LDL-C decrease between ages 8-15 years, and then rise until ages 65-70. In males a marked decline (from 164 mg/dl at age 10-11 to 152 mg/dl at age 16-17) is seen in the concentration of total cholesterol (Fig. 2), followed by an increase which continues to ages 65-70. For LDL-C a lesser decline is observed in adolescent and postadolescent males, but it increases gradually from 93 mg/dl at ages 16-17 to about 150 mg/dl at ages 65-69.

VLDL-C concentrations rise gradually in both sexes up to the sixth decade, although the increment in VLDL-C is greater in males than in females, resulting in higher VLDL-C levels in males ages 25-54. As shown in Figure 1, the slopes of the age-related increases in total cholesterol and LDL-C are greater for males than for females, until ages 45-50. The older cohorts of females continue to show increments in total and LDL-C values until ages 65-70.

For LDL-C values a first crossover between male and female cohorts occurs in the second decade, and for total cholesterol this is seen in the third decade; the male cohorts maintain higher levels than the females throughout the next three decades. A second crossover occurs in the sixth decade when females again show higher levels of total cholesterol and LDL-C.

As indicated in Figure 2, the total cholesterol falls in both sexes at the onset of adolescence. However, the underlying lipoprotein distribution curves differ according to sex. In females the drop in total cholesterol is due to a fall in LDL-C, with no change in VLDL-C or HDL-C. In males the nadir of the total chol-

TABLE IV

Mean Plasma Total Cholesterol, VLDL-C, LDL-C, and HDL-C, in White Children Ages 6-19 years, in Selected North American Populations [a]

Age	N	Total cholesterol[b] mg/dl	VLDL-C[b] mg/dl	LDL-C[b] mg/dl	HDL-C[b] mg/dl
Males					
6-7	67	155.9 ± 2.5	6.9 ± 0.6	95.0 ± 2.5	56.0 ± 1.3
8-9	71	155.6 ± 2.6	9.6 ± 0.8	90.5 ± 2.4	55.6 ± 1.5
10-11	93	164.0 ± 2.9	10.4 ± 0.8	96.8 ± 2.8	57.3 ± 1.2
12-13	742	159.5 ± 1.8	9.0 ± 0.5	95.4 ± 1.7	55.9 ± 1.1
14-15	129	154.8 ± 2.3	10.7 ± 0.7	95.5 ± 2.1	49.2 ± 1.0
16-17	160	152.1 ± 1.8	13.5 ± 0.7	93.2 ± 1.6	45.6 ± 0.8
18-19	67	156.6 ± 3.3	13.8 ± 0.9	99.3 ± 3.2	43.7 ± 1.0
Females					
6-7	58	162.2 ± 2.4	9.8 ± 1.1	100.5 ± 3.3	50.1 ± 1.7
8-9	60	166.1 ± 2.7	10.6 ± 1.1	100.0 ± 2.9	55.7 ± 1.4
10-11	101	161.3 ± 2.3	11.8 ± 0.8	98.1 ± 2.1	51.5 ± 1.1
12-13	102	160.8 ± 2.3	10.6 ± 0.7	97.7 ± 2.0	53.0 ± 1.0
14-15	122	154.9 ± 2.4	10.6 ± 0.6	93.5 ± 2.1	51.0 ± 1.0
16-17	164	159.7 ± 2.1	12.3 ± 0.6	95.2 ± 2.0	52.8 ± 1.0
18-19	53	165.7 ± 4.1	11.1 ± 1.1	101.8 ± 3.5	53.2 ± 1.6

[a]Source: LRC Prevalence Study, Visit 2 Random Sample.
[b]Mean ± SEM.

esterol distribution occurs slightly later than in females and is mainly due to a fall in HDL-C.

Sex Hormone Effects

A high proportion of the white women in the LRC Prevalence Study reported use of some form of sex hormones. The prevalence of sex hormone users was 50% in the 20-24 year age group, and 30-40% in the 50-64 year age groups (11). It is presumed that those over age 45 ("older" women) were predominantly intra- or postmenopausal and were receiving replacement estrogens, whereas most of the women below age 45 years ("younger" women) are presumed to be taking oral contraceptives.

Data from Visit 1 participants show that total cholesterol levels were moderately higher and triglyceride levels were markedly higher in the younger women taking hormones, as compared with nonhormone users (13). Older hormone users showed small and inconsistent alterations in triglyceride and a modest but consistent reduction in cholesterol.

The smaller numbers of female users and nonusers of sex hormones recalled to Visit 2 showed essentially similar cholesterol and triglyceride relationships as those observed in Visit 1 participants. Figure 3 and Table V show that the higher total cholesterol level of the younger users primarily reflects a higher LDL-C, with VLDL-C and HDL-C making only slight contributions.

In the older users, LDL-C increases to a lesser degree than in the nonusers, resulting in a markedly higher LDL-C in the hormone nonusers beyond age 50, which accounts for the

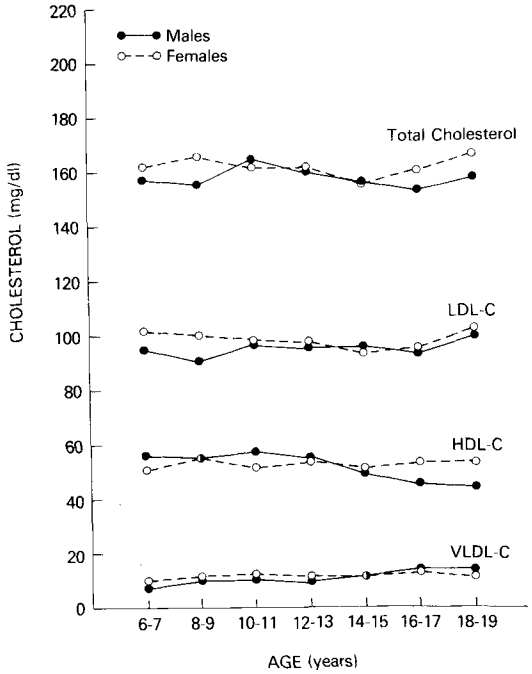

FIG. 2. Mean plasma total cholesterol, LDL-C, HDL-C, and VLDL-C, by two-year age groups for 763 males and 676 females, ages 6-19 years. LRC Prevalence Study, Visit 2 random sample.

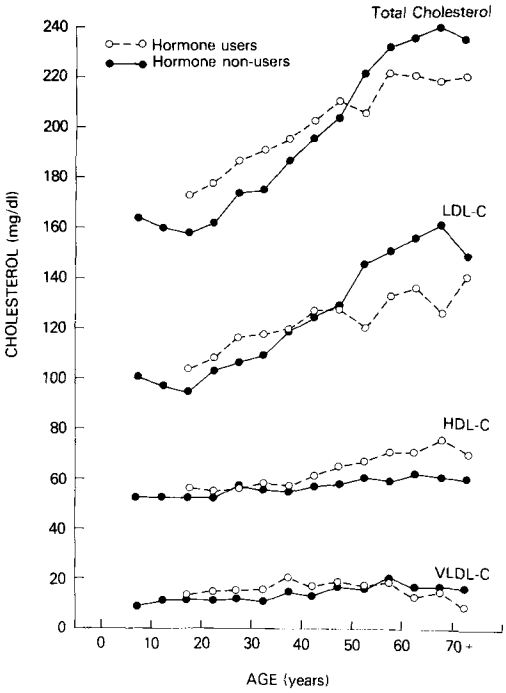

FIG. 3. Mean plasma total cholesterol, LDL-C, HDL-C and VLDL-C by 10-year age groups in 732 females taking and 2601 not taking sex hormones. LRC Prevalence Study, Visit 2 random sample.

higher total cholesterol seen in the same group.

HDL-C, which is only slightly higher in the younger hormone users than in the nonusers, rises sharply in the users after ages 35-39 and reduces the lowering effect of the LDL-C on total cholesterol levels.

This finding of higher HDL-C concentrations in sex hormone users is in contrast to those described by Arntzenius et al. (14), who found lower HDL-C concentrations in oral contraceptive (OC) users ages 40-41 yr than in nonusers. This difference was independent of smoking habit.

Variation in findings in different studies may be related in part to constituents and doses of estrogen and progestin within the OCs (15-17). Plasma total cholesterol and VLDL-C are positively related to the dose of the estrogen component, and significantly elevated HDL-C levels are present only in users of OCs containing greater than 50 μg ethinyl estradiol or mestranol (15). Therefore, different studies on the effect of sex hormones on plasma lipids and lipoproteins cannot be compared unless the type and amount of hormones used are comparable.

U.S.-U.S.S.R. Comparisons

Comparisons of plasma lipid levels in the males (white, ages 40-59) in the two Soviet LRCs and four U.S. LRCs showed that total cholesterol was consistently higher in the Soviet men, ranging from ca. 15 mg/dl higher in the 40-44 age band, to 6-10 mg/dl higher in the other three age bands. Conversely, triglyceride levels were markedly higher in the U.S. males, ranging from 30 mg/dl higher at ages 40-46 to 25 mg/dl higher at ages 50-59 (5).

Examination of the lipoprotein concentrations in the two samples revealed several interesting comparisons. HDL-C values were generally constant, but with a slight rise in the 55-59 age band, in U.S. males; such an age trend was less apparent in the Soviets. HDL-C was consistently higher in the U.S.S.R. than in the U.S. samples at all ages, and in an amount that accounts for a large fraction of the observed differences in total cholesterol (5).

The entire distribution curve for HDL cholesterol was shifted to the right in the Soviet sample with a tendency toward greater skewing at the higher values. This resulted in a 95th percentile value for the Soviet sample of 86 mg/dl, a value not reached in the U.S. sample. It is also noteworthy that more than 2% of the Soviet examinees had HDL-C levels of at least 100 mg/dl (5).

The purpose of this report has been to highlight key findings that are emerging from the initial analysis of data gathered in 14 populations under the auspices of the Lipid Research Clinics Prevalence Study, and to alert the research community to the diversity of reports that are anticipated as soon as the entire study has been completed (e.g., the Soviet LRCs only recently completed data collection, and the Israeli LRC Prevalence Study is still in progress). Here only three aspects of the data have been touched upon as examples of the myriad of research possibilities arising from the LRC Prevalence Study.

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TABLE V

Mean Plasma Total Cholesterol, VLDL-C, LDL-C, and HDL-C, in White Females, by Sex Hormone Usage [a]

Age	N	Total cholesterol[b] mg/dl	VLDL-C[b] mg/dl	LDL-C[b] mg/dl	HDL-C[b] mg/dl
Users					
15-19	20	173.0 ± 9.3	13.7 ± 2.5	103.6 ± 8.5	56.1 ± 3.0
20-24	101	178.0 ± 3.4	15.2 ± 0.9	108.3 ± 3.3	54.7 ± 1.4
25-29	132	187.2 ± 2.6	15.3 ± 1.0	115.9 ± 2.5	56.1 ± 1.3
30-34	97	190.7 ± 3.3	15.6 ± 0.9	117.4 ± 3.1	58.0 ± 1.7
35-39	50	195.6 ± 6.1	20.4 ± 2.0	118.9 ± 5.4	57.0 ± 2.2
40-44	63	202.9 ± 5.0	17.2 ± 1.5	127.8 ± 4.7	61.1 ± 2.5
45-49	72	211.8 ± 4.2	19.0 ± 1.4	127.5 ± 4.0	65.5 ± 2.1
50-54	70	205.9 ± 3.5	18.7 ± 1.1	119.7 ± 3.3	67.6 ± 2.1
55-59	60	222.4 ± 4.9	19.2 ± 1.9	132.5 ± 4.7	70.8 ± 2.3
60-64	32	220.2 ± 5.2	13.5 ± 1.3	136.6 ± 5.0	70.4 ± 2.8
65-69	25	218.0 ± 5.9	15.6 ± 2.7	126.4 ± 5.7	76.1 ± 4.7
70+	10	220.3 ± 7.1	9.1 ± 2.1	140.8 ± 8.1	70.7 ± 3.8
Non-users					
15-19	276	158.3 ± 1.6	11.7 ± 0.5	94.9 ± 1.4	52.0 ± 0.7
20-24	96	162.1 ± 3.4	11.9 ± 0.7	98.1 ± 2.8	52.2 ± 1.5
25-29	181	173.9 ± 2.2	12.0 ± 0.6	106.0 ± 2.0	56.0 ± 1.0
30-34	235	174.8 ± 1.9	10.8 ± 0.6	109.0 ± 1.7	55.4 ± 0.8
35-39	243	187.5 ± 2.2	14.3 ± 0.7	118.8 ± 2.1	54.8 ± 0.9
40-44	245	196.1 ± 2.1	13.9 ± 0.5	125.2 ± 2.0	57.1 ± 1.0
45-49	249	204.3 ± 2.3	16.8 ± 0.8	129.7 ± 2.1	57.7 ± 1.1
50-54	178	222.7 ± 3.1	16.4 ± 0.9	146.1 ± 2.9	60.1 ± 1.1
55-59	172	232.0 ± 3.0	21.2 ± 1.3	151.8 ± 2.9	59.1 ± 1.3
60-64	111	235.7 ± 4.6	17.6 ± 2.3	156.3 ± 4.5	62.0 ± 1.6
65-69	101	239.6 ± 4.6	17.6 ± 1.6	161.6 ± 4.7	60.5 ± 1.8
70+	127	224.8 ± 3.0	16.2 ± 1.3	148.9 ± 2.9	60.1 ± 1.5

[a]Source: LRC Prevalence Study, Visit 2 Random Sample.
[b]Mean ± SEM.

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Effects of Estrogens and Progestins on High Density Lipoproteins

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ABSTRACT

High density lipoprotein (HDL) levels are known to be higher in women than in men, and to increase with estrogen use. To assess the effects of estrogens on HDL subspecies, analytic ultracentrifuge measurements of HDL were compared in 11 menopausal estrogen users and 16 controls. The difference in mean schlieren patterns between the groups showed a significantly higher level of HDL with flotation rate ($F^{\circ}_{1.20}$) > 1.5 (predominantly HDL_2) in the users. This was similar to the difference in HDL seen between nonusers of hormones and age-matched males. A previous study had shown that users of combination oral contraceptives had increased levels of HDL with $F^{\circ}_{1.20} \leq 3.5$ (primarily HDL_3) suggesting that the estrogen effect on HDL is altered by the presence of added progestin. The progestin effect was studied here in more detail in two women with type V hyperlipoproteinemia treated with norethindrone acetate. Reduction in serum triglyceride was accompanied by a reduction in HDL, predominantly in the less dense species (HDL_2). Among groups of oral contraceptive and noncontraceptive estrogen and progestin users whose HDL-cholesterol levels have been reported recently, there was a direct correlation ($r = 0.86$, $p < .001$) between mean HDL-cholesterol and triglyceride levels. Endogenous hormonal influences on HDL were assessed by serum hormone and lipoprotein measurements at weekly intervals during two consecutive menstrual cycles in four healthy females. An increase in HDL of highest flotation rate ($F^{\circ}_{1.20}$ 5-9) was seen, which corresponded with the time of ovulation, raising the possibility of pituitary as well as gonadal hormone effects on HDL.

INTRODUCTION

It has long been recognized that premenopausal women have higher levels of high density lipoprotein (HDL) than men (1-6), and that administration of estrogens to either sex may increase total HDL (6-8). The effects on HDL of another of the major female gonadal hormones, progesterone, have not been identified. There has been renewed interest in the influence of these hormones and of combination estrogen-progestin oral contraceptives on HDL because of the increased incidence of coronary disease reported in "pill" users (7-11), and the strong inverse relationships between levels of HDL and coronary risk (12,13).

Data are presented and reviewed here which indicate that exogenous estrogens, progestins and combinations have differing effects on specific subfractions of HDL and on HDL-cholesterol. Also, fluctuations of HDL during the normal menstrual cycle are shown in relation to endogenous estrogen, progestin, and luteinizing hormone levels.

METHODS

Subjects

Several groups of subjects were studied. Estrogen effects on HDL were analyzed in 11 menopausal women aged 44-66 who were using conjugated estrogens (0.625-1.25 milligrams per day) and in 16 menopausal women of comparable age who had not used estrogens or contraceptives for at least six months. The measurements were performed previously as part of a study of lipids and lipoproteins in a sample of the population of Modesto, California (14). For calculation of male-female differences in HDL, analytical ultracentrifuge data from the Modesto study population were also used. In the age group 27-46 years, there were 40 men and 29 women who were not using estrogens or oral contraceptives, and in the age group 47-66 years, there were 40 men and 25 women who were not using hormones. Blood samples were obtained within 8 hr of a light, fat-free breakfast or breakfast and lunch.

The influence of oral contraceptives and noncontraceptive estrogen and progestin use on serum HDL-cholesterol and triglyceride were studied in 4,978 healthy female volunteers enlisted in the Walnut Creek Contraceptive Drug Study. Details of this study and a description of the cohort have been published (15,16). In the present analysis, two "miscellaneous" treatment groups were omitted, leaving 17 groups of hormone users (n = 1382) and a group of 3422 nonusers.

HDL were also analyzed in two women ages 58 and 62 with type V hyperlipoproteinemia being treated with the progestin, norethindrone acetate. Blood samples were taken after overnight fast at intervals indicated in Results.

Finally, weekly measurements of HDL were

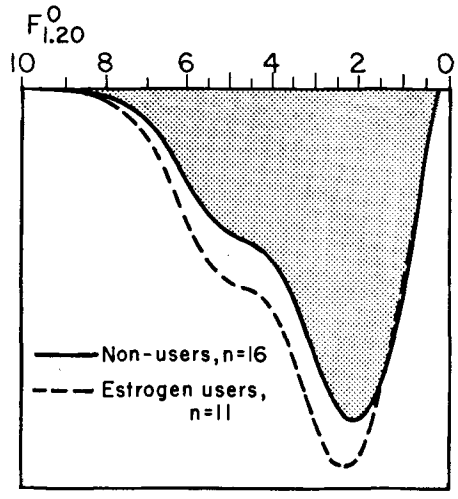

FIG. 1. HDL as measured by analytic ultracentrifugation in estrogen users and nonusers. The curves are the computer-derived means of individual curves in each group.

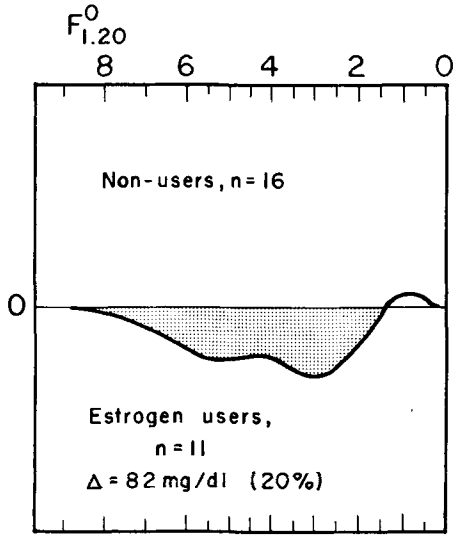

FIG. 2. Differences in HDL between estrogen user and nonuser groups. The curve represents the computer-derived difference between the mean HDL curves for the two groups shown in Fig. 1. The area under the baseline (shaded) represents the increase in lipoproteins in the user group.

carried out on blood samples obtained after an overnight fast during the course of the two sequential menstrual cycles in each of four healthy volunteers ages 22-26. The subjects were asked not to vary their diet or exercise levels during the course of the study.

Methods of Measurement

Analytical ultracentrifuge measurements of serum HDL were performed as described previously (17). Computer techniques were employed to generate individual and mean corrected schlieren patterns, to plot curves representing differences between pairs of schlieren patterns, and to calculate concentrations of total HDL and HDL of specified flotation rates (17).

HDL-cholesterol was measured by a modification of the heparin-manganese chloride method (18,19).

Total serum cholesterol and triglyceride concentrations were measured using either the Technicon Autoanalyzer (AA II) (18), or enzymatic methods (20,21).

Concentrations of serum estradiol, progesterone and luteinizing hormone (LH) were measured by radioimmunoassay techniques.

RESULTS

Estrogen Effects on HDL

The mean corrected schlieren patterns of HDL measured in the analytic ultracentrifuge in estrogen users and controls are shown in Figure 1. Estrogen users had higher levels of HDL with flotation rate ($F^0_{1.20}$) greater than 1.5. The differences are shown more precisely by subtracting the curve of the nonusers from that of the users (Fig. 2). This yields a "difference curve" with three peaks: two which are higher in the faster floating region, and one which is lower in the estrogen users in the slower floating region. The positive difference, 82 mg/dl or 20% of the control level, was significant at $p < 0.05$, while the negative difference was not statistically significant.

It is possible to compare these differences in HDL with those between men and women (nonusers of hormones) in the same population (14,22) (Fig. 3). The same three flotation peaks are present, the two higher in women corresponding to those higher in the estrogen users, and the third with slower peak flotation rate lower in both groups. These three peaks recently have been shown by Anderson et al. to be due to the presence of three subfractions of HDL separable by equilibrium density gradient ultracentrifugation (22) and have been designated as HDL_{2b}, HDL_{2a}, and HDL_3. Thus, estrogen use appears to be associated with higher levels of HDL_{2b} and HDL_{2a}, but not HDL_3 and the difference in HDL between users

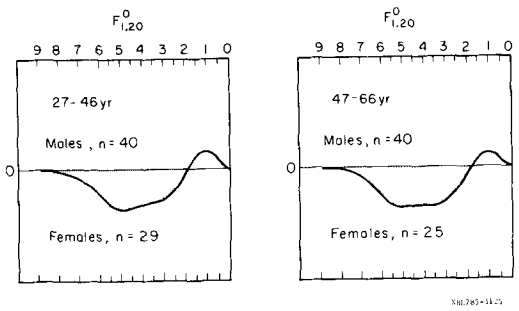

FIG. 3. Differences in HDL between men and women (nonusers of hormones) in age groups 27-46 and 47-66 in the Modesto population study (14). Mean HDL difference curves were computer-plotted as in Fig. 2. The shaded areas represent the lipoproteins higher in women than men.

and nonusers resembles the difference in HDL between women and men.

Norethindrone Acetate Effects on HDL and Triglyceride

The effect of estrogen on HDL (Fig. 2) is in contrast to the findings previously reported in users of oral contraceptives, namely an increase in HDL of $F^{0}_{1.20}$ 0-3.5, but not in faster floating HDL (23). On the basis of the HDL subclassification described above, the increase appears to include predominantly HDL_3 and to a lesser extent HDL_{2a}, but not HDL_{2b}.

The most likely cause of the different results in estrogen and contraceptive users was an effect of the added progestin on HDL. The effect on HDL of one of the progestins, norethindrone acetate, was studied in detail in two female subjects with type V hyperlipoproteinemia. Figure 4 shows serum total HDL as measured in the analytic ultracentrifuge and serum triglyceride in one of the subjects before, during, and following treatment with norethindrone acetate. The initial values were obtained when she was using conjugated estrogens. Upon withdrawal of estrogen, there was a reduction in serum triglyceride and total HDL, predominantly in the HDL_2 region. Within one week of introduction of norethindrone acetate, there was a further reduction in these measurements, progressing slightly with time and increased dosage to involve a reduction in HDL_3 as well. Drug withdrawal resulted in a return in all measurements towards baseline levels.

In the second subject, serum triglyceride fell from 4994 mg/dl to 2772, 2344, and 2073 mg/dl after one, two and three weeks of treatment with norethindrone acetate, 5 mg per day. Serum total HDL fell from 299 mg/dl to 229, 195, and 168 mg/dl at the same three points. As in the first subject, the reduction in HDL was predominantly in less dense HDL ($F^{0}_{1.20}$ 2-9), and in this case there was also a slight increase in HDL of $F^{0}_{1.20}$ 0-2.

Since norethindrone acetate treatment resulted in simultaneous lowering of triglyceride and HDL in these patients, the relationship of serum triglyceride and HDL-cholesterol was further examined using the Walnut Creek Oral Contraceptive Drug Study population (Fig. 5) (see Methods). A strong positive correlation is seen among the user groups, suggesting that use of contraceptive steroids, estrogens, and progestins results in parallel changes in serum HDL-cholesterol and triglyceride.

HDL and the Menstrual Cycle

Having investigated the effects of exogenous sex hormones on HDL, we turned to the study of changes in HDL as influenced by endogenous hormones in the normal menstrual cycle. Due to variation in cycle length and timing of hormonal peaks, it is not possible to group the data

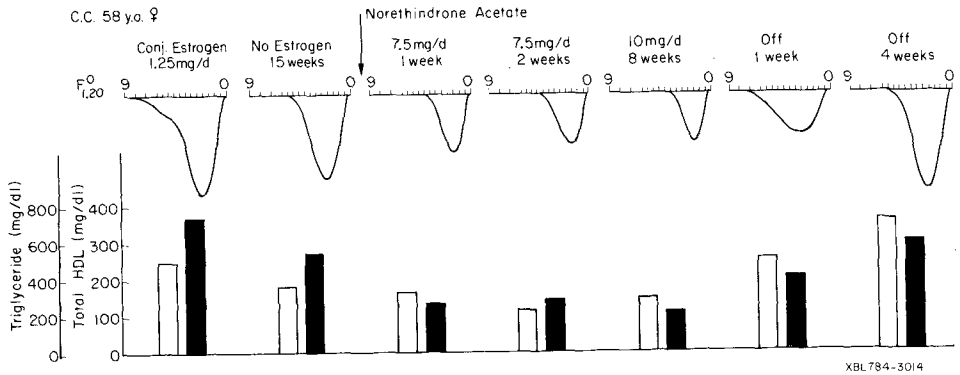

FIG. 4. Serum triglyceride and total HDL as measured by analytic ultracentrifugation in a subject with type V hyperlipoproteinemia and taking estrogen or norethindrone acetate.

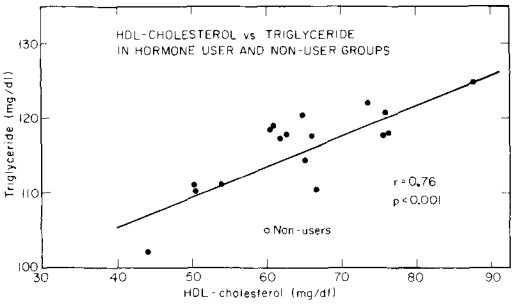

FIG. 5. Mean serum triglyceride plotted against mean HDL-cholesterol for each of the 17 hormone user groups (•) and the nonuser group (○) in the Walnut Creek Contraceptive Drug Study population (16).

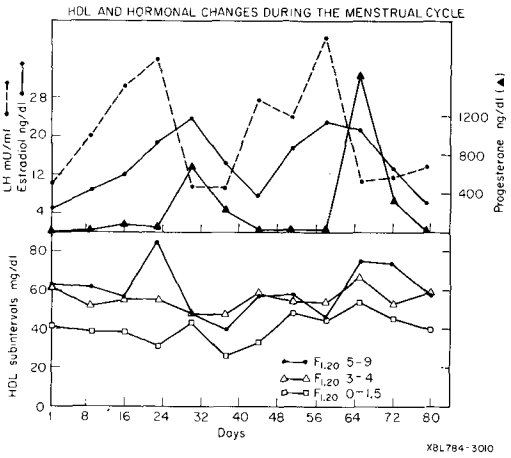

FIG. 6. Measurements of serum estradiol, progesterone and LH (upper panel), and HDL (lower panel) during two consecutive menstrual cycles in a healthy female. Cycles begin with the first day of menstruation at days 1 and 35. HDL concentrations, measured by analytic ultracentrifugation, are shown in three flotation intervals: $F^{\circ}_{1.20}$ 0-1.5 (contained within the HDL_3 subgroup), $F^{\circ}_{1.20}$ 3-4 (contained within HDL_{2a}), and $F^{\circ}_{1.20}$ 5-9 (contained within HDL_{2b}) (cf. Ref. 22).

for the four subjects studied. Fig. 6 shows the HDL (lower panel) and gonadal hormone levels (upper panel) in one subject during two sequential menstrual cycles, the results being representative of those in the other subjects. Ovulation occurs at the time of the midcycle peak in level of LH, after which progesterone concentration increases markedly. Estrogen levels before and after ovulation are comparable. Concentration of HDL of $F^{\circ}_{1.20}$ 0-1.5 and 3-4 showed no systematic changes during the cycle, while levels of HDL of $F^{\circ}_{1.20}$ 5-9, representative of HDL_{2b}, increased at or just after the time of ovulation and then declined rapidly. Due to the complexity of the various hormone patterns and the fact that other gonadal and pituitary hormones were not measured, it is not possible to link the midcycle increase in HDL_2 to any specific hormone change, but the temporal association with LH is suggestive.

DISCUSSION

Exogenous gonadal hormones (6-8) and derivatives (16, 23, 24-26) may exert major effects on serum HDL. Interest in these effects derives not only from the possible consequences of altered HDL in the large number of women using such preparations, but also from the insights that may be gained regarding the influence of endogenous gonadal hormones on the control of HDL levels.

Estrogens are known to increase HDL, and in the present paper this increase has been shown to involve primarily the less dense HDL_2 subfractions. Synthetic progestins may lower HDL, at least in women with hypertriglyceridemia. Again the major effect is on HDL_2. In combination with estrogen, progestins tend to shift the increase of HDL towards the more dense HDL_3 subspecies (23), and specific progestins may increase or decrease HDL-cholesterol (16). That these effects are pharmacologic is self-evident, but it is instructive to identify the possible differences between pharmacologic and physiologic hormone actions that might be involved in influencing HDL.

Estrogens, commonly administered as synthetic estrogen derivatives, or as conjugated "natural" estrogens (primarily estrone and equilin), result in supra-normal HDL levels. This may be due to specific drug effects, to unphysiologically high serum estrogen levels, or possibly to alterations in the normal pituitary-gonadal feedback relationships. It has not been determined whether elevated endogenous estradiol or estrone levels, as seen with estrogen-producing ovarian tumors, have a similar effect on HDL.

In the case of progestins, the situation is even more complex due to the number of pharmacological actions associated with these drugs, namely, progestational, androgenic, estrogenic, anabolic, and anti-estrogenic (16, 27). In a previous publication (16), we have suggested that the effects on HDL-cholesterol of progestins in oral contraceptives appear to bear some relation to the progestins' relative anti-estrogenic or estrogenic effects, but other properties such as androgenicity cannot be dismissed, particularly since androgens are

known to lower HDL levels (6,8). Furthermore, as with other cross-sectional data, it is not possible to rule out patient selection factors which might have influenced HDL levels in the treatment group.

A final aspect of the pharmacology of exogenous hormone effects on HDL is the direct correlation with effects on serum triglyceride and presumably VLDL. Although preliminary analyses (Wingerd and Krauss, unpublished) indicate that within groups of hormone users the expected inverse relation between HDL and triglyceride (28) is generally seen, the present results and those of others (8) suggest that the overall metabolic relationships between HDL and VLDL are influenced by hormone use, possibly by parallel effects on synthesis and/or catabolism of these lipoproteins.

Studies of lipoproteins in the normal menstrual cycle, while confirming an increase in HDL in mid-cycle (29,30), have not helped to define the hormonal determinants of this increase except to suggest that factors other than estrogen level are likely to be involved. It is not known what effects endogenous progesterone (as opposed to synthetic progestins) may have on HDL, but it may be that the postovulatory surge in progesterone has a role in reducing HDL toward baseline levels. A possible role of pituitary gonadotropin in contributing to the ovulatory peak in HDL must also be considered.

Since HDL levels are known to increase with age in women (16,29) it is difficult to sort out effects due to menopause per se. It may be, however, that loss of progesterone effect or enhanced gonadotropin levels might contribute to the increase in HDL in older women.

Since the inverse relationship in HDL and coronary risk has recently received renewed interest (12,12), it would seem appropriate to comment on the possible role that hormone-induced changes in HDL might have in relation to this risk. An argument has recently been brought forth that HDL_2, by virtue of its correlation with total HDL and HDL-cholesterol, represents the HDL components most likely to correlate with coronary disease incidence (31). Thus, in terms of HDL alone, estrogens would theoretically have an ameliorating effect on coronary risk, and the majority of progestins and contraceptives a neutral or negative effect. It has been shown, however, that estrogen use increases the incidence of coronary events in either sex (32-35), and recently high endogenous estradiol/testosterone ratios have been identified in men with accelerated coronary disease (36). Any putative "protective" role of enhanced HDL in estrogen users might well be reversed by other estrogen effects, such as increases in VLDL or changes in other lipoprotein fractions (8), or in blood pressure (37). Similar considerations hold true for the effects of oral contraceptives, although here it is tempting to suggest that specific preparations associated with reduced HDL-cholesterol might contribute to increased coronary disease in pill users.

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HDL-Cholesterol Levels in the Multiple Risk Factor Intervention Trial (MRFIT) By the MRFIT Research Group[1,2]

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ABSTRACT

Preliminary data from the Multiple Risk Factor Intervention Trial (MRFIT) have been examined for evidence that the program has an influence on plasma HDL-cholesterol. The overall mean level of this lipoprotein in the initial cohort of 1,084 men was not altered by two years of participation in this risk factor reduction project. However, changes did occur, both upwards and downwards, in some individuals. There were significant negative associations between change in HDL-cholesterol and changes in body mass, VLDL-cholesterol, LDL-cholesterol, and serum thiocyanate (a measure of cigarette smoking exposure); and there was a small positive association with change in reported alcohol intake. Multiple regression analysis revealed each of these associations to be independent of the others. The fat-controlled diet designed to lower total serum cholesterol did not decrease HDL-cholesterol levels. We conclude that conventional risk reduction programs are not likely to lower circulating HDL-cholesterol, and that program components such as weight reduction and smoking cessation may increase the levels.

INTRODUCTION

The Multiple Risk Factor Intervention Trial (MRFIT) is a randomized collaborative study to test the hypothesis that a six-year program for lowering three major coronary risk factors will reduce mortality from coronary heart disease (CHD) among men at above average risk (1). Beginning in November 1973, men aged 35-57 were recruited in 22 clinical centers. All were initially free of overt coronary heart disease, but judged to be at above average risk. Eligibility for the trial was based on a combined risk score calculated from the serum cholesterol level, the diastolic blood pressure and the reported cigarette smoking history. This approach allowed some participants with average or even below average serum cholesterol levels to enter the trial if the blood pressure and smoking frequency were sufficiently high. Men with serum cholesterol levels exceeding 350 mg/dl were excluded to avoid the ethical concern of withholding drug treatment from such individuals, and severe hypertension was also a cause for exclusion. Study participants had to be willing to enter a six-year study and to be randomized to either study group.

A series of three baseline examinations to determine eligibility culminated in the randomization of participants to two groups of equal size, termed Special Intervention (SI) and Usual Care (UC). Participants in the SI group were advised to follow a standard fat-controlled eating pattern designed to lower serum cholesterol concentration, and weight reduction was sought among those who were overweight. Participants who smoked cigarettes were encouraged to quit. Participants who were hypertensive entered a stepped-care program of weight reduction, moderate sodium restriction, and conventional pharmaceutical agents (2). A variety of group and individual counseling techniques was used in the intervention efforts (3).

The recruitment phase of the MRFIT was successfully completed in February of 1976 with 12,886 men entered into the trial. The progress of the trial during the subsequent two years of intervention has been satisfactory, with high rates of followup, and with reductions, among SI participants, in the levels of the three major risk factors (3).

This paper is a preliminary examination of a particular component of the MRFIT data: the changes in HDL-cholesterol among SI participants during the first two years of the trial. The importance of this examination stems from the epidemiological observations implicating HDL-cholesterol as a possible CHD risk-lowering factor (4,5) and from the fact that relatively little is known about the determinants of circulating HDL-cholesterol. Therefore, it is pertinent to consider the influence that efforts to lower conventional CHD risk factors might have on HDL-cholesterol, and particularly whether dietary recommendations directed at lowering total serum cholesterol concentration might affect this lipoprotein.

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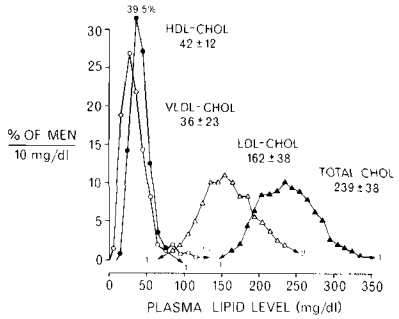

FIG. 1. Frequency distributions of fasting plasma HDL-cholesterol level and other cholesterol fractions in the initial cohort of MRFIT participants at baseline (N=2151). Mean ± SD is provided; the relatively high mean for total plasma cholesterol is a consequence of the criteria for selection of MRFIT participants.

These issues are explored in this report, an enlargement on a pilot study carried out after the first year of intervention (6). In addition, information concerning the determinants of circulating levels of HDL is sought by regression analyses of the relation between other risk variables and HDL-cholesterol levels.

EXPERIMENTAL PROCEDURES

The baseline findings reported here are for the 2,151 participants who had completed the second annual examination by the time of this analysis. The longitudinal findings are those of the 1,084 of these participants in the Special Intervention (SI) group. Although this number of observations is large by ordinary standards, the sample is only the first one-sixth of all MRFIT participants.

The dietary specifications of the fat-controlled eating pattern included (for the period of observation reported here) reduction of cholesterol intake to less than 300 mg daily and restriction of total fat to the range 30-35% of calories with less than 10% of calories as saturated fat and with 10% as polyunsaturated fat. No lipid-lowering drugs were prescribed by MRFIT physicians. The pharmaceutical agents prescribed for hypertensive participants (after a trial of weight reduction, where appropriate) included hydrochlorothiazide or chlorthalidone as first line drugs, followed sequentially by reserpine (or Aldomet), hydralazine and guanethidine (2).

The chemical methods were patterned on those of the Lipid Research Clinics (7). Blood specimens were drawn in EDTA at the second baseline exam after an overnight fast. The plasma specimens were stored at +4 C and shipped at this temperature from each clinical center to the Central Laboratory in San Francisco, where analysis was carried out within ten days of the venipucture. Plasma cholesterol and triglyceride concentration were analyzed on the Autoanalyzer II, using calibrators provided by the CDC Lipid Standardization Laboratory to give cholesterol results comparable to those of the LRC and of the Abell-Kendall Reference Method. HDL-cholesterol concentration was measured in the supernatant following precipitation of the low and very low density lipoproteins by heparin and manganese. For participants with triglyceride levels less than 300 mg/dl, VLDL-cholesterol was estimated by the Friedewald technique (8), that is, dividing the triglyceride concentration (in mg/dl) by five. LDL-cholesterol was then calculated as the difference between the total cholesterol and the other lipoprotein fractions. For participants whose triglyceride concentration exceeded 300 mg/dl, preparative ultracentrifugation was used to provide a more accurate VLDL-cholesterol value.

Serum thiocyanate, a measure of cigarette smoking exposure (9), was determined by the automated method of Butts et al. (10). The habitual number of alcoholic drinks consumed per week was assessed by questionnaire. Diastolic blood pressure was the average of two readings taken with the random zero muddler device after sitting for 5 min in a quite room. Adherence to the MRFIT fat-controlled diet was rated excellent, good, fair or poor by a nutrition specialist four months prior to the second annual visit, using a semistructured subjective interview.

TABLE I

HDL-Cholesterol Levels in Special Intervention Participants over the First Two Years of the MRFIT (N=1084)

	Plasma HDL-cholesterol (mg/dl)		
	Baseline	Year 2	Difference
Mean	42.1	42.7	+0.6
S.D.	11.6		10.3

RESULTS AND DISCUSSION

The frequency distribution of total cholesterol and of each lipoprotein cholesterol fraction at baseline are shown in Figure 1. The distribution of HDL-cholesterol concentration is roughly bell-shaped and has a mean of 42 mg/dl. The value for mean total cholesterol is 239 mg/dl, the sum of the three lipoprotein-cholesterol fractions.

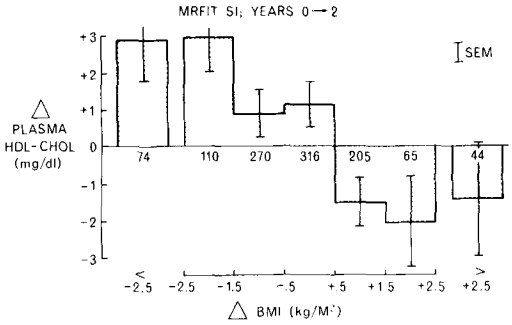

FIG. 2. Mean change (±SEM) in plasma HDL-cholesterol level according to change in degree of obesity, expressed as body mass index (BMI). A decrease in BMI of one unit in a 6-foot man is equivalent to a weight loss of 3.3 Kg.

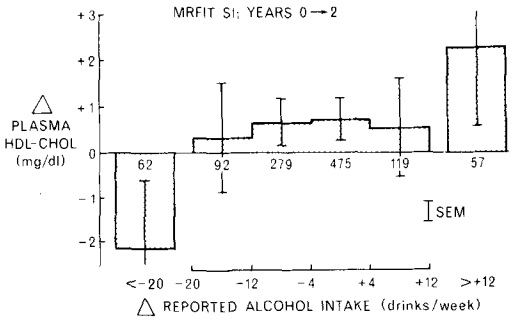

FIG. 3. Change in plasma HDL-cholesterol level according to change in reported habitual alcohol intake.

Table I shows mean HDL-cholesterol levels, among the 1,084 special intervention portion of the cohort, at baseline and at the second annual examination. The mean change, an increase of .6 mg/dl, is not significantly different from zero. However, the standard deviation of the change was relatively high—10 mg/dl—implicating major deviations from baseline, both upwards and downwards, in some individuals. The degree of spread in the distribution of values for change in HDL-cholesterol level was probably not due to laboratory variations alone because the precision of the analysis is relatively high; repeated determinations on a single specimen have a standard deviation in our laboratory of only 3 mg/dl (i.e., CV=6.7%). Biologic sources of variance may also have influenced the plasma HDL-cholesterol levels of some individuals. These were sought by examining the relationship between change in HDL-cholesterol level and change in other relevant variables over the two-year period.

Bivariate Analyses

Figure 2 shows change in plasma cholesterol level according to change in body mass index. Participants who had a decrease in body weight, shown on the left, had an increase in plasma HDL-cholesterol level. Conversely, those who gained weight had a decrease in HDL-cholesterol. These findings are consistent with prior evidence, largely cross-sectional, that HDL levels are inversely related to body weight (11-15). However, the magnitude of the mean HDL-cholesterol change in the MRFIT was small. Causal inference (that the weight loss leads to the increase in HDL) is supported by the dose-response pattern, but it is also possible that the changes in weight and the HDL-cholesterol were not causally linked, both being influenced by an unidentified third factor.

Figure 3 shows change in HDL-cholesterol according to change in reported habitual alcohol intake. On the average, persons reporting a marked decrease in alcohol intake had a slight decrease in HDL-cholesterol and those with a marked increase in alcohol intake had a slight increase in this lipoprotein. The great majority of participants reported lesser changes in alcohol intake and had mean changes in HDL-cholesterol level that were negligible. Thus, the longitudinal findings in this cohort do not demonstrate the strong association between reported alcohol intake and HDL-cholesterol level noted in previous cross-sectional (14,16) and longitudinal (17) studies.

The mean change in plasma HDL-cholesterol level according to change in serum thiocyanate level is shown in Figure 4. Serum thiocyanate, designated SCN, is a biochemical test which provides an objective measure of cigarette smoking habits (9). The groups of participants who had substantial decreases in the level of serum thiocyanate were found to have increased mean HDL-cholesterol levels. Participants whose serum thiocyanate changed less than 80 μMol/L in either direction had no appreciable change in mean HDL-cholesterol. The implication that persons who quit smoking have a small rise in plasma HDL-cholesterol was also shown in this study by self-report data on smoking habits, and the observation is consistent with previous cross-sectional studies (14,18). Of course, this does not prove that quitting smoking causes circulating HDL-cholesterol level to increase; the association between the two events could be due to an unidentified confounding factor.

Figure 5 shows change in HDL-cholesterol level according to the degree of adherence to the MRFIT eating pattern. There was no significant HDL-cholesterol change at any level of

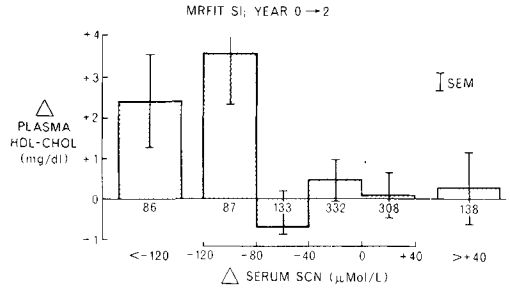

FIG. 4. Change in plasma HDL-cholesterol level according to change in serum thiocyanate level. Serum thiocyanate in an index of tobacco exposure (9).

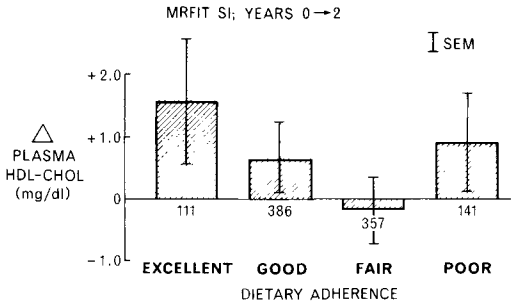

FIG. 5. Change in plasma HDL-cholesterol level according to estimated degree of adherence to the MRFIT fat-controlled diet.

adherence, although there was a tendency toward a small increase in the group with excellent adherence. Since degree of adherence had the expected relationship with the magnitude of the decrease in *LDL*-cholesterol (not shown), the absence of an observed association with HDL-cholesterol suggests that the MRFIT diet does not have a major influence on this fraction.

Multivariate Analyses

The bivariate categorical analyses in Figures 2 through 5 do not take into account the confounding correlations that may exist among these and other variables. For example, losing weight and quitting smoking both tend to be associated with an increase in HDL-cholesterol. Yet people who quit smoking are likely to gain weight.

The relationship between each factor and HDL-cholesterol, independent of the other factors, is examined by multiple regression analysis (Table II). The multivariate regression coefficients are measures of the association between the change in the independent variable specified and the change in HDL-cholesterol level (as the dependent variable) when all the other variables are held constant. The use of standardized coefficients (coefficients which have been multiplied by the standard deviation of the independent variable) allows the relative importance among the associations to be estimated; the larger the coeffieicnt, the stronger the association.

The regression analysis shows the negative association between change in body mass index and change in HDL-cholesterol to be independent of the other change variables listed. The multivariate analysis also confirms the existence of statistically significant independent associations between change in HDL-cholesterol and changes in reported alcohol intake (positive) and serum thiocyanate (negative).

TABLE II

Longitudinal Associations with Change in Plasma HDL-Cholesterol as the Dependent Variable over the First Two Years of the Trial among 1,084 Special Intervention Participants[a]

Independent variable	Correlation coefficient	Standardized multiple regression coefficient
Δ Body mass index	-.12[b]	-.09[c]
Δ Reported alcohol intake	.05	.09[b]
Δ Serum thiocyanate	-.07	-.09[b]
Adherence to MRFIT diet	.04	.03
Δ Diastolic BP	+.11[b]	.10[b]
Δ VLDL-cholesterol	-.17[b]	-.16[b]
Δ LDL-cholesterol	-.14[b]	-.13[b]
baseline HDL-cholesterol	-.47[b]	-.44[b]

[a]Baseline selection criterion variables (serum cholesterol, DBP and cigarettes/day) were also included in the regression model as partial compensation for biases created by using these variables as criteria for selecting study participants. All these variables had small and nonsignificant coefficients.

[b]$p < .001$.

[c]$p < .01$.

TABLE III

Cross-sectional Associations with Plasma HDL-cholesterol as the Dependent Variable among 1,084 SI Participants at Baseline

Independent variable	Standardized multiple regression coefficient
Body mass index	-.26[a]
Reported alcohol intake	.23[a]
Serum thiocyanate level	-.10[b]
Diastolic BP	.03
Total serum cholesterol	.05

[a]$p<.001$.
[b]$p<.01$.

Change in diastolic blood pressure was also included in the regression model because it was found unexpectedly to be positively associated with change in HDL-cholesterol. Changes in LDL- and VLDL-cholesterol, included because of their obvious relevance, had significant negative regression coefficients; this agrees with the inverse association noted in previous cross-sectional studies (19). These observations are encouraging in a general way, allowing speculation that efforts to lower LDL- and VLDL-cholesterol may be harmonious with those which will raise HDL-cholesterol level. (The large negative coefficient for baseline HDL-cholesterol is due to the statistical phenomenon of regression toward the mean.)

With the exception of the estimated adherence to the MRFIT diet, each of the variables in Table II has a significant association with HDL-cholesterol change when all the other variables are held constant. This means, for example, that the negative association between change in body weight and change in HDL-cholesterol is at least partly independent of the association each has with VLDL-cholesterol.

The cross-sectional data at baseline are not a prime focus of this presentation because the observed associations may include bias resulting from the process through which the participants were selected. The findings are presented in Table III using multiple regression analysis to provide a partial compensation for such bias. With the exception of blood pressure, the cross-sectional findings are qualitively similar to the longitudinal ones, suggesting that they have a biological basis.

Despite the consistency of these associations, causality remains to be established, especially as there are independent variables not included among those listed which might be confounding these relationships. A prime example is physical activity, which may be related to both HDL-cholesterol and body weight (13). The influence of this factor, and the search for others, will be the subject of future MRFIT analyses.

CONCLUSIONS

Preliminary experience with a subgroup of MRFIT participants studied over the first two years of the trial has revealed change in several variables to be independently related to change in HDL-cholesterol level. For the most part, these associations fit with observations available from previous studies of cross-sectional design, and are therefore likely to have a biological, rather spurious, basis. Causality remains to be established, but one inference is that weight loss and smoking cessation increase the level of HDL-cholesterol. Taking the group as a whole, neither the fat-controlled diet nor the multifactor intervention program altered mean levels of this lipoprotein.

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Leucine as an In Vitro Precursor to Lipids in Rat Sciatic Nerve

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ABSTRACT

The in vitro incorporation of leucine, isoleucine and pyruvate into lipids was compared and the possibility that leucine might serve as an *in situ* precursor to the corresponding *iso* fatty acids in the rat sciatic nerve was studied. The relative incorporation of ^{14}C from leucine into lipids vs. nonlipids was 20%, and the incorporation of label into total lipids from leucine was one-half that from pyruvate. The incorporation of label from leucine and pyruvate into sterols was nearly equivalent, but the incorporation of label into all other lipid classes from leucine was less than that from pyruvate, and the incorporation of label from isoleucine into lipids was much less in all cases. No detectable label from leucine was incorporated into brached chain fatty acids. It is concluded that leucine may be a substantial in vitro precursor to all major lipids in peripheral nerve, especially sterols. The possibility and significance of a leucine catabolic pathway in the cytosol in relation to availability of 3-hydroxy-3-methylglutaryl CoA for sterol biosynthesis is discussed.

INTRODUCTION

The liver is generally accepted as the major site of amino acid degradation (1), but it is well known that the branched chain amino acids are preferentially degraded in extrahepatic tissues (2). This unique partition of metabolic pathways on an organ level is thought to be largely due to a low level of branched chain amino acid transaminase activity in the liver (3), but the metabolic significance is unclear. Leucine has been suggested as a major source of serum and tissue cholesterol in studies performed with skeletal muscle (4,5) and adipose tissue (5). In vitro studies using rat aorta have indicated that leucine may be an important *in situ* precursor of certain lipids, especially cholesterol in that tissue (6). In support of these findings is the fact that one of the breakdown products of leucine metabolism is 3-hydroxy-3-methylglutaryl CoA (HMG CoA), an intermediate involved in cholesterogenesis.

The oxidation of branched chain amino acids may be altered by the nutritional state (2,7-9) and by hormones (2). The circulating level of the branched chain amino acids is increased during short term fasting (9) and in obese man (10) and in diabetic animals and man (11,12). The branched chain amino acids are key precursors to *iso* and *anteiso* fatty acids (13). As a precursor to metabolic intermediates, leucine may become especially important in altered metabolic states, because the flux of intermediates through various metabolic pathways may be changed. It is known, for example, that in vitro, sciatic nerves from streptozotocin diabetic rats release greater amounts of $^{14}CO_2$ from [^{14}C-1]leucine than sciatic nerves from control animals (14). We undertook this study to compare the relative degree to which ^{14}C-labeled leucine, isoleucine and pyruvate would incorporate label into specific lipids in the rat sciatic nerve. Pyruvate was used as a nonamino acid precursor because it is decarboxylated in mitochondria by a similar mechanism as the 2-keto derivatives of the branched chain amino acids. In separate experiments the incorporation of label from leucine into branched chain fatty acids in the sciatic nerves of diabetic and nondiabetic rats was also studied.

METHODS

Male Wistar rats weighing 200-250 g were fed ad libitum Lab Blocks (Allied Mills, Inc., Chicago, IL) and water. Rats were decapitated with a guillotine (Harvard Apparatus Co., Dover, MA), the nerves were immediately excised from each side along with their major branches, and were dissected free of connective tissue (14). Nerves were placed in a balanced salt solution (15) until sufficient tissue was collected for incubation. About 30 min elapsed between excision and incubation. The two nerves from each rat were randomly and equally distributed between incubation vessels. Each vessel contained several nerves representing ca. 100 mg tissue in 6 ml. Incubation was carried out in sealed Erlenmeyer flasks containing 5.5 mM glucose and 0.1 mM ^{14}C-labeled precursor in a balanced salt solution (15) equilibrated with O_2 and CO_2 (95:5), pH 7.4, 37 C. The flasks were agitated at 42 cycles/min in a Dubnoff metabolic shaker.

Uniformly labeled L-leucine, L-isoleucine and sodium [^{14}C-2]pyruvate were purchased from New England Nuclear Corp., Boston, MA. The concentration of radioactivity used in the incubation medium was 0.1 μCi/ml (0.1Ci/mole) in all experiments except those involving branched chain fatty acid synthesis in which

the concentration of radioactivity was 1.0μCi/ml (1Ci/mole).

Following incubation, the nerves were blotted dry on filter paper and extracted with chloroform-methanol (16). Extracts were washed three times with upper phase (16). The lipid extracts were evaporated to dryness with a stream of nitrogen in a water bath at 50 C, and the lipid residues were weighed.

In the experiments involving branched chain fatty acids only, diabetic and nondiabetic rats were studied. All other experiments were limited to normal rats. Diabetes was produced in the rats by I.P. injection of streptozotocin (120 mg/kg). They were considered diabetic when 4+ glycosuria was manifest, plasma glucose levels exceeded 300 mg/100 ml, and the animals were losing weight, or gaining less than 1 g/day in the last five days (14). Nerves were excised and treated as described above.

Neutral lipids consisting of cholesteryl esters, triglycerides, free fatty acids, diglycerides and free sterols were separated by thin layer chromatography (TLC) from total lipids on silica gel (Supelcosil 12A, Supelco Inc., Bellefonte, PA). Polar lipids remained at the origin. Standard lipid mixtures containing hydrocarbons, steryl esters, methyl esters, free fatty acids, free cholesterol, fatty alcohols and diglycerides were spotted on both edges of plates, and the plates were developed in petroleum ether-ethyl ether-acetic acid (70:30:0.5). Lipid bands were visualized under ultraviolet light after spraying the TLC plates lightly with 2',7'-dichlorofluorescein, quantitatively transferred to counting vials and counted in a liquid scintillation spectrometer. The scintillation fluid consisted of 10 ml of toluene containing 5 g/l PPO (2,5-diphenyloxazole) and 0.3 g/l dimethyl-POPOP)1,4-*bis*[2,4-(4-methyl-5-phenyloxazoyl)] benzene). Sample quenching was monitored with internal and external standards and was not variable.

Since the free sterols were difficult to separate from diglycerides by TLC, aliquots of lipid extracts containing 2 to 8 mg of ^{14}C-labeled total lipids and an added internal standard of [^{3}h]cholesterol of known radioactivity were deacylated in 2 ml of 0.2M methanolic NaOH overnight at room temperature in Teflon-lined screw-cap vials. The reaction mixture was extracted three times with 2 ml portions of petroleum ether, and the combined extracts were evaporated to a convenient volume with a stream of nitrogen. The free sterols were then isolated by TLC as described above for neutral lipids. Based on the amount of [^{3}H]cholesterol recovered, the ^{14}C incorporated into sterols was corrected. The amounts of ^{14}C label in individual sterols of the sterol pool was not determined.

Polar lipids were separated by two-dimensional TLC (17) on silica gel (Supelcosil 42A, Supelco Inc., Bellefonte, PA). Radioactivity in individual spots was determined as described above.

Fatty acid analysis of lipids was carried out on the total neutral and polar lipid fractions which were isolated as described above. Fatty acid methyl esters were prepared by base-catalyzed methanolysis as described in the deacylation procedure above, in which the lipids were treated with 0.2N NaOH. This method was used in order to avoid the conversion of plasmalogens to dimethyl acetals which interfere with the gas chromatographic analysis of the branched chain fatty acids. After the reaction was complete, the reaction mixture was neutralized with dilute acetic acid to a phenolphthalein end point, and the fatty acid methyl esters were extracted with petroleum ether which was evaporated with a stream of nitrogen to a convenient volume.

In order to determine the degree to which branched chain fatty acids were labeled, the saturated fatty acid methyl esters were isolated by TLC on $AgNO_3$-impregnated silica gel (18). This fraction was analyzed by two gas chromatographic methods to determine radioactivity in individual fatty acid species. The fatty acids were identified according to relative retention times with standard fatty acid mixtures containing even and odd carbon fatty acid methyl esters from 14:0 through 26:0 and *iso* 14:0, *iso* 15:0, *anteiso* 15:0, *iso* 16:0, *iso* 17:0 and *anteiso* 17:0. Initially, a Nuclear-Chicago flow through radioactivity detector interfaced to a Beckman GC-45 gas chromatograph fitted with a 2 m x 4 mm ID glass column packed with 10% Silar-10C on 100:120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA) was used. The helium carrier gas flow rate was 100 ml/min, and the propane quenching gas flow rate was 10 ml/min. The split ratio between the flame ionization detector and the radioactivity detector was 1:15. The gas chromatographic column was programmed from 150 to 220 C at 10 C per min. Detector and injector temperatures were both 250 C. Because no detectable radioactivity was found associated with the branched chain fatty acids using this method, a more sensitive method was attempted in which the fatty acid methyl esters were separated on a Perkin Elmer 801 gas chromatograph utilizing a 4 m x 4 mm ID glass column packed with 10% Apeizon L on 100:120 mesh Supelcoport (Supelco Inc., Bellefonte, PA), and each peak was collected as a

fraction and counted in a liquid scintillation spectrometer. Gas chromatographic conditions were: injector 290 C, column 230 C, detector 300 C.

RESULTS AND DISCUSSION

Labeled leucine has often been used as a protein precursor in order to determine rates of protein synthesis (19,20), but it is not generally considered a significant precursor to lipids. Classically, labeled acetate has been used in studies involving the biosynthesis of lipids. Caston and Singer (21) showed in vivo that in frog sciatic nerves, leucine was the only amino acid appreciably incorporated into lipids. In addition, they found that the relative incorporation of ^{14}C from leucine into total lipids vs. nonlipids was 20%. We found virtually the same relative incorporation into lipids and nonlipids in rat sciatic nerve in vitro. Additionally, this percentage was much lower in other tissues tested (aorta, 2%; skeletal muscle, 4%; adipose tissue, 7%).

The label incorporated in vitro into rat sciatic nerve total lipids from [^{14}C]leucine was only one-half that from equimolar [^{14}C]-pyruvate (Table I). This resembles previous observations in the rat aorta where the label incorporated into total lipids from leucine was one-third that from pyruvate (6).

These findings show that in tissues such as peripheral nerve and aorta, leucine may be a substantial lipid precursor. This is contrasted by the adult rat brain in which leucine appears to be lipogenic only to a small degree (22), suggesting a metabolic difference between peripheral nerve and brain.

TABLE I

In Vitro Incorporation of ^{14}C Labeled Leucine, Isoleucine and Pyruvate into Total Lipids of Rat Sciatic Nerve[a]

^{14}C-precursor	Radioactivity (dpm/mg tissue/3 hr)
Leucine	197 ± 32.8[b]
Isoleucine	65 ± 9.3
Pyruvate	420 ± 89.1

[a]Rat sciatic nerves were incubated in Gey and Gey's balanced salt solution containing 5.5 mM glucose and 0.1 mM of the ^{14}C-precursor in an atmosphere of 95% O_2 + 5% CO_2 at 37 C and pH at 7.4.
[b]Standard error. Each mean represents seven observations.

The differences in the amounts of radioactivity incorporated into total lipids from each precursor can be explained in part by the metabolic fates of the products formed. Leucine may be converted to acetyl CoA through HMG CoA and isoleucine to propionyl CoA and acetyl CoA. Label from propionyl CoA is less likely than acetyl CoA to be incorporated into lipids. The incorporation of label into lipids from pyruvate is probably a reflection of carbohydrate utilization. Since the incubation medium also contained 5.5 mM unlabeled glucose, this undoubtedly resulted in dilution of the pyruvate pool and decreased the apparent labeling of total lipids. In Table II the amounts of label incorporated in vitro into lipid classes of nerve are shown. It is clear that the

TABLE II

In Vitro Incorporation of ^{14}C from Labeled Leucine, Isoleucine and Pyruvate into Lipid Classes of Rat Sciatic Nerve[a]

Lipid Class[b]	^{14}C-precursor Leucine	Isoleucine	Pyruvate
	(dpm/mg tissue/3 hr)		
Sterols	58.4 ± 6.0[c]	17.7 ± 1.2	66.0 ± 7.6
Triglycerides	29.5 ± 2.6	9.2 ± 1.7	146.8 ± 10.5
Phosphatidyl serine	4.5 ± 0.1	2.1 ± 0.3	10.1 ± 0.4
Sphingomyelin	5.7 ± 0.6	2.7 ± 0.8	11.4 ± 3.4
Phosphatidyl choline	44.4 ± 7.9	16.8 ± 2.6	86.6 ± 8.1
Phosphatidyl ethanolamine	12.0 ± 1.6	6.4 ± 0.5	37.0 ± 2.4
Cerebroside	7.7 ± 0.8	2.8 ± 0.4	14.7 ± 2.5
Total neutral lipids	118.1 ± 5.7	33.2 ± 1.3	254.0 ± 10.5
Total polar lipids	78.4 ± 3.5	31.9 ± 1.5	166.5 ± 9.3

[a]Rat sciatic nerves were incubated in Gey and Gey's balanced salt solution containing 5.5 mM glucose and 0.1 mM of the ^{14}C-precursor in an atmosphere of 95% O_2 + 5% CO_2 at 37 C and pH 7.4.
[b]Other lipid classes were analyzed but were found to contain less than 4% of the radioactivity. These were: cholesteryl esters, free fatty acids, diglycerides, phosphatidic acid, lysophosphatidyl choline, sulfatides and several unidentified lipids.
[c]Standard error. Each mean represents seven observations.

amount of label incorporated into sterols from leucine and pyruvate was nearly equivalent, but the contribution of ^{14}C from leucine to the other lipid classes was much less than that from pyruvate. Thus, in the peripheral nerve, leucine may be a metabolically significant precursor to cholesterol and other lipids. This corroborates studies done on the aorta (6), skeletal muscle (4,5) adipose tissue (5), kidney and intestine (4).

The relative importance of intermediates derived from leucine in metabolism is unclear. The rate of decarboxylation of leucine in several tissues is accelerated in fasting (8,9) and diabetes (14,23,24). In such states the supply of metabolic intermediates from leucine would be greater and could be utilized at accelerated rates in the biosynthesis of lipids and other components. The biosynthesis of branched chain fatty acids from branched chain amino acids has been observed in rat skin (25); however, in the rat sciatic nerve, we were unable to detect any ^{14}C label in the odd numbered carbon *iso* fatty acids. Apparently the substrate specificity of rat sciatic nerve fatty acid synthase is such that it will accept only acetyl CoA as a primer and will not readily accept other possible primers such as isovaleryl CoA, and this property is not altered in diabetes. In contrast, a purified enzyme system from rat adipose tissue incorporated label from the C_4 and C_5 short chain *iso* and *anteiso* acyl CoA derivaties into the corresponding fatty acids (26), and certain bacteria preferentially synthesize branched chain fatty acids (13).

HMG CoA, an intermediate of cholesterogenesis and ketogenesis, may be generated from acetyl CoA and is also a catabolic product of leucine. According to a shunt pathway proposed by Popjak (27), HMG CoA may be regenerated from 3,3-dimethylallylpyrophosphate, an intermediate of cholesterogenesis. If such a pathway is operative in peripheral nerve, it would enhance the incorporation of label from steroidogenic precursors into other lipids. Recent studies have clearly shown that HMG CoA is synthesized from acetyl CoA by separate pathways in the cytosol and mitochondria of rat and chicken liver and to a lesser extent in kidney (28). The enzymes involved are compartmentalized and are molecularly distinct (28-30). Accordingly, cholesterogenesis occurs in the cytosol of many cells, but ketogenesis from Acetyl CoA is exclusive to the mitochondria of liver and kidney, even though HMG CoA lyase is present in the mitochondria of virtually all cells (28). This is because mitochondrial acetyl CoA of extrahepatic and nonkidney cells cannot be metabolically converted to mitochondrial HMG CoA, since no HMG CoA synthase is present (28). It is thought that whatever HMG CoA that might be generated in the mitochondria of cells outside the liver and kidney arises from the catabolism of leucine, since mitochondrial HMG CoA synthase appears to be exclusive to liver and kidney and is not present in the mitochondria of other tissues (28).

The equivalent labeling of sterols by leucine and pyruvate in nerve and aorta (6) could be attributed to the direct participation of HMG CoA generated from leucine. Since cholesterogenesis is exclusive to the cytosol, the preferential labeling of sterols over other lipid classes by leucine would require a cytosolic mechanism for generating HMG CoA from leucine, because the inner mitochondrial membrane is impermeable to HMG CoA, and the carbon skeleton of the latter could exit only after conversion to acetoacetate which would be converted to acetyl CoA in the cytosol. Such a pathway is improbable, because extrahepatic tissues tend to utilize acetoacetate and 3-hydroxybutyrate, and these tissues are not ketogenic (28). It also would not explain the preferential labeling of sterols over other lipid classes by leucine, because a common precursor would be utilized, acetyl CoA.

Some evidence is available for a cytosolic pathway in which leucine may be converted to HMG CoA which would be available for cholesterogenesis. Cytosolic branched chain 2-keto acid dehydrogenase activity has been reported in rat liver, where it accounted for almost 10% of the total homogenate activity (31). Similarly, this activity has been reported in bovine liver cytosol, and it was shown that the enzyme system is probably molecularly distinct from that in the mitochondria (32). May and Buse have recently found higher cytosolic activities in rat liver (up to 20% of the total homogenate) under certain experimental conditions (personal communication). Unfortunately, similar studies have not been reported in nerve. The possibility of a cytosolic pathway by which leucine may be degraded and preferentially incorporated into sterols via HMG CoA is intriguing. The fact that the leucine/pyruvate incorporation ratio in neural and aortic (6) sterols was close to 1:1 but was 1:2, or less in other lipid classes would appear to support such a hypothesis.

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Fatty Acid Biosynthesis in the Developing Endosperm of *Cocos nucifera*[1]

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ABSTRACT

Endosperm tissue of developing coconut endosperm incorporated [^{14}C]acetate and [^{14}C]-malonate into [^{14}C]C_8-C_{18} fatty acids. The distribution of [^{14}C] label into the various fatty acids mimicked the distribution of endogenous fatty acids at early and middle stages of endosperm development. Although [^{14}C]C_8-C_{18} fatty acids were taken up rapidly by endosperm tissue slices, no elongation occurred; [^{14}C]stearic acid was not desaturated to oleic. Cell free preparations have also been obtained from this tissue that readily incorporated [^{14}C]malonyl-CoA into a range of [^{14}C] fatty acids in the presence of ACP and NADH at pH 7.0. Employing this system, a number of experiments were designed to determine the mechanism of chain length termination. In contrast to intact tissue slice experiments, cell-free extracts yielded as principal products palmitic and stearic acid, although up to 20% were shorter chain acids. A number of possible mechanisms for chain length termination were proposed and tested.

The coconut (*Cocos nucifera*) is one of the most important sources of edible oil. The oil is found in the kernel (endosperm) enclosed by a strong hard shell (endocarp), a fibrous fruit coat (mesocarp) and a smooth skin (exocarp). The mature fruit takes 12-13 months to develop and contains about 70-75% oil in its endosperm (1).

Although most plant lipids contain predominantly C_{16} and C_{18} fatty acids, coconut oil contains over 50% C_{12} and C_{14} fatty acids (1). As part of a wider study on the mechanism by which plants regulate the chain length of their fatty acid end products, we have examined fatty acid biosynthesis in the developing endosperm tissue of *Cocoa nucifera*. Some preliminary results were recently reported (2). This paper will describe both experiments carried out with tissue slices and with cell-free extracts from developing coconut endosperm.

MATERIALS AND METHODS

Materials

[1-^{14}C]Acetate acid, sodium salt (58.6 Ci/mol) and [2-^{14}C]-malonic acid (22.1 Ci/mol) were obtained from New England Nuclear, Boston, MA. [1-^{14}C]Decanoic acid (14.3 Ci/mol), [1-^{14}C]lauric acid (32 C8/mol), [1-^{14}C]myristic acid (45 Ci/mol), [1-^{14}C]-palmitic acid (58 Ci/mol), [1-^{14}C]stearic acid (58 Ci/mol) and [1-^{14}C]oleic acid (54 Ci/mol) were obtained from the Radiochemical Centre, Amersham. The radiopurity of each of these compounds was checked by gas liquid chromatography-radiochromatography before their use as substrates.

[1,3-^{14}C]Malonyl-CoA (54 Ci/mol), [1-^{14}C] octanoyl-CoA (29 Ci/mol), [1-^{14}C]decanoyl-CoA (58 Ci/mol), [1-^{14}C]lauroyl-CoA (58 Ci/mol) and [1-^{14}C]-palmitoyl-CoA (58 Ci/mol) were obtained from Dhom Products Ltd., North Hollywood, CA. [1-^{14}C]Acetyl-CoA (58 Ci/mol) was from the Radiochemical Center, Amersham. [1-^{14}C]Myristoyl-CoA (54.6 Ci/mol) and [1-^{14}C]stearoyl-CoA (50.9 Ci/mol) were purchased from New England Nuclear, Boston. Nucleotides, malonyl-CoA, G-6-P and G-6-P dehydrogenase were from Sigma Chemical Company, St. Louis, MO Tricine (N-Tris(hydroxymethyl)methylglycine), L-α-glycerol phosphate and dithiothreitol were from Calbiochem., Oak Grove Village, IL. Sorbitol was from Grand Island Biological Company, Grand Island, NY. *Escherichia coli* acyl carrier protein (ACP) and [0-^{14}C]lauroyl-ACP were a gift from Dr. J.B. Ohlrogge. ACP was isolated by the method of Alberts, Majerus and Vagelos (3) and purified up to the initial acid precipitation step. [0-^{14}C]Stearoyl-ACP, prepared by the enzymatic method of Jaworski and Stumpf (4), was a gift from Dr. Tom McKeon. It contained ca. 40% [0-^{14}C]palmitoyl-ACP.

PCS (phase combining system for liquid scintillation counting) was from Amersham/Searle Corporation, Arlington Heights, IL. Ethylene glycol monomethyl ether was obtained from Matheson, Coleman and Bell,

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East Rutherford, NJ. Organic solvents of analytical grade were obtained from Mallinckrodt Chemical Works, St. Louis, MO. Precoated Silica Gel G plates for thin layer chromatography (TLC) were purchased from Analtech Inc., Newark, DE. Ten percent EGSS-X on Gas-Chrom (100/120 mesh) was from Applied Science Laboratories, State College, PA, and 10% DEGS-PS on Supelcoport (80/100 mesh) was from Supelco, Bellefonte, PA.

Tissue slice incubations. Freshly picked coconuts were received by air from Professor N.P. Kefford, Department of Botany, University of Hawaii, Honolulu, and from Mr. R. Miyashita, Department of Parks and Recreation, Honolulu. They were immediately used on arrival or kept at 4 C for not more than 2-3 days.

Endosperm slices, about 1 mm thick and 5 mm wide, were cut with a razor blade. One gram of these slices was immersed in an incubation mixture containing 50 μmoles of potassium phosphate buffer (pH 7.5), 30 μmoles of $KHCO_3$ and 0.34 μmole of [1-^{14}C] sodium acetate or 0.90 μmole o [1-^{14}C] malonic acid or 0.15 μmole of a long chain [1-^{14}C] fatty acid (C_{10}-C_{18}), in a total volume of 1.2 ml. The long chain fatty acids were added dissolved in 10 μl of ethylene glycol monomethyl ether. The mixture was shaken in a 25 ml Erlenmeyer flask at 25 C for 5-6 hr with air as the gas phase.

Analysis of tissue slice incorporation products. At the end of the incubation period with tissue slices, 0.1 ml of 1 N H_2SO_4 was added. The supernatant was removed and the tissue slices were rinsed with 2 x 2 ml of buffer. They were then extracted with 6 ml of chloroform/methanol, 2:1 (v/v) for 16 hr at 25 C. The tissue slices were filtered off and washed with 2 ml of chloroform/methanol, 2:1 (v/v) mixture. In the final two-phase system, the chloroform phase was washed with 1 ml of distilled water or 1 ml of 0.1 M malonic acid (for mixtures containing [2-^{14}C] malonic acid) and then evaporated to dryness under nitrogen. The residue was redissolved in 200-250 μl of benzene. A sample was removed for radioactive counting in 10 ml of PCS-xylene, 2:1 (v/v) scintillation fluid using a Beckman LS-230 liquid scintillation counter.

Preparation of acetone powder and soluble enzyme extracts. Preliminary experiments with fresh endosperm tissue extracts were difficult to carry out because of the presence of massive amounts of lipid. Therefore, fresh tissue was extracted with cold acetone (-20 C) to delipidize and dehydrate the tissue. The resulting white powder could be stored indefinitely at -20 C.

Endosperm tissue (200-400 g fresh weight) was cut into small pieces and placed in a Waring blender prechilled at -20 C. About 2 volumes of prechilled (-20 C) acetone were added, and the tissue was homogenized with several short (20 sec) periods of blending. The mixture was quickly filtered under suction and the residue returned to the blender for re-extraction with fresh, chilled acetone. This process was repeated until most of the lipids were removed and the residue appeared as a fine white powder. About 3-4 extractions were required for 6-7 month matured endosperm and 6-7 extractions for 11-12 month matured endosperm. The acetone-extracted residue was then resuspended in peroxide-free diethylether and filtered. This procedure was repeated 2-3 times to remove traces of acetone. The partially dried powder was finally spread out on aluminum foil in a vacuum desicator and the ether removed under vacuum. The dry powder was weighed and stored at -20 C.

When required, 0.2 g of the acetone powder was added to 2.5-30.0 ml of 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol, stirred for 10 min at 4 C and then centrifuged at 12,000 g for 20 min. This clear, slightly viscous supernatant was used as the acetone powder extract.

To remove endogenous cofactors from the acetone powder extract, 0.56 g of ammonium sulfate were slowly added to 1 ml of extract. The turbid solution was shaken and left for about 20 min at 0 C and then centrifuged at 30,000 g for 30 min. The sediment was redissolved to 1 ml of buffer solution.

The acetone powder extract (20 ml) was also separated into a solution and a particulate fraction by centrifuging at 100,000 g for 3 hr in a Beckman Model L ultracentrifuge. The 100,000 g sediment was washed by resuspension in 6 ml of buffer solution and recentrifuged. The final pellet was resuspended evenly in 0.6 ml of buffer solution.

Preparation of microsomal fraction from fresh coconut endosperm. Fresh endosperm tissue (35 g) was added to 52 ml (1.5 volume) of homogenizing medium made up of 0.5 M sorbitol-0.1 M potassium phosphate buffer (pH 7.5-1 mM dithiothreitol-1 mM EDTA.) The tissue was homogenized 2 x 5 sec in a Sorval Onmimix homogenizer at maximum speed and then squeezed through 4 layers of cheese cloth. The filtrate was centrifuged at 800 g for 5 min to remove unbroken cells and debris. The supernatant was centrifuged further at 10,000 g for 20 min. The upper lipid layer was carefully removed, washed with 10 ml of the homogeniz-

ing medium and the washing was combined with the 10,00 g supernatant. The combined 10,000 g supernatant and lipid layer washing were centrifuged further at 100,000 g for 1 hr. The resultant sediment (microsomal fraction) was washed by resuspension, recentrifugation, and finally resuspended in 2 ml of the homogenizing medium.

[^{14}C]Fatty acid synthesis by extracts. Routine fatty acid synthesis experiments were carried out with 100 µl of extract (ca. 0.5 mg protein), 25 µmoles potassium phosphate buffer (pH 7.0), 90 µg *E. coli* ACP, 0.75 µmole $KHCO_3$, 0.34 µmole ATP, 44 nmoles $MgCl_2$, 24 nmoles $MnCl_2$, 68 nmoles NADPH, 0.17 µmole G-6-P, 0.01 units G-6-P dehydrogenase and 0.2 µCi [1,3-^{14}C]-malonyl-CoA (3.7 nmoles) or 0.2 µCi [1-^{14}C]acetyl-CoA (3.4 nmoles) in a total volume of 0.3 ml. In studying the effect of pH on the activity, 25 µmoles of the different pH values were added to the mixture, and no further adjustment was made to the final pH of the mixture. In later experiments using [1,3-^{14}C]malonyl-CoA as substrate, $KHCO_3$, NADPH and its regenerating system (G-6-P/G-6-Pdehydrogenase) were omitted. The reaction mixture was incubated at 24 C for 1 hr.

Formation of neutral [^{14}C]acylglycerols from [^{14}C]fatty acyl derivatives by a microsomal fraction. One-hundred µl of washed 100,000 g sediment (4 mg/ml protein) from fresh young endosperm was incubated with 25 µmoles potassium phosphate buffer (pH 7.0), 0.09 µmole of L-α glycerol phosphate and 1 nmole of [1-^{14}C]acyl-CoA (0.05 µCi) in a total volume of 0.3 ml. In two other tubes, [0-^{14}C]-stearoyl-ACP (25,000 cpm, 2.5 nmoles) and [0-^{14}C] lauroyl-ACP (20,000 cpm, 0.5 nmoles) replaced the [1-^{14}C]acyl-CoA as substrate and and 0.01 µmole of CoA was added as an additional cofactor. Incubation was carried out at 24 C for 1 hr after which the [^{14}C] fatty acids and neutral [^{14}C]acylglycerols were extracted as described by Mancha et al. (5) and separated on TLC.

Assay for utilization of [1-^{14}C]acyl-CoA. The ability of acetone powder extracts to utilize [1-^{14}C]acyl-CoA substrates was determined with an incubation mixture containing ca. 0.5 mg protein from an ammonium sulfate precipitate (0-80% saturation) of the extract, 25 µmoles potassium phosphate buffer (pH 7.0), 0.34 µmole ATP, 0.09 mg *E. coli* ACP, 44 nmo nmoles $MgCl_2$, 68 nmoles NADH, 9 nmoles malonyl-CoA and 2 nmoles [1-^{14}C]-acyl-CoA (specific radioactivities given above). After incubating for 1 hr at 24 C, 1 mg coconut endosperm endogenous lipids and 0.5 mg palmitic acid were added as carrier lipids, and the mixture was extracted for neutral lipids and free fatty acids with petroleum ether as described by Mancha et al. (5).

Analysis of radioactive products from cell-free experiments. At the conclusion of an incubation, the reaction was stopped by the addition of 0.3 ml 40% KOH. The reaction tubes were capped and the mixtures directly saponified at 80 C for 1 hr. They were then cooled, acidified with 0.5 ml of 8 N H_2SO_4 and the [^{14}C] fatty acids extracted with 2 x 1 ml of chloroform. The chloroform extract was washed with 1 ml of water of 1 ml of 0.1 M malonic acid (for [1,3-^{14}C] malonyl-CoA incubations). It was then evaporated by dryness under nitrogen and the residue redissolved in 200-250 µl of benzene. Aliquots were counted in a liquid scintillation counter and the remainder methylated with diazomethane to form methyl esters for gas liquid chromatography (GLC) analysis.

In experiments where the radioactive products were separated into neutral lipids/free fatty acids, acyl-ACPs and acyl-CoAs, the procedure of Mancha et al. (5) was followed. The neutral lipids/free fatty acis, extracted with petroleum ether saturated with 50% aqueous isopropanol, was further separated into mono-, di- and triacylglycerols and free fatty acids by TLC. Since little complex lipids, if any, were expected to be formed, the acyl-CoA fraction was not subjected to the alumina column treatment but was directly saponified and converted to methyl esters.

Chemical α-oxidation of [^{14}C]fatty acids. Chemical α-oxidation was carried out by the procedure of Harris et al. (6).

Thin layer chromatography. Appropriate samples were chromatographed on Silica Gel G plates using the solvent system diethylether/benzene/ethanol/acetic acid, 40:50:2:0.1 (v/v) (5) to separate mono-, di- and triacyl glycerols and free fatty acids. Nonradioactive marker compounds were cochromatographed on the same plate and detected by exposure to iodine vapor. Radioactive areas on the plate were detected by scanning in a Model 7201 Packard radiochromatogram scanner. The gel in the radioactive areas was scraped into scintillation vials and directly counted. For further analysis, the gel was extracted with 4 x 1 ml of chloroform/methanol, 2:1 (v/v) and the lipid was saponified and methylated as described below.

GLC analysis. Radioactive lipids extracted from incubated tissue slices or from TLC plates were saponified in 1 ml of 1 N KOH in 90% ethanol for 1 hr at 80 C. The mixture was then acidified with 0.2 ml of 8 N H_2SO_4. One ml of

water was added and the mixture was extracted with 2 x 2 ml of chlofororm. The extract was reduced to about 0.5 ml under nitrogen and methylated with an excess of freshly prepared ethereal solution of diazomethane.

Radio-GLC analysis of the methyl esters was carried out with a Varian Aerograph Model 920 instrument fitted with a thermal conductivity detector coupled to a Nuclear-Chicago Biospan (4998) radioactivity detector. Routine analysis was carried out on a 5 ft x 1/4 in. stainless steel column packed with 10% DEGS-PS on 80/100 Supelcoport. C_8-C_{14} fatty acids were separated at 140 C for 14 min after which the column temperature was raised to 160 C. Some analyses were also made on a 10% EGSS-X on 100/120 Gas-Chrom column packing under the same operating conditions.

Extraction of endogenous coconut lipid. Endosperm tissue (30-50 g) from freshly opened coconuts was homogenized in 10 volumes of chloroform/methanol, 2:1 (v/v) mixture using a Polytron blender. The mixture was left overnight at 4 C. It was then filtered, and the residue was washed with small volumes of chloroform/methanol mixture. The chloroform layer in the combined extract was washed with 1/5 volume of methanol/water, 1:1 (v/v). A sample was dried in a tared weighing vessel. The remaining extract was evaporated to dryness in a rotary evaporator and then made up to 25 ml with benzene. A sample was saponified and methylated with diazomethane for GLC analysis, and another sample was estimated for triacylglycerols by the triacylglycerol C-37 Rapid Stat Kit (Pierce Chemical Co., Rockford, IL).

RESULTS

Effect of stage of development of [1-^{14}C]acetate and [2-^{14}C]malonate incorporation into fatty acids by endosperm tissue slices. The development of the fruit of the coconut palm was described by Child (1). In the first six months, the fruit increases in volume while the cavity or embryo sac remains filled with liquid endosperm. At this stage, solid endosperm (kernel) begins to form, Initially at the end opposite the stalk but gradually extending to form a layer all around the interior. Rapid development of this endosperm layer occurs in the next 3-4 months from an initial thin jelly-like layer to form a firm solid kernel. The kernel continues to become progressively harder with intracellular deposition of oil until hard white flesh is attained at full maturity at 13 months. About 75% of the dry weight and 84% of the oil deposit are already formed by the ninth month (1).

It is difficult to define precisely the age of endosperm tissue employed in these studies. The coconuts used here were roughly at three stages of development, identified as: Stage A (ca. 6-7 months), Stage B (ca. 11-12 months) and Stage C (ca. 13-14 months). In the fully ripe coconut, the husk (mesocarp) had started to dry out. Table I shows the fatty acid composition of endogenous lipids extracted from the endosperm of these types of coconuts. As the coconut endosperm develops from an early stage of cellular proliferation to active deposition of oil droplets at later stages, fatty acid synthesis may show shifts in the nature of the products formed. This is shown in Table I where it is seen that the endogenous lipids of young endosperm tissue have a fatty acid composition with 61% of the acid as C_{16} and C_{18} acids. However, in the almost ripe coconut, most of the oil had already been deposited (1) and the fatty acid composition of the endogenous lipids showed a predominance of C_{12} and C_{14}acids. Clearly in the transition of development between Stages A and B, the tissue is synthesizing $C_{10\text{-}14}$ fatty acids, but at or after Stage B the synthesis of these acids has been completed.

Slices of endosperm tissue from Stage A and Stage B coconut tissue incorporated [1-^{14}C]-acetate and [2-^{14}C]malonate into fatty acids (Table II), although incorporation was low. Endosperm tissue from Stage C coconut had very low activity with [1-^{14}C]acetate and no activity at all with [2-^{14}C]malonate. The incorporation of [1-^{14}C]acetate and [2-^{14}C]-malonate into fatty acids was, therefore, related to the stage of development of the endosperm. As predicted in Table I, Stage A and Stage B endosperm tissues incorporated [^{14}C]acetate predominantly into [^{14}C]short chain fatty acids (C_8-C_{14}) while the [^{14}C]products with Stage C tissue were almost exclusively C_{16} and C_{18} fatty acids. The fatty acids synthesized from malonate at each stage of development closely resembled those synthesized from acetage except for the formation of a small amount of C_{20} fatty acid. It is possible that this C_{20} fatty acid was formed by chain elongation of existing fatty acids in the presence of [2-^{14}C]malonate.

To determine whether [^{14}C]fatty acids in the incubated slices were synthesized de novo or by chain elongation of preexisting endogenous chains present in the extract, the extracted [^{14}C]products from several incubations were pooled together. [^{14}C]Palmitic acid was isolated by preparative GLC from these products and degraded by chemical oxidation

TABLE I

Fresh Weight and Endogenous Lipid Content of Coconut Endosperm at Different Stages of Development

Stage of development[a]	Fresh weight of endosperm (g)	Total endogenous lipid (g)	Composition of fatty acids in endogenous lipids (%)[b]							
			8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2
A	81	2.9	4	4	14	17	18	0	29	14
B	270	80.4	8	11	35	22	10	3	8	3
C	270	90.1	5	7	32	25	13	4	10	4

[a]Estimated stage of development of the coconut as described in the text.

[b]Extraction of endogenous lipids and estimateion of fatty acid composition are described in the Methods section.

TABLE II

Incorporation of [1-^{14}C]Acetate and [2-^{14}C]Malonate into Fatty Acids by Endosperm Tissue Slices from Coconuts at Different States of Development[a]

Stage of development[b]	Radioactiave substrate	Total nmoles incorporated into faty acids	Distribution of ^{14}C into fatty acids (%)								
			8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	20:0
A	Acetate	9.98	35	11	19	8	5	3	3	14	0
B	Acetate	3.67	18	8	42	12	7	0	9	5	0
C	Acetate	0.44	0	0	0	8	61	0	14	17	0
A	Maloate	1.56	21	15	19	7	7	3	9	11	7
B	Malonate	3.04	10	13	28	16	11	0	11	3	8
C	Malonate	0	---	---	---	---	---	---	---	---	---

[a]Endosperm tissue slices (1 g) were incubated with 0.34 μmole of [1-^{14}C]acetate of 0.90 μmole of [2-^{14}C]-malonate. The lipid products were extracted and analyzed as described in the Methods section. Fatty acid composition of the endogenous lipids of endosperm tissue are given in Table I.

[b]See definition of Stages A, B, and C under Results and Discussion.

TABLE III

Uptake of [1-^{14}C] Fatty Acids ad Esterification into Triacylglycerols by Endosperm Tissue Slices[a]

[1-^{14}C] Fatty acid substrate	Percent of substrate taken up	Distribution of ^{14}C uptake (%)[b]	
		Free fatty acids	Triacylglycerols
10:0	78	26	74[c]
12:0	82	47	53
14:0	39	98	2
16:0	49	96	4
18:0	17	99	1
18:1	49	99	1

[a]Stage A *coconut* endosperm tissue slices (1 g) were incubated with 150 nmoles of [1-^{14}C] fatty acid. Experimental details are described in the Methods section.

[b]Radioactive lipids extracted from incubated endosperm tissue slices were separated by TLC.

[c]Included 5% DG and 8% MG.

(6). The results indicated that the [^{14}C]-palmitic acid formed from [1-^{14}C] acetate was synthesized de novo. The same results were obtained with [^{14}C] palmitic acid formed from [2-^{14}C] malonate. Thin layer chromatography of [1-^{14}C] acetate incorporation products showed that the [^{14}C] fatty acids synthesized were present as mono- and triacyl-glycerols as well as free acids, namely, 14% MG, 32% TG, 54% FFA. No diglycerides were detected.

Ability of endosperm tissue to metabolize exogenously added [1-^{14}C] fatty acids. It was of interest to determine whether coconut endosperm had the capacity to metabolize exogenously added fatty acids. Table III shows that uptake of [1-^{14}C] fatty acids by Stage A tissue slices occurred very readily. The efficiency of uptake appeared to decrease with the chain length of the substrate. It is possible that a portion ofthe substrate taken up moved into a nonmetabolic pool by directly partitioning into the lipid phase of the tissue slices. However, when the extracted [^{14}C] lipids were separated on TLC with a diethylether/benzene/ethanol/-acetic acid, 40:50:2:0.2 (v/v) solvent system, it was found that those obtained from incubations with [1-^{14}C]decanoic and [1-^{14}C]lauric acids consistently contained a considerable proportion of [^{14}C]-neutral acylgycerols, showing that the tissue was capable of activating these acids and transferring them to suitable endogenous acceptors. Although the experiment reported in Table II showed a very low incorporation of [1-^{14}C]-myristic acid into triacylglycerols, the same incubation with endosperm slices from another coconut gave a higher proportion (25%) of radioactivity as triacylglycerols. With the long chain C_{16} and C_{18} fatty acids, there was consistently little incorporation into triacylglycerols.

Radio-GLC analysis showed that the original [1-^{14}C] fatty acid remained unchanged. Thus, although the endosperm tissue was capable of activating the medium chain fatty acids to acyl CoAs for esterification into neutral acylglycerols, these activated forms were not elongated, desaturated, or metabolized further by the tissue slices. Presumably, the [^{14}C] acyl-CoAs could not enter the de novo-elongation ACP track (7) because of the absence of a direct acyl-ACP liguase or an acyl-CoA/ACP transacylase and, therefore, could not be elongated and desaturated.

Incorporation of [1,3-^{14}C] malonyl-CoA into fatty acids by extracts of coconut endosperm - properties of the system. Further characterization of lipid biosynthesis in endosperm tissue required the preparation of cell-free extracts capable of incorporation of [^{14}C] substrates into fatty acids. When incubated with [1,3-^{14}C] malonyl-CoA, extracts of an acetone powder preparation of coconut endosperm incorporated radioactivity into products extractable with lipid solvents. With the incubation system described in the methods section, about 40-60% of [1,3-^{14}C]malonyl-CoA were incorporated by a fresh extract of acetone powder from Stage A and Stage B endosperm. Very little activity (3% incorporation) was obtained with acetone powder extract from Stage C tissue. When [1-^{14}C] acetyl-CoA was the substrate, very little activity (less than 3% incorporation) was obtained with all extracts. These low activities probably relate to acetyl-CoA carboxylase activity, which was very low in these extracts. Although maximum incorporation was obtained after 40 min incubation under the specified conditions, a 60 min time was selected for all incubations. The amount of incorporation was proportional to the enzyme protein in the acetone powder extract up to a concentration of 170 μg pro-

TABLE IV

Effect of Various Cofactors on Incorporation of [1,3-^{14}C] Malonyl-CoA into Fatty Acids

Incubation mixture[a]	Percentage of activity in complete mixture
Experiment 1	
Complete	100
–All cofactors	4
–ACP	4
–$KHCO_3$	110
–ATP	85
–$MgCl_2$	123
–$MnCl_2$	90
Experiment 2[b]	
Complete	100
–NADH, NADPH	18
–NADH	57
–NADPH	99

[a]Incubation conditions are described in the Methods section. Enzyme source was an ammonium sulfate (0-80% saturation) precipitate of an acetone powder extract of young endosperm (Stage A – Materials and Methods for definition of stages of development).

[b]$KHCO_3$, ATP, $MgCl_2$ and $MnCl_2$ were omitted from Experiment 2.

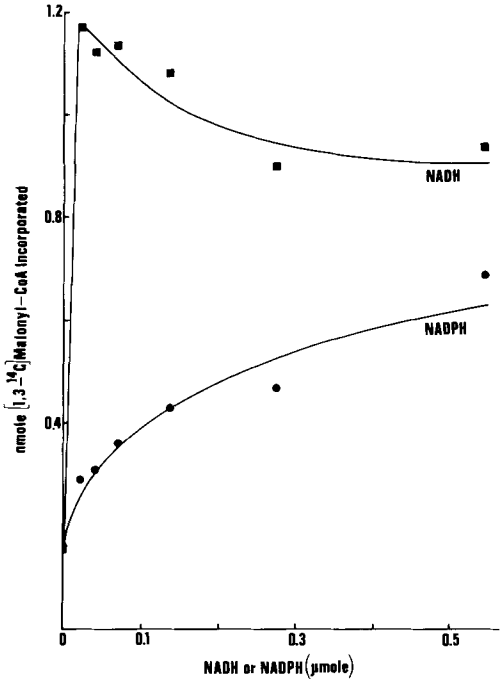

FIG. 1. Effect of increasing amounts of NADH or NADPH on incorporation of [1,3-^{14}C] malonyl-CoA by an ammonium sulfate fraction of acetone powder extracts of Stage A endosperm tissue. Details in Methods section.

tein/0.3 ml incubation mixture. Optimal activity was obtained at a pH of ca. 7.0. At pH 6.5, only 20% of the activity at pH 7.0 was observed and at pH 8.0, 80% of the optimal activity occurred. Under optimal conditions, [^{14}C] malonyl-CoA was incorporated at a rate of about 2.4 nmoles/hr/mg protein. The incubation system showed an absolute requirement for added ACP after removal of endogenous cofactors by ammonium sulfate precipitation (Table IV). It was also stimulated by reduced pyridine nucleotide. Figure 1 shows that the system utilized NADH more readily, although at higher concentrations NADPH was also a suitable reductant.

To determine whether the enzyme system was particulate or soluble, the acetone powder extract was centrifuged at 100,000 g for 3 hr. Ninety-eight percent of the activity was recovered in the 100,000 g supernatant and only 2% from the washed 100,000 g sediment. An extract prepared from fresh endosperm tissue was also active in incorporating [1,3-^{14}C] malonyl-CoA into [^{14}C] fatty acids. Most of this activity (87%) was found in the soluble fraction, and only 3% of the total activity was recovered in the 100,000 g sediment. The procedure was designed to preserve organelles such as mitochondria and plastids.

Nature of incorporation products. Table V shows the distribution of [^{14}C] fatty acids in the total [^{14}C] products formed from [1,3-^{14}C] malonyl-CoA by extracts from acetone powder and from fresh endosperm tissue. In all of them, long chain [^{14}C] fatty acids (C_{16}-C_{18}) accounted for over 60% of the radioactivity. The percentage of the shorter chain [^{14}C]-fatty acids (C_8 and C_{10}) was variable between different experiments probably in part because of the difficulty of complete recovery of these volatile acids without adopting special procedures; but they were always absent from products obtained with acetone powder extracts of Stage B endosperm. [^{14}C] Fatty acids were also obtained from incubation mixtures from which *E. coli* ACP was omitted; presumably synthesis occurred employing only endogenous coconut ACP. To obtain enough radioactive material for analysis, 10 incubations (no added ACP) were simulataneously carried out and the [^{14}C] products pooled. It is seen in Table V that with limiting amounts of endogenous coconut ACP, there was a significant increase in the proportion of [^{14}C] stearic acid and [^{14}C] oleic acids formed. Whether this shift is related either to the use of subminimal concentrations of ACP or to the participation of endogenous ACP with a specificity different

TABLE V

Distribution of Radioactivity in Fatty Acids Produced by Various Extracts from [1,3-^{14}C]Malonyl-CoA

Type of extract	nmoles Substrate incorporated/ mg protein	Distribution of ^{14}C in fatty acids (%)						
		8:0	10:0	12:0	14:0	16:0	18:0	18:1
Acetone powder extract:								
Stage A endosperm	3.20	6	6	9	12	50	13	4
Stage A endosperm[a]	0.60	0	0	4	12	47	26	11
Stage B endosperm	2.46	0	0	15	16	67	12	0
Fresh Stage A endosperm-								
100,000 g supernatant	9.43	6	6	8	13	56	11	0
100,000 g sediment	2.44	1	3	4	6	64	22	0

[a]*E. coli* ACP omitted from incubation mixture and 10 incubations pooled.

TABLE VI

Distribution of [^{14}C] Fatty Acids in Different Lipid Classes

Enzyme preparation[a]	Lipid class[b]	Percentage of [^{14}C] incorporated							
		8:0	10:0	12:0	14:0	16:0	18:0	18:1	Total
Acetone powder extract	FFA	0	0	1	1	33	11	11	57
	Acyl-ACP	4	2	3	5	24	0	0	38
	Acyl-CoA	0	1	0	0	4	0	0	5
100,000 g supernatant	FFA	0	0	0	0	12	5	0	17
	Acyl-ACP	6	6	8	12	40	6	0	79
	Acyl-CoA	0	1	0	0	2	1	0	4
100,000 g sediment	NL/FFA	0	0	1	1	25	14	0	41
	Acyl-ACP	1	2	4	5	35	6	0	53
	Acyl-CoA	-	1	0	0	4	1	0	6

[a]Stage A endosperm tissue was employed.

[b]Radioactive products were separated by the Mancha procedure (6) into lipid classes NL, neutral lipids; FFA, free fatty acids; and acyl-ACPs and acyl-CoAs. Details in text.

TABLE VII

Formation of Esterified [^{14}C]Fatty Acids from [1,3-^{14}C]Malonyl-CoA in the Presence of Microsomal Fraction

Extract[a]	Incorporation nmoles/hr/mg protein	Radioactivity in lipid classes (%)[b]					
		Neutral lipid fraction				Acyl-ACP	Acyl-CoA
		TG	DG	MG	FFA		
Acetone powder extract	11.25	0	0	0	31	62	7
Microsomal fraction + acetone powder extract	4.69	19	4	11	9	46	11
100,000 g supernatant of *fresh endosperm*	37.8	0	0	0	17	79	4

[a]Actone powders and fresh endosperm tissue were from young coconut (Stage A). Incubation conditions were the same as described in Materials and Methods except that 22.2 nmoles of [1,3-^{14}C]malonyl-CoA were employed.

[b]^{14}C-Products were separated into the three lipid classes by the Mancha procedure (6) followed by TLC of the neutral lipid fraction. Details in text.

from that of *E. coli* ACP remains for further investigation to determine.

The composition of the [^{14}C]fatty acid synthesized as a function of various conditions was also examined employing acetone powder extracts of Stage A endosperm: (a) incubation of 24 C, 30 C and 40 C; (b) incubation at pH 7.0 and at pH 8.0; (c) addition of 0.09 mg, 0.18 mg and 0.36 mg *E. coli* ACP to the incubation. In (a) and (b), there was no significant shift in the pattern of [^{14}C]fatty acids produced compared with that produced under the standard incubations conditions described in the Methods section. With increasing ACP concentrations, however, the pattern of [^{14}C]synthesized fatty acids shifted to shorter chain lengths, i.e., C_{8-12}.

To determine whether the [^{14}C]fatty acids were synthesized de novo or by mere elongation or preexisting chains in the extract, [^{14}C]-palmitic acid isolated from incubation products was purified by preparative GLC and degraded by the $KMnO_4$ chemical α-oxidation procedure (6). The results indicated that the [^{14}C]-palmitic acid obtained in the incubation mixture was synthesized de novo from [1,3-^{14}C]-malonyl-CoA via an ACP pathway.

The [^{14}C]lipids were also separated into neutral lipids-free fatty acids, acyl-ACPs and acyl-CoAs by the Mancha procedure (5). The results are shown in Table VI. It is seen that the medium chain [^{14}C]fatty acids (C_8-C_{14}) were mainly, if not exclusively, present as acyl-ACP derivatives. The [^{14}C]palmitoyl moiety was present in all three fractions. [^{14}C]Acyl-CoA was a trace product.

Formation of neutral [^{14}C]acylglycerols by 100,000 g sediment of fresh endosperm tissue.

The petroleum ether extract of acidified incubation mixtures did not differentiate between free fatty acids and neutral acylglycerols. The extract was, therefore, chromatographed on TLC as previously described (2). Table VII shows that all the [^{14}C]products (extracted with petroleum ether from acidified mixtures) synthesized by the extracts obtained from acetone powder or with 100,000 g supernatant of fresh endosperm were free fatty acids. On addition of a microsomal fraction (100,000 g sediment of fresh endosperm) to the incubation mixture, these [^{14}C]fatty acids were also found in mono-, di- and triacylglycerols. Incubation with microsomal fraction alone also produced [^{14}C]fatty acids in the neutral acylglycerols. There was no apparent chain length specificity towards the esterification of these [^{14}C]fatty acids formed from [1,3-^{14}C]-malonyl-CoA since the same pattern of fatty acid composition was seen both in the [^{14}C]-

TABLE VIII

Formation of Neutral [^{14}C] Acylglycerols from [^{14}C] Acyl-CoA and [^{14}C] Acyl-ACP by a Microsomal Fraction[a]

Substrate	Percent of [^{14}C] Substrate incorporated	Distribution of [^{14}C] incorporated (%)			
		TG	DG	MG	FFA
[1-^{14}C] Acyl-CoA					
8:0	78	55	9	23	13
10:0	69	37	9	41	13
12:0	100	39	8	42	11
14:0	46	24	18	51	7
16:0	59	15	21	59	5
18:0	100	13	14	67	6
[0-^{14}C] Acyl-ACP[b]					
12:0	34	5	54	30	11
18:0	93	56	32	11	1
18:0 + CoA	72	53	31	14	2

[a]Details are described in the Methods section.

[b][0-^{14}C] Stearoyl-ACP substrate contained about 40% [0-^{14}C] palmitoyl-ACP. Because of the extensive transfer of the stearoyl groups to NL, obviously palmitoyl-ACP was also an effective donor substrate.

acylglycerols and in the total [^{14}C] fatty acids produced. The microsomal fraction thus appeared to possess the enzymes for neutral lipid biosynthesis.

Substrate specificity for microsomal transacylating activity. To determine the nature of the substrates for the formation of neutral acylglycerols by the microsomal fraction, incubation of microsomal preparations with different [1-^{14}C] acyl-CoAs was carried out. L-α-Glycerol phosphate was added as a possible acceptor for the acyl groups. [^{14}C] Acyl-ACPs were also tested as possible substrates in the presence and absence of CoA. Due to the limited availability of such substrates, only [^{14}C] stearoyl-ACP and [^{14}C] lauroyl-ACP were tested. [^{14}C] Stearoyl-ACP also contained ca. 40% [^{14}C] palmitoyl-ACP.

Table VIII shows that both [1-^{14}C] acyl-CoAs and [^{14}C] acyl-ACPs were esterified onto neutral acylglycerols. Since the omission of L-α-glycerol phosphate did not lower the acylation reactions, experiments without this acceptor were not included in Table VIII. With [^{14}C] acyl-ACP as substrate, transesterification onto neutral acylglycerols occurred in the absence of CoA showing that acyl-ACP served as the direct donor in the transfer process.

Ability of acetone powder extract to utilize [1-^{14}C]acyl-CoA. The formation of the whole range of [^{14}C] fatty acids from [1,3-^{14}C]-malonyl-CoA by endosperm extracts (Table V) and its dependence upon ACP (Table IV) suggested that these [^{14}C] fatty acids were synthesized along the ACP track (7). It was of interest to determine whether the extract could utilize [1-^{14}C] acyl-CoA added to it and whether the chain length of the substrate had any effect on the process. [1-^{14}C] Acyl-CoA was incubated with an ammonium sulfate precipitate of an acetone powder extract of Stage A endosperm in the presence and absence of *E. coli* ACP. ATP, NADH and malonyl-CoA were added as cofactors for possible elongation processes.

TLC of the extracted [^{14}C] products showed over 96% as free fatty acids, and GLC of the methyl esters confirmed that the original [^{14}C] fatty acyl groups remained unchanged. Thus, the [1-^{14}C] acyl-CoA substrates were merely hydrolyzed to free acids by an acyl-CoA hydrolase but were not elongated or desaturated. These results are in complete agreement with those obtained when [^{14}C] fatty acids were incubated with tissue slices, namely, the absence of any elongation or modification of the acyl chain of the acyl-CoAs.

DISCUSSION

The purpose of this investigation was to obtain basic information concerning the biosynthesis of fatty acids in coconut endosperm. Possible mechanism of chain length termination could then be tested.

Coconut endosperm lipids are composed of over 50% C_{12} and C_{14} fatty acids (1). [^{14}C]-Fatty acids synthesized from [1-^{14}C] acetate or [2-^{14}C] malonate by intact endosperm slices were similar in having over 50% C_{12} and C_{14} acids. In contrast, the [^{14}C] fatty acids obtained with cell-free extracts showed a markedly different pattern. Since palmitic acid was always the major component (over 50%) while C_{12} and C_{14} fatty acids seldom exceeded 20%, presumably the mechanism controlling the high

proportion of C_{12} and C_{14} fatty acids in intact tissues was not functioning in these cell-free extracts.

The fatty acid synthesizing activity in coconut endosperm extracts did not appear to be membrane or organelle associated, since it was not sedimented by centrifugation at 100,000 g under conditions where organelle structure would be preserved. It had an absolute ACP requirement for activity. In the absence of exogenously added ACP, the system, operating with only the endogenous coconut ACP in the extract, showed minimal activity (Table IV); but the [^{14}C]fatty acids synthesized showed an increase in the proportion of stearic and oleic acids (Table V). Presumably, this may relate to the limited number of fatty acid chains initiated by the low level of available ACP and a shift towards elongation of preexisting acyl-charged endogenous ACP. In the presence of excess *E. coli* ACP (from 0.09 mg to 0.36 mg), the pattern of [^{14}C]fatty acids synthesized was shifted to shorter chain fatty acids. Changes in either temperature (from 24 C to 40 C) of pH did not influence the type of [^{14}C]fatty acids synthesized.

One may speculate as to the nature of this control. It may operate either by a switching system composed of a medium chain acyl-ACP thioesterase-acyl-CoA synthetase combination (7) or by an acyl-ACP/CoA transacylase which would be specific for the medium chain length fatty acids so that these acids would be switched off the ACP track onto a CoA track and then used as acyl donors (8). Another possibility could be the direct transacylation of lauroyl-ACP and myristoyl-ACP to glycerol phosphate to form neutral acylglycerols. A fourth possibility could involve the specificity of the coconut ACP, that is, medium chain acyl coconut ACPs could be a more effective substrate than the medium chain acyl *E. coli* ACPs. Finally, there could be a separate synthetase system for C_{12} and C_{14} fatty acids with a different cellular localization, e.g., associated with the microsomes or special organelles which eventually convert to oil droplets.

Only 3% of the total activity in the cell-free extracts was recovered in the once-washed microsomal fraction (100,000 g sediment). The [^{14}C]fatty acids synthesized by this residual activity were mainly C_{16} and C_{18} acids. Thus, the microsomal fraction probably had no specific fatty cid synthetase system associated with it. Similarly, the C_{16}-C_{18} [^{14}C]fatty acids synthesized with endogenous coconut ACP (Table V) can be explained by its limiting effect on the system as explained above. Certainly there was no evidence suggesting a strong shift to the synthesis of shorter chain fatty acids by the employment of endogenous ACP.

Analysis of [^{14}C]fatty acids among the three lipid classes separated by the Mancha procedure (5) showed that the short and medium chain fatty acids remained as acyl-ACP derivatives (Table VI). This indicated that the coconut extracts had no acyl-ACP hydrolase activity towards C_8-C_{14} fatty acyl-ACP compounds. Ohlrogge et al. (9) recently reported a similar lack of acyl-ACP hydrolase activity for C_8-C_{14} fatty acids in plant systems. A specific acyl-ACP hydrolase-acyl-CoA synthetase switching system would, therefore, probably not be involved, An acyl-ACP/CoA transacylase would also be an unlikely mechanism of control since acyl-CoAs with or without ACP were not elongated or modified by tissue extracts.

Table VII clearly shows that the transacylating activity for esterification of acyl-CoA to neutral acylglycerols was located in the microsomal fraction. However, there was no specificity towards the type of fatty acids esterified since C_8-C_{14} as well as C_{16}-C_{18} acids were detected in the neutral acylglycerols in the same proportions as in the total [^{14}C]fatty acids synthesized. Table VIII shows that the microsomal fraction was capable of transacylating all the acyl-CoAs from C_8 to C_{18}. It was also capable of direct transacylation of lauroyl-ACP, palmitoyl-ACP and stearoyl-ACP onto neutral acylglycerols without the mediation of CoA. It is not possible to comment on the specificity of this transacylation of acyl-ACPs since a complete range of [^{14}C]acyl-ACP substrates was not available for comparative studies. Thus, while the microsomal fraction was capable of direct transfer of acyl groups from acyl-ACP to neutral acylglycerols, the role of this reaction as a mechanism for chain termination of C_{12} and C_{14} fatty acids in coconut endosperm tissue cannot be properly assessed. It should be noted, however, that intact coconut endosperm tissue slices could incorporate [1-^{14}C]decanoic and lauric acids but not [^{14}C]myristic, palmitic and stearic acids into neutral [^{14}C]-acylglycerols; these acyl chains remained, however, unchanged (Table III). This suggests that these acids were not entering the ACP track but were being introduced into the acylglycerols perhaps as acyl-CoA derivatives.

In summary, five possible mechanism have been tested with a variety of endosperm systems. These include (a) a specific acyl-ACP hydrolase-Acyl-CoA synthetase switching system, (b) a specific acyl-CoA transacylase, (c)

a specific acyl-ACP transacylase, (d) a difference in specificity of endogenous acyl coconut ACP vs. acyl *E. coli* ACP, and (e) a specific fatty acid synthetase which terminates at the C_{12}-C_{14} level. We believe that these mechanisms have been eliminated by direct experiments. Further work with this difficult tissue will hopefully reveal the actual control mechanism.

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COMMUNICATIONS

Fatty Acid Positional Specificity in Phospholipids of L1210 Leukemia and Normal Mouse Lymphocytes

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ABSTRACT

The positional distribution of fatty acids in the choline and ethanolamine phosphoglycerides of the L1210 murine leukemia cells was determined and compared to that of normal mouse lymphocytes. The major phospholipids of both cell types had appreciable degrees of positional specificity as evident from the higher percentage of saturated fatty acids in position 1 and of polyunsaturated fatty acids in position 2. The L1210 cells had less arachidonate and more linoleate in position 2 of choline and ethanolamine phosphoglycerides as compared to the normal lymphocytes. However, there were similar proportions of saturated, monoenoic and polyenoic fatty acids in positions 1 and 2 of the phospholipids of the L1210 leukemia cells and the lymphocytes. These data demonstrate that fatty acid positional specificity is retained in the major phospholipids of this rapidly growing tumor.

INTRODUCTION

Previous observations on hepatoma and other tumors have demonstrated some loss of fatty acyl positional specificity in choline phosphoglycerides (1-4) and other phospholipids (4). It is important to explore this topic further since the reported decrease in specificity may have important implications regarding potential differences in membrane function between normal and neoplastic cells. In the present study, we have compared the fatty acyl positional distributions in L1210 lymphoblastic murine leukemia cells and normal mouse lymphocytes in order to determine whether a rapidly growing leukemia also exhibits a loss of specificity.

MATERIALS AND METHODS

Methods for transplanting, harvesting and handling L1210 cells and lipid extraction have been described (5). Normal lymphocytes were obtained from DBA/2 mice which had not been injected with L1210 leukemia cells. The animals were sacrificed by cervical dislocation, and the thymus was removed and washed with the capsule intact. Suspensions of single cells were produced by gently teasing the tissue through a screen (200 mesh) into phosphate-buffered saline with glucose (100 mg/100 ml). The suspension was sedimented for 2 min, and the supernatant solution containing the cells was washed 3 times prior to counting. All steps were carried out at 4 C. Butylated hydroxytoluene (0.005%) was added to lipid extracts. Phospholipids were separated using one dimensional thin layer chromatography (TLC) on Silica Gel G plates prewashed with ethyl acetate and developed in $CHCl_3/CH_3OH/CH_3COOH/-H_2O$ (100:50:14:6). Segments of the silica gel containing the choline or ethanolamine phosphoglycerides were scraped from the chromatoplate and extracted immediately with $CHCl_3-/CH_3OH$ (1:1) for 45 min (6), then washed with 9 ml 0.04N HCl. Phospholipase A_2 was used to remove the fatty acid residues from the 2-position of an aliquot of the choline and ethanolamine phosphoglycerides. About 0.3 mg of the purified phosphoglycerides from the cells was dissolved in 2 ml diethyl ether. Then, 0.1 mg phospholipase A_2 (from Crotalus adamanteus venom, Sigma Chemical Co., St. Louis, MO) in 0.5 ml of 0.1 M borate buffer (pH 7.0) containing 2.5 mM Ca^{++} was added to the ether solution (7). This mixture was shaken vigorously for 2 hr in an Eberbach shaker bath at 37 C. TLC was used to confirm the completeness of degradation. The resultant fatty acids from position 2 and the lysophospholipids were extracted using $CHCl_3/CH_3OH$ (2:1) (8) and separated by TLC on Silica Gel G plates using a solvent system containing $CHCl_3/CH_3-$

$OH/CH_3COOH/H_2O$ (160:50:2:6). The lysophospholipids were saponified and the fatty acids from position 1 were contained in the saponifiable fraction. The fatty acids were methylated (9), and the methyl esters were separated on a 1.8 m x 6.4 mm metal column containing 10% SP-2330 on chromosorb W (AW) using a Hewlett-Packard 5710 gas liquid chromatograph. When aliquots of the intact L1210 choline or ethanolamine phosphoglycerides were saponified, methylated and analyzed, the fatty acid percentages were the same as the sum of the percentages at positions 1 and 2 divided by 2.

RESULTS AND DISCUSSION

Choline and ethanolamine phosphoglycerides make up about 80% of the L1210 cell phospholipids, and they are the major ones into which radioactivity is incorporated when labeled fatty acids are incubated with the cells (5). Table I demonstrates that the fatty acid composition of these L1210 phosphoglycerides is not randomly distributed. A majority of the fatty acids located in position 1 of both phosphoglycerides is saturated, whereas most of the fatty acids in position 2 are polyunsaturated. Palmitate, stearate and oleate were located to a greater extent in position 1, while linoleate, arachidonate and the polyunsaturates containing 22 carbon atoms were present to a greater extent in position 2. Such a distribution in which position 1 is enriched with saturated fatty acids and position 2 contains a higher percentage of polyenoic fatty acids characterizes the phospholipids of many normal mammalian tissues (10) including normal lymphocytes from pigs, rabbits and calf thymus (11).

In order to determine if there was any quantitative loss of fatty acid positional specificities of the phospholipids of the neoplastic L1210 leukemia cell, studies were carried out with normal lymphocytes obtained from the same strain of mouse. Position 1 of the phospholipids from the normal lymphocytes was occupied mostly by saturated fatty acids, and the most abundant class of fatty acid in position 2 was polyunsaturated. When the phospholipids from the L1210 leukemia and normal lymphocytes were compared, there were differences in the relative proportions of some individual acids, Position 2 of both phospholipids of the normal lymphocytes contained proportionately more arachidonate and less linoleate than position 2 of the L1210 cell. There was also a lower proportion of palmitate in position 2 of choline phosphoglycerides and of oleate in position 1 of ethanolamine phosphoglycerides of the L1210 cell as compared to the normal lymphocyte. However, with the exception of a somewhat greater percentage of polyunsaturates in position 2 of ethanolamine phosphoglycerides from the normal lymphocytes, both of the cell types had similar fatty acid distributions.

A lesser degree of positional specificity of the phospholipid fatty acids of tumors as compared to normal tissues has been reported in several instances. In the studies of Ruggieri and Fallani, position 1 of the choline phosphoglycerides from the Yoshida hepatoma contained 66.8% saturated fatty acids as compared to 95.3% in position 1 of normal liver (1). Position 2 of the Yoshida hepatoma contained only 46.7% polyunsaturated fatty acids as compared to 76.6% in the liver (1). This difference was in part due to less arachidonate and polyenoic fatty acids of 22 carbon chain length in the tumor. A similar loss of specificity was evident in position 2 of choline and ethanolamine phosphoglycerides of hepatoma 7288 CTC studied by Wood (4). That there might be a relationship between neoplasia and a loss of fatty acid positional specificity was further suggested by studies indicating that a less undifferentiated Morris hepatoma had similar positional specificities to normal liver (1). Bergelson and Dyatlovitskaya also found that most of the fatty acids of hepatoma 27 choline phosphoglycerides were distributed less specifically than in normal liver (2). For example, 90% of the fatty acids in position 1 of the normal rat liver were saturated as compared to only 44.4-70.4% in samples of the hepatoma. Similarly, 80.5% of the fatty acids in position 2 of the liver lecithin were polyunsaturated as compared to 16.0-36.8% in samples of the hepatoma. There was also some loss of fatty acid positional specificity in nephroma RA as compared to rat kidney (2). It should be noted that even though the tumors had an apparent loss of specificity, in most cases they still contained a majority of saturated fatty acids in position 1 and a majority of unsaturated fatty acids in position 2. This was also true in an extensive study of the positional specificity in the Ehrlich ascites tumor cell (12). In this case, however, there were no comparative studies of the distribution in the counterpart control tissue.

Based upon these findings, it has been suggested that tumor cells have an abnormality in the mechanism which produces the asymmetrical distribution of fatty acids in phosphoglycerides (13). If such a defect were a general

TABLE I

Positional Distribution of Fatty Acyl Groups in the Major Phospholipid Fractions of the L1210 Cell

	L1210 Leukemia				Normal lymphocytes			
	Choline phosphoglycerides		Ethanolamine phosphoglycerides		Choline phosphoglycerides		Ethanolamine phosphoglycerides	
	Position		Position		Position		Position	
	1	2	1	2	1	2	1	2
	%							
Classes								
Saturated	58.4[a]	27.0	51.9	17.2	55.1	37.7	53.3	11.2
Monoenoic	25.1	19.4	16.7	13.7	29.0	15.9	26.0	10.5
Polyenoic	12.0	47.4	12.8	58.5	3.4	42.0	18.4	76.6
Individual Acids								
16:0	27.3	14.8	13.1	6.5	29.9	30.4	11.1	7.1
16:1	2.0	3.1	1.1	1.3	1.0	1.8	n.d.[b]	0.9
18:0	29.2	7.7	34.9	6.5	22.4	3.7	42.2	3.2
18:1	20.2	15.9	15.1	11.8	18.7	13.8	26.0	9.6
18:2	6.6	29.5	4.7	17.3	2.0	10.1	1.9	5.0
20:0[c]	1.3	2.5	2.4	2.3	n.d.	n.d.	n.d.	0.2
20:1	3.0	0.4	0.5	0.6	9.2	0.4	n.d.	n.d.
20:2	2.8	3.5	2.2	3.1	2.4	1.6	0.8	1.6
20:3	0.2	1.9	n.d.	2.0	n.d.	2.6	n.d.	3.6
20:4	0.6	7.6	2.6	17.3	0.1	22.2	8.7	41.9
22:4[d]	1.4	2.2	2.4	5.2	0.2	2.2	2.3	5.5
22:5	0.3	1.3	0.3	5.2	n.d.	1.4	1.4	6.5
22:6	0.1	1.4	0.6	8.4	n.d.	1.8	2.9	12.4
Others[e]	5.0	8.2	20.1	12.5	14.1	8.0	2.7	2.5

[a]The values are the mean of two determinations. The sum of the classes for each position is less than 100% due to unidentified acids.
[b]Not detected.
[c]Includes 18:3.
[d]Includes 24:0.
[e]Includes <14:0, 14:0 and unidentified.

characteristic of tumor cells, it should be magnified in the rapidly growing and highly undifferentiated L1210 leukemia cell which kills its host in 1-2 weeks. Our studies comparing this tumor with normal lymphocytes from the same strain of mice demonstrate that a moderately high degree of fatty acid positional specificity is retained in the L1210 cell phospholipids. The persistence of this degree of selectivity in the rapidly growing L1210 cell indicates that a major decrease in fatty acid positional specificity is not a uniform characteristic of neoplastic cells.

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Composition of Lipids Bound to Pure Cytochrome P-450 of Cholesterol Side-chain Cleavage Enzyme from Bovine Adrenocortical Mitochondria

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ABSTRACT

Phospholipids bound to highly purified cytochrome P-450 from bovine adrenocortical mitochondria, part of the enzyme complex responsible for catalyzing the conversion of cholesterol to pregnenolone, have been examined for comparison with the bulk phospholipids of the mitochondria from the same tissue. In both cases, the major phospholipids are phosphatidylcholine (PC) (37%) and phosphatidylethanolamine (PE) (56%), as well as smaller amounts of sphingomyelin and diphosphatidylglycerol. The fatty acid compositions of the four classes of phospholipids and of the neutral lipids bound to the pure enzyme are indistinguishable from those of the respective mitochondrial lipids. They are also similar to those of mitochondria from other organs except for high levels of arachidonate and low levels of diphosphatidylglycerol.

INTRODUCTION

The enzyme cytochrome P-450 responsible for side chain cleavage of cholesterol (cholesterol → pregnenolone) called P-450 scc, occurs in mitochondria from steroid-forming organs and catalyzes the rate-determining step in steroid synthesis – the step specifically stimulated by the trophic hormones ACTH and LH (1). Extraction of this cytochrome P-450 from mitochondrial membrane requires detergent presumably because it is membrane-bound. Highly purified P-450 scc is soluble and contains ca. 80 nmoles of phospholipid per nmole of enzyme (850,000 MW) (2). Removal of phospholipid denatures the enzyme, although some enzymatic activity can be restored by renaturation in the presence of phospholipid and heme (3); this requirement for phospholipid shows some specificity (3). In addition, the soluble enzyme with bound phospholipid can be incorporated into membranes composed of purified or synthetic phospholipids (4). Because of the requirement of phospholipid for reconstitution of P-450 enzyme activity, it was decided to determine the composition of the bound phospholipid and that of the whole mitochondrial membrane system from bovine adrenal cortex.

METHODS

The procedure for extracting and separating classes of phospholipids by two dimensional thin layer chromatography (TLC) (5,6), methods of hydrolysis (7), isolation of neutral lipids by TLC (6), methylation of fatty acids (8) and separation and identification of these acids by gas liquid chromatography (9) and mass spectroscopy (10) are given elsewhere. Methods for preparing bovine adrenocortical mitochondria, homogeneous cytochrome P-450 (3) and for determining phospholipid as in-

TABLE I

Phospholipid Composition of Bovine Adrenocortical Mitochondria and Cytochrome P-450

Phospholipid	Mictochondria[a]	P-450[b]
	(%) Total inorganic phosphate)[a]	
Phosphatidylcholine	38.3 ± 4.1	36.8 ±5.2
Phosphatidylethanolamine	57.2 ± 3.2	57.7 ± 4.1
Diphosphatidylglycerol	6.0 ± 1.4	4.0 ± 0.9
Sphingomyelin	2.5 ± 0.3	1.5 ± 0.3

[a]The phospholipid content of mitochondria was 480 ± 32 n moles/mg protein (mean and range of 6 determinations) and that of P-450 was 94 ± 14 n moles/mg protein (mean and range of 8 determinations).

[b]Values are means and ranges for determinations on two separate preparatiaons.

TABLE II

Percent Fatty Acid Composition of Phospholipid Fractions and Neutral Lipid from P-450 scc

Fatty acid	Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Diphosphatidylglycerol	Neutral lipids	Total mitochondrial phospholipid
12:0	4.4	0.7	0.4	3.9	4.6	0.5
14:0	6.5	1.1	0.3	6.6	7.5	0.6
14:1	7.6	1.1	0.3	1.4	1.7	0.6
15:0	0.4	0.6	4.3	1.1	1.3	2.6
16:0	39.6	27.3	5.4	31.0	32.5	15.0
16:1	2.6	1.8	1.0	1.3	1.6	1.3
17:0	0.3	1.2	4.4	1.1	1.3	3.0
17:1	0.3	1.0	1.8	1.7	1.5	1.5
18:0	14.9	21.3	40.4	24.4	22.3	32.4
18:1	4.3	24.8	13.4	12.8	3.8	17.7
18:2	2.5	9.2	3.8	11.9	3.4	6.6
20:0	0.5	0.4	1.2	0.3	0.7	2.2
20:1/2[a]	0.2	0.7	2.9	0.2	0.1	2.0
20:3	0.6	1.5	0.7	0.2	0.1	1.0
20:4	0.4	6.4	15.4	0.5	0.2	9.5
22:0	2.9	0.1	0.4	0.5	1.3	0.3
22:1	2.9	0.2	0.7	0.3	0.5	0.4
22:4	---	0.4	3.1	0.1	0.5	2.0
24:0	2.5	0.1	0.2	0.5	1.5	0.1
24:1	0.6	---	---	---	0.5	0.5
26:0	6.0	0.2	0.2	0.4	3.0	0.2
26:1	---	---	---	---	9.8	---

[a]The fatty acids 20:1 and 20:2 are measured together.

organic phosphate have all been published (3). The mitochondria prepared by the above method show less than 5% microsomal contamination as shown by enzyme markers and confirmed by electron microscopy (data not shown). Inner mitochondrial membrane was prepared by a published method (11).

RESULTS

Table I shows that cytochrome P-450 scc contains two principal classes of phospholipid – phosphatidylcholine (PC) and phosphatidylethanolamine (PE) together with smaller amounts of diphosphatidylglycerol and sphingomyelin. An earlier study revealed no evidence of diphosphatidylglycerol in adrenocortical mitochondria (12); using larger amounts of mitochondria, small amounts of these phospholipids were found in keeping with an earlier report (13). The phospholipid of the mitochondrion is similar in composition to that associated with the enzyme (Table I). The mitochondrial phospholipids from bovine adrenal closely resemble those from rat whole adrenal (13). The neutral lipids included cholesteryl esters (52.8%) and cholesterol (14.1%) with smaller amounts of hydrocarbons, fatty acids and glycerides.

Table II shows the fatty acid composition of the P-450 phospholipid which resembles that of mitochondrial phospholipid from whole rat adrenal to a remarkable degree (13) and is also indistinguishable from that of the mitochondrial phospholipid from bovine adrenal cortex (not shown); in duplicate determinations comparing the fatty acid compositions of the four classes of phospholipids from bovine adrenocortical mitochondria with those extracted from homogeneous P-450 from the same source revealed that the same fatty acids were present and the proportions of the various fatty acids were the same within the limits of experimental error. A previous detailed study in the rat was based upon whole adrenal (13), so that the small differences observed between the rat and beef may be partly explained by the contribution of the medulla in the studies on the rat.

Folch extracts of the supernate and pellet from sonicated mitochondria (i.e., before cholate extraction) and of inner mitochondrial membrane were all similar in lipid composition to the enzyme and whole mitochondria (data not shown).

DISCUSSION

Much interest centers around the role of phospholipid in the functions of membrane-bound enzymes. It has been suggested that the phospholipid may be important in regulating enzyme activity or in electron transport (14). It appears that interaction between protein and phospholipid may result in the development of a special functional compartment of phospholipid around the enzyme - the so called boundary lipid (15). The P-450 discussed here is surrounded with lipid indistinguishable in composition from the mitochondrial lipid, although this does not exclude a functional specialization of the boundary lipid (12). It is interesting to note that reconstitution of enzyme activity from stripped protein appears to require both PC and PE (3); pure P-450 has been incorporated in enzymatically active form into membranes using a wide variety of single species of PC and PE as well as combinations of these classes (4). Finally, it should be added that similarity between lipid of enzyme and that of mitochondria cannot be attributed to randomization caused by cholate since submitochondrial fractions not treated with cholate (including inner membrane where P-450 scc is found (11)) show the same lipid composition (Results).

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METHODS

Separation of Triglycerides by Chain Length and Degree of Unsaturation on Silica HPLC Columns

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ABSTRACT

Triacylglycerols can be separated by both chain length and number of double bonds using micro particulate silica high pressure liquid chromatography columns with isooctane, diethyl ether, and acetic acid solvent mixtures. The separations obtained are the reverse of those observed with μ-Bondapak C_{18} columns (Waters Associates); i.e., longer chain length triglycerides elute from the column earlier than their shorter chain homologs, and saturated triglycerides elute before the more unsaturated ones. Base line separation was obtained between tristearin, triolein, trilinolein, and trilinolenin.

INTRODUCTION

Silica high pressure liquid chromatography (HPLC) columns are generally considered useful for separation of classes of lipid compounds, whereas reverse phase columns with octadecylsilyl or alkyl phenyl groups bonded to silica particles are used to separate homologous compounds found within a class. During our HPLC studies of estolide triglycerides (1), we noted that, in addition to class separation, silica columna were providing separations by chain length and degree of unsaturation between the classes. This prompted us to investigate the possibility of separating normal homologous

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

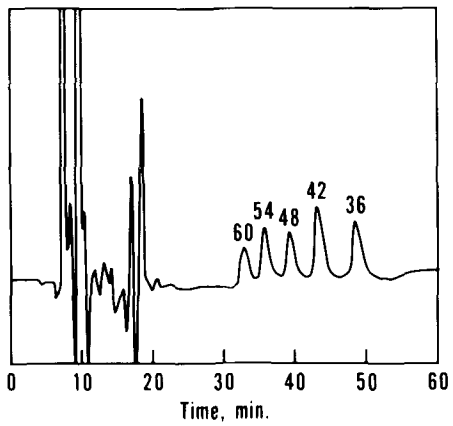

FIG. 1. HPLC chromatogram of saturated triglyceride mixture. The 60 cm x 3.8 mm μ-Porasil column was eluted with 98:1:1 isooctane/ethyl ether/acetic acid at 1.0 ml/min.

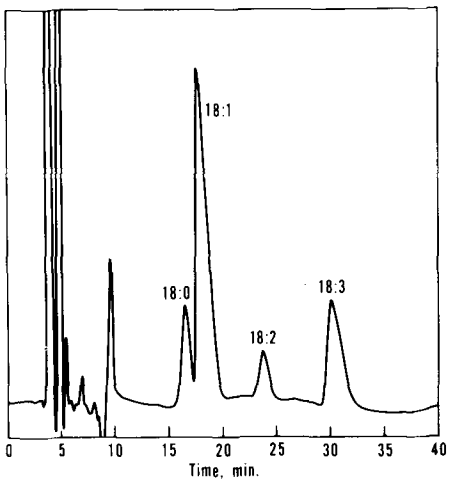

FIG. 2. HPLC chromatogram of C_{54} triglyceride mixture. The 30 cm x 3.8 mm μ-Porasil column was eluted with 98:1:1 isooctane, ethyl ether, and acetic acid at 1.0 ml/min. A = tristearin; B = triolein; C = trilinolein; D = trilinolenin.

triglycerides on the silica columns.

MATERIALS AND METHODS

Chromatograms were obtained with a Waters Associates (Milford, MA) ALC-201 liquid chromatograph using one or two 30 cm x 3.8 mm μ-Porasil columns. Samples of 5-10 μl of 10% $CHCl_3$ solutions were injected by means of a U6K septumless loop injector. The Waters differential refractometer was used as a detector. Samples were run isocratically with a mixture of isooctane/diethyl ether/acetic acid (99:1:1) on the μ-Porasil column and with mixtures of acetronitrile/acetone (2:1) on the μ-Bondapak C_{18} column (Waters Associates). The flow rates used were 1.0 or 2.0 ml/min, respectively.

RESULTS AND DISCUSSION

The separation of normal saturated triglycerides (triarachidin, tristearin, tripalmitin, trimyristin, and trilaurin) is shown in Figure 1. Base line resolution between the saturated triglycerides was achieved using two 30-cm μ-Porasil columns in tandem. One column was sufficient to resolve a mixture of C_{54} triglycerides: tristearin, triolein, trilinolein, and trilenolein (Figure 2). These separations are the reverse of those reported on μ-Bondapak C_{18} columns where the shorter chain length and more unsaturated components eluted first (2). On reverse phase columns, we were unable to elute triarachidin and tristearin because they became insoluble in the eluting solvent. Interestingly, tripalmitin and triolein had nearly identical retention volumes to each other on both the μ-Porasil and μ-Bondapak C_{18} columns.

Since we had noted that the relative retentions of methyl oleate and methyl palmitate were the same on μ-Bondapak C_{18} and that the methyl palmitate elutes before methyl oleate on the μ-Bondapak alkylphenyl columns, we suspected that it might be possible to separate triolein and tripalmitin on silica columns using benzene, toluene, or xylene in the solvent system instead of diethyl ether. A mixture of benzene and acetic acid (99.9:0.1) eluted triolein slightly before tristearin and tripalmitin. With xylenes and acetic acid (99.9:0.1) triolein, tristearin, and tripalmitin had retention volumes of 9, 10, and 11 ml, respectively; however,the eluting peaks were much broader than they were when eluted from the same columns with isooctane solvents.

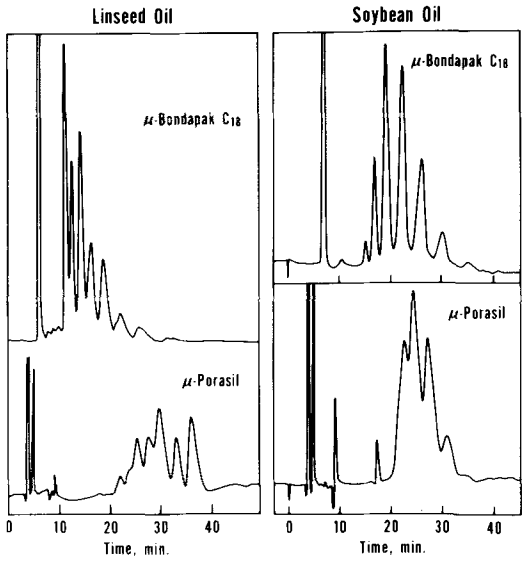

FIG. 3. Comparison of normal and reverse phase HPLC chromatograms of soybean and linseed oils. HPLC conditions: μ-Bondapak C_{18} eluted with acetonitrile/acetone (2:1) at 1.0 ml/min; μ-Porasil eluted with isooctane/ether/acetic acid (99:1:1) at 1.0 ml/min.

Figure 3 shows a comparison of the HPLC chromatograms obtained for soybean oil and linseed oil on normal and reverse-phase columns. The separations obtained on the μ-Porasil columns were the reverse of those obtained on the μ-Bondapak column. Improved column efficiencies on the micro particulate silica columns could lead to useful separations because silica columns, in general, have higher capacities than reverse phase columns, and the triglycerides are much more soluble in the solvents used in the HPLC analysis.

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Dietary Polyunsaturated Fat Versus Saturated Fat in Relation to Mammary Carcinogenesis

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ABSTRACT

High levels of dietary fat have been shown to promote the development of mammary tumors induced in rats by 7,12-dimethylbenz(α)anthracene, and polyunsaturated fats were found to be more effective than saturated fats. In further studies it was found that diets containing3% sunflowerseed oil (polyunsaturated fat) and 17% beef tallow or coconut oil (saturated fats) enhance tumorigenesis as much as a diet containing 20% sunflowerseed oil. Rats on these diets developed at least twice as many tumors as those fed diets containing either 3% sunflowerseed oil or 20% of the saturated fats alone. These results are in accord with human epidemiological data which show that breast cancer mortality in different countries is positively correlated with total fat intake but not with intake of polyunsaturated fat. Total fat intake varies greatly in different countries, but most human diets probably contain levels of polyunsaturated fat at least equivalent to 3% sunflowerseed oil.

The first study carried out in our laboratory to investigate effects of dietary fat on mammary carcinogenesis showed that tumors induced by 7,12-dimethylbenz(α)anthracene (DMBA) developed more readily in rats on a semipurified diet containing 20% (w/w) corn oil, compared to rats on a similar high fat diet containing 20% coconut oil or a low fat diet containing 0.5% corn oil (1). On reviewing the literature, it was found that experiments in a number of laboratories had shown that rats and mice fed high fat diets were consistently more susceptible to mammary tumorigenesis than those fed corresponding low fat diets. This was observed both with spontaneous tumors (2,3) and with tumors induced by different carcinogens (4,5). Most of these studies were carried out during the 1940s and early 1950s, and during the 10 years prior to our study, very little effort was devoted to investigating the role of diet in carcinogenesis. However, renewed interest in these effects of dietary fat on mammary carcinogenesis in animals was stimulated by the realization that epidemiological data for human populations show a strong positive correlation between mortaility from breast cancer and dietary fat intake in different countries of the world (6-8).

Our original study with the animal model was based on the hypothesis that dietary fat might influence tumorigenesis by altering the distribution and/or metabolism of DMBA, the carcinogenic hydrocarbon used to induce the tumors. This possibility was suggested by the lipophilic nature of DMBA and its tendency to accumulate and persist in adipose tissue (7). However, subsequent experiments showed that a high corn oil diet stimulated tumorigenesis only when it was fed after treatment of the rats with DMBA (8,9). This suggested that the dietary corn oil was acting as a promoting agent rather than influencing initiation of the tumors.

This conclusion is supported by other experimental evidence. As noted above, high fat diets were found to enhance the development of spontaneous tumors as well as tumors induced by different carcinogens, indicating that the effect is independent of the initiating stimulus. Furthermore, recent studies by Chan et al. (10) have shown that the fat effect is not limited to tumors induced by lipophilic carcinogens, but can also be demonstrated with mammary tumors induced by the water-soluble, direct-acting carcinogen, N-nitrosomethylurea. In addition, experiments by Rao and Abraham (11) and by Hopkins and West (12) have provided evidence that dietary fat can influence the growth of transplantable mammary carcinoma in host animals.

If these findings in experimental animals are relevant to the development of breast cancer in humans, the evidence that dietary fat acts as a promoting agent has important implications for cancer prevention. It is becoming generally accepted that environmental factors such as diet have an important influence on carcinogenesis, but there is still a strong tendency to think that this influence is mediated largely through environmental or dietary carcinogens. Prevention of cancer by identification and elimination of the many possible carcinogens or procarcinogens in the environment would seem to be an almost impossible task. However, if dietary fat acts as a promoting agent, a substantial reduction in breast cancer incidence could

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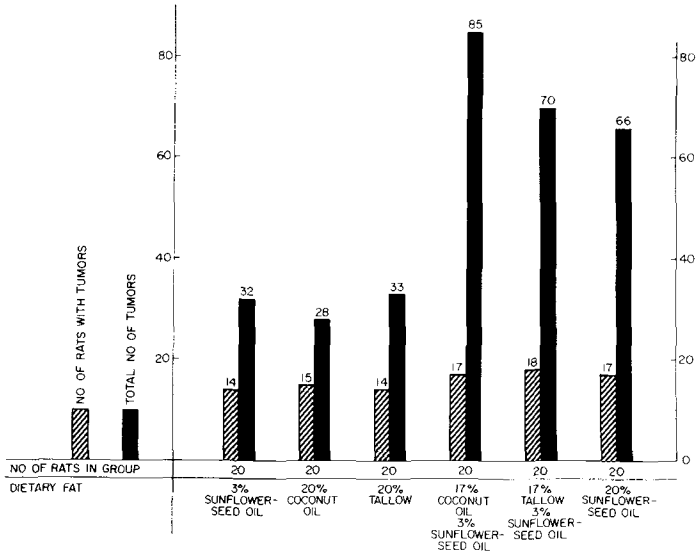

FIG. 1. Effects of type and level of dietary fat on development of mammary tumors in female Sprague-Dawley rats treated with DMBA. The rats were maintained on Purina Chow and were given 5 mg of DMBA in 0.5 ml of sesame oil by stomach tube at 50 days of age. One week later they were transferred to semipurified diets similar to those used in earlier studies (13), with fat contents as indicated. The rats were autopsied 4 months after receiving the DMBA. Both palpable and nonpalpable tumors are included in the results shown.

perhaps be achieved by simply reducing the fat content of the diet, without regard to the initiating stimulus. It might also be possible to reduce the likelihood of metastasis after removal of a breast tumor by decreasing the fat content of the diet and thus providing a less favorable environment for proliferation of cancer cells derived from the primary tumor.

Such considerations encouraged us to continue our studies of the effect of dietary fat on mammary carcinogenesis in rats, in order to obtain more information regarding the exact dietary components responsible for the effect, and the nature of the mechanisms involved. These studies have included feeding trials with a variety of different dietary fats and oils (13), which confirmed the indication from our first experiment that unsaturated fats enhance mammary tumorigenesis more effectively than saturated fats (1). Polyunsaturated fats such as cottonseed oil and sunflowerseed oil nearly doubled the number of tumors in rats treated with DMBA, when fed as 20% (w/w) of the diet, whereas fats such as coconut oil, butter or tallow, fed at the same level, produced little if any increase in tumor yield over that obtained with a low fat diet. However, there did not seem to be a direct correspondence between degree of unsaturation and mammary tumor yield. For example, rats fed lard or olive oil developed about the same number of tumors as those fed corn oil or soybean oil, although the latter oils are much more highly unsaturated (13). A similar observation was made by Dayton et al. (14), who found that a high oleic safflower oil enhanced tumorigenesis in DMBA-treated rats to the same extent as a high-linoleic safflower oil.

If these observations in the animal model are applicable to human populations, one might expect to see a relationship between human breast cancer mortality and the type, as well as the amount of fat consumed in different counties. On the basis of the data available, it is not possible to obtain a quantitative measure of the degree of unsaturation of dietary fat available to the various nations of the world. However, a rough idea may be obtained by comparing the proportions of vegetal fats in different national food supplies, since vegetal fats generally tend to be more unsaturated than animal fats.

Breast cancer mortality in different countries shows a positive correlation with intake of animal fat, but little or no correlation with intake of vegetal fat. Furthermore, the correlation with animal fat intake is not as strong as that with total fat intake (15). Comparative studies of Japanese and American diets by Insull et al. (16) have shown that Japanese consume a more unsaturated type of fat than Americans, and their adipose tissue fats contain a higher proportion of linoleic acid than those of Americans. However, breast cancer incidence

(17) and mortaility (18) are much lower in Japanese women than in American women. These data do not seem to fit with the observed effects of different dietary fats on mammary tumorigenesis in the animal model, and therefore cast doubt on the model as a predictor of factors which may influence breast cancer development in humans.

This apparent discrepancy appears to have been resolved by recent studies in our laboratory on rat mammary carcinogenesis. Results of these experiments are illustrated in Figure 1. Rats treated with DMBA and fed a high fat diet containing 20% sunflowerseed oil developed about twice as many mammary tumors as those fed either a low fat diet containing 3% sunflowerseed oil or high fat diets containing 20% coconut oil or beef tallow. These results were predictable on the basis of our earlier observations (13). However, when 3% sunflowerseed oil was fed with 17% of either coconut oil or tallow, the yield of tumors increased to the level obtained by feeding 20% sunflowerseed oil (Fig. 1).

These findings indicate that there is a requirement for polyunsaturated fat in mammary tumorigenesis, which is not satisfied by fats such as coconut oil or beef tallow, but can be provided by adding 3% sunflowerseed oil to these fats. This may explain why fats such as lard and olive oil are nearly as effective as more unsaturated fats in promoting mammary tumorigenesis, since they would provide as much polyunsaturated fatty acid (linoleic acid) as 3% sunflowerseed oil, when fed as 20% (w/w) of the diet. The results of Dayton et al. (14) could also be explained on this basis, since the high oleic safflower oil used in their experiments would provide an even higher level of linoleic acid than either lard or olive oil. Our experiments also demonstrate that, in addition to the need for polyunsaturated fat, there must also be an overall requirement for a high fat diet, since neither 3% sunflowerseed oil in the present experiments nor 5% corn oil in earlier studies (13) were capable by themselves of enhancing the tumor yield.

These observations on the animal model can now be reconciled with the data on human populations. The total fat available for dietary purposes varies greatly in different countries (19) but is more likely to be derived from vegetal than animal sources in countries where fat intake is low. Thus, it seems probable that most human diets supply polyunsaturated fat at the level needed to demonstrate the promoting effect of dietary fat in the animal model. The observed positive correlation between dietary fat and breast cancer mortality in humans is

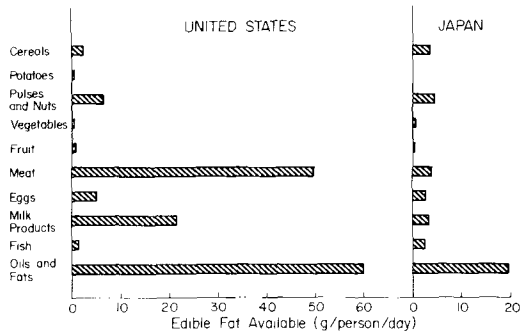

FIG. 2. Sources of available dietary fat in the United States and Japan, based on data published by FAO (19).

therefore more likely to be related to total fat intake, which also influences mammary tumorigenesis in animals, as discussed above. The observation that human breast cancer mortality shows the best correlation with total fat intake and no correlation with vegetal fat intake (15,20) is thus in accord with our recent observations on the animal model. This has helped to restore our confidence that the model does provide information of relevance to the etiology of human breast cancer.

If one accepts this premise, it follows that reduction in dietary fat intake should be accompanied by a fall in breast cancer incidence and mortality. This is one of the reasons for including a reduction in fat intake as one of the dietary goals for the United States (21). Sources of available dietary fat in the United States are shown in Figure 2, and similar data for Japan are given for comparison. It can be seen that 90% of the available fat in the American diet is derived from meat, milk products, and oils and fats such as margarines, shortenings and salad oils. Any attempts to achieve a substantial reduction in dietary fat intake would undoubtedly involve these components of the diet.

ACKNOWLEDGMENT

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Membrane-Bound Phospholipid Desaturases

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ABSTRACT

This review covers studies on membrane-bound phospholipid desaturases in yeast and rat liver carried out in this laboratory. In yeast the desaturase system was shown to effect the direct desaturation of dioleoyl-lecithin to dilinoleoyl-lecithin. In rat liver the desaturase was capable of converting 2-eicosatrienoyl-lecithin to 2-arachidonoyl-lecithin. Both systems required reduced pyridine nucleotides, O_2 and cytochrome b_5. Eicosatrienoyl-lecithin desaturase along with eicosatrienoyl-CoA desaturase of rat liver microsomes was solubilized with detergents and purified 7-8-fold from the microsomal pellets. Both activities were reconstituted in the presence of deoxycholate on addition of the other components of the cytochrome b_5-electron transport chain (cytochrome b_5 and NADH-cytochrome b_5 reductase) to the solubilized desaturase; addition of lecithin further stimulated the activities. The demonstration of desaturation of eicosatrienoyl-lecithin by a solubilized and partially purified desaturase provides strong evidence for the direct desaturation of the lecithin substrate without prior conversion to the acyl-CoA thiolester.

Long chain unsaturated fatty acids are formed in eukaroytes by direct aerobic desaturation of acyl chains in a reaction requiring both oxygen and an electron donor such as NADH or NADPH. Recent studies have shown that the microsomal-bound enzyme system responsible for the desaturation reaction consists of three proteins: NADH-cytochrome b_5 reductase, cytochrome b_5 and a terminal cyanide sensitive desaturase (1-13). The postulated roles of these components in the overall desaturation process are illustrated in Figure 1. Briefly, the reducing equivalents from the electron donors are transferred to the desaturase by way of the cytochrome b_5 reductase and cytochrome b_5, and the desaturase thus reduced activates molecular oxygen for the desaturation reaction.

Desaturation of acyl chains in the form of thioesters (either CoA or ACP derivates) is well established, but evidence provided by Gurr et al. (14) and Baker and Lynen (15) suggested that desaturation of phospholipid-linked fatty acids can also occur. Direct desaturation of phospholipids apart from that of acyl thioesters has now been demonstrated conclusively in the yeasts *Torulopsis utilis* (16) and *Candida lipolytica* (17,18) as well as in rat liver (19). Studies to be presented here will be limited largely to those done in our laboratory on desaturation of phospholipids.

The first phospholipid desaturase system studied in this labroatory was that of the yeast, *C. lipolytica*.. To test the possibility of direct desaturation of phospholipids, we incubated either [^{14}C,^{32}P]lecithin or [^{14}C,^{32}P]phosphatidyl ethanolamine with yeast microsomes in the reaction mixture containing oxygen and reduced pyridine nucleotides (17). These doubly labeled phospholipids were obtained from cells of *C. lipolytica* grown in the presence of [^{14}C]acetate and [^{32}P]orthophosphate (17) and were used as substrates in order to eliminate the possibility of hydrolytic breakdown of the substrate and/or resynthesis by transacylation. When ^{14}C- and ^{32}P-labeled

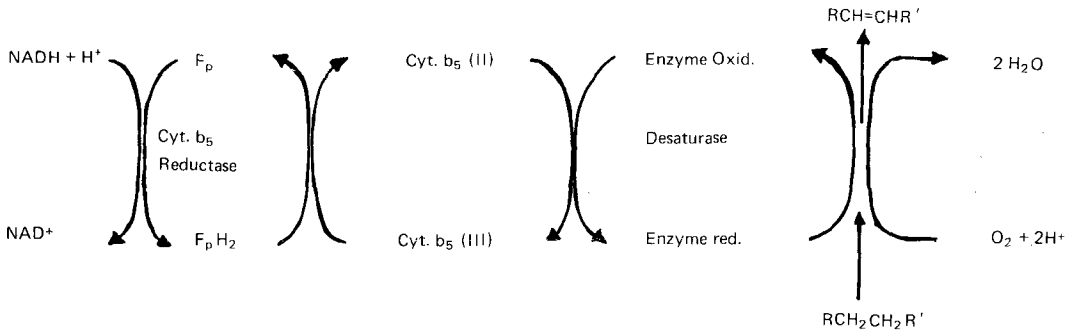
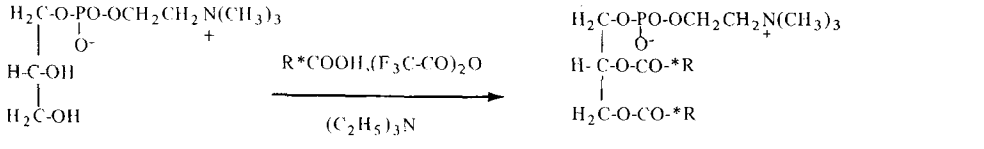


FIG. 1. Pathways of microsomal electron transport coupled to desaturation of acyl chains.

a) Chemical Procedure

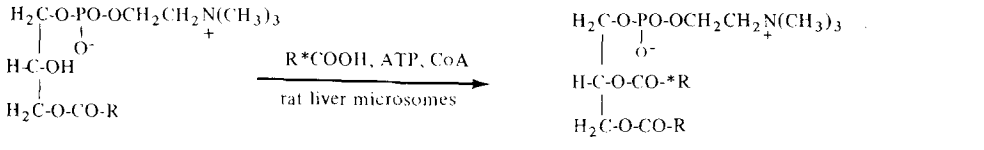


sn-3-glycerophoshorylcholine

1,2-[^{14}C]diacyl-*sn*-3-glycerophosphorylcholine

R*COOH = [^{14}C]oleic acid
[^{14}C]linoleic acid
[^{14}C]eicosatrienoic acid

b) Enzymatic Procedure



1-acyl-*sn*-3-glycerophosphorylcholine

1-acyl-2-[^{14}C]acyl-*sn*-3-glycerophosphorylcholine

R*COOH = [^{14}C]oleic acid
[^{14}C]linoleic acid
[^{14}C]eicosatrienoic acid

FIG. 2. Preparation of ^{14}C-labeled lecithin substrates by a) chemical procedure (20); and b) enzymatic acylation procedure (18,19,21).

lecithin or phosphatidyl ethanolamine were incubated with *C. lipolytica* microsomes in the presence of oxygen and reduced pyridine nucleotides, no breakdown of either substrate was observed. This was shown by the relatively constant ^{14}C/^{32}P ratios of the substrates and the products reisolated from the incubation mixture. For lecithin, the ^{14}C/^{32}P ratios of the substrate and the product were 1.2 and 1.3, respectively; for phosphatidyl ethanolamine, the ratios were 0.74 and 0.79.

Both phospholipids isolated after the incubation, however, differed from the respective substrates in that they contained an increased amount of linoleic acid. The proportion of linoleic acid in both lecithin and phosphatidyl ethanolamine increased by 5-10%. The increased amount of linoleate in the reisolated products appeared to be due to direct desaturation of the phospholipid substrates.

One of the difficulties with these early studies was that the phospholipid substrates, particularly the lecithin substrate, already contained a high proportion of linoleic acid. To get around this problem, we prepared specifically labeled phospholipid substrates (Fig. 2). 1-Acyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine was prepared biosynthetically from [1-^{14}C]oleic acid with rat liver microsomes (18,19,21), and 1,2-di[^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine by acylation of the $CdCl_2$ complex of *sn*-glycero-3-phosphorylcholine with [1-^{14}C]oleic acid in the presence of trifluoroacetic anhydride and triethylamine (20). Both the biosynthetically prepared and the chemically synthesized lecithins were desaturated by the yeast microsomal system (18). With both substrates the reaction proceeded without a lag period and was linear for about 15 min. The 1,2-di[^{14}C]oleoyl-glycerophosphorylcholine appeared to be desaturated more rapidly than the 1-acyl-2-[^{14}C]-oleoyl substrate (Table I) but, as described below, the former substrate can undergo desaturation at both 1- and 2-positions. With either substrate no conversion to free fatty acids, other labeled phospholipids, or water-soluble products was observed. These observations confirm the results obtained with the ^{14}C- and ^{32}P-labeled phospholipid substrates indicating that no breakdown of the phospholipids occurs under desaturating conditions, as is shown also by the fact that desaturation of both lecithin species proceeds without a detectable lag period. Furthermore, the specific activity of the lecithin desaturase, under optimal conditions, is comparable to that of the oleoyl-CoA desaturase.

The conversion of 1-acyl-2-[^{14}C]oleoyl-glycerophosphorylcholine to the 2-[^{14}C]linoleoyl derivative required molecular oxygen and a reduced pyridine nucleotide (Table II).

TABLE I

Relative Rates of Desaturation of Lecithins and Coenzyme A Esters

Substrate	Rate of desaturation[a]		
	C. *lipolytica*	Normal rats	Starved-refed rats
	pmol/min/mg protein		
Lecithins			
1-[^{14}C]stearoyl-2-acyl[b]	0	0	0
1-Acyl-2-[^{14}C]oleoyl	65 ± 15	---[c]	---
1,2-Di-[^{14}C]oleoyl	83 ± 10	20[d]	---
1-Acyl-2-[^{14}C]eicosatrienoyl	---	20 ± 3	50 ± 8
1,2-Di-[^{14}C]eicosatrienoyl	---	10	---
1-Acyl-2-[^{14}C]linoleoyl	---	2	---
Coenzyme A esters			
[1-^{14}C]stearoyl	---	370 ± 40	2,330 ±200
[1-^{14}C]oleoyl	73 ± 9	30[d]	---
[1-^{14}C]eicosatrienoyl	---	53 ± 7	140 ± 6
[1-^{14}C]linoleoyl	---	5	---

[a]Determined in a reaction mixture (1 ml) containing either 30 μM ^{14}C-labeled lecithin or fatty acyl-CoA (0.03-0.1 μCi), 100 mM potassium phosphate (pH 7.2), 100 mM sucrose, 10 mM NADH and microsomes containing 0.5 to 2 mg of protein. Triton X-100 or deoxycholate (0.1%) was added with the lecithin substrate only. Incubations were for 15 min at 25 C with microsomes of *C. lipolytica* (18) and at 37 C with those of rat liver (19). Reactions were terminated by extraction of the lipids with methanol/chloroform (2:1, v/v). The recovered lipids were methanolyzed (22) and the resulting fatty acid methyl esters analyzed by $AgNO_3$-TLC as described previously (17,19).

[b]This substrate was prepared biosynthetically from [1-^{14}C]stearic acid with rat liver microsomes (18,19) in presence or absence of added lysolecithin. The [^{14}C]stearoyl group was found to be 98% in the C-1 position after treatment of the [^{14}C]stearoyl-lecithin with phospholipase A_2.

[c]The dash signifies "not determined."

[d]The product was presumably *cis*-6,9-octadecadienoate.

Oxidized nucleotides could not substitute for reduced; however, under the conditions of the experiment, no significant preference was shown between NADH and NADPH. The phospholipid desaturase was inhibited by KCN, the activity being abolished by 5mM KCN. The presence of carbon monoxide in the gas phase, however, had no inhibitory effect. The inhibition by cyanide and the noninhibition by carbon monoxide thus indicates the involvement of a cytochrome b_5-linked microsomal electron transport system and not a P-450-linked system in the desaturation of phospholipids.

The positional distribution of ^{14}C-labeled fatty acids in the 1,2-di-[^{14}C]oleoyl lecithin substrate and in its desaturation products was determined by treatment with snake venom phospholipase A_2 (18). The composition of ^{14}C-labeled fatty acids in both the 1- and 2-positions of the synthetic substrate was 93.5% oleate and 6.5% linoleate. [^{14}C]Linoleate was present as a contaminant in the [^{14}C]oleic acid used in the synthesis of di-[^{14}C]oleoyl lecithin substrate. The lecithin isolated after the desaturation contained an increased amount of linoleic acid at both the 1- and 2-positions, but to a greater extent at the

TABLE II

Cofactor Requirements of Lecithin Desaturases

Assay conditions	Rate of desaturation[a]	
	C. *lipolytica*[b]	Rat liver[c]
	%	
Complete	100	100
- NADH	6	14
- O_2	15	5
Complete + 1 mM KCN	46	89
Complete + 5 mM KCN	9	45
Complete + CO	150	105

[a]Determined as described in Table I. The rate of desaturation of 1-acyl-2-[^{14}C]oleoyl-glycerophosphorylcholine by *C. lipolytica* microsomes in the complete system was 50 pmol/min/mg; that of 1-acyl-2-[^{14}C]-eicosatrienoyl-glycerophosphorylcholine by microsomes of starved-refed rats in the complete system was 55 pmol/min/mg.

[b]Data of Pugh and Kates (18).

[c]Data of Pugh and Kates (19).

TABLE III

Apparent Kinetic Constants of Lecithin Desaturases[a]

Lecithin	*C. lipolytica*[b]						Starved-refed rats		
	Grown at 25 C			Grown at 10 C					
	Km (app) x 10^4		V	Km (app) x 10^4		V	Km (app) x 10^4		V
	M		pmol/min/mg	M		pmol/min/mg	M		pmol/min/mg
1-Acyl-2-[^{14}C]oleoyl	2.5		1300	2.5		710	---		---
1,2-Di-[^{14}C]oleoyl	9.5		3600	---		---	---		---
1-Acyl-2-[^{14}C]eicosatrienoyl	---[d]		---	---		---	3.6		257

[a]Determined in a reaction mixture (1 ml) containing 100 mM potassium phosphate (pH 7.2), 100 mM sucrose, 10 mM NADH, 0.1% Triton X-100, 1-2 mg microsomal protein and varying amounts of ^{14}C-labeled lecithin up to 100 μM. Incubations were for 15 min at 25 C with microsomes of *C. lipolytica* and at 37 C with those of rat liver. Apparent K_m and V values were calcualted from double-reciprocal plots according to Lineweaver-Burk.

[b]Data of Pugh and Kates (18).

[c]Data of Pugh and Kates (19).

[d]*The dash signifies "not determined."*

2-position: namely, net increases of 8% and 16% in the 1- and 2-position, respectively. These results show that, in this organism, phospholipid desaturation takes place at both position-1 and position-2 of lecithin, but more rapidly at position 2.

Previous work from this laboratory has shown that the lipids of *C. lipolytica* contain lower proportions of oleic and higher proportions of linoleic acids when the organism is grown at 10 C than when the organism is grown at the normal growth temperature of 25 C (23,24). Microsomal membranes prepared from cells of *C. lipolytica* grown at 10 C and 25 differed similarly in the proportions of oleic and linoleic acids present. Membranes prepared from cells grown to mid-log phase at 25C contained 50% linoleic and 30% oleic acids (molar ratio 18:2/18:1, 1.7), whereas those grown at 25 C contained 40% linoleic and 40% oleic acids (molar ratio 18:2/18:1, 1.0).

When phospholipid desaturase activity was measured in the two membrane fractions (18), the activity of the 10 C membrane (20-25 pmol/min/mg) was lower than that of the 25 membrane (50-80 pmol/min/mg) at the standard assay temperature of 25C. The lower phospholipid desaturase in the 10 C membrane was due neither to an unusual temperature sensitivity of the enzyme (see below), nor to an altered apparent K_m for the lecithin substrate (Table III).

Arrhenius plots of phospholipid desaturase activity for the two membrane preparations showed that the activity of the 10 C cells was lower than that of the 25 C cells at all temperatures studied over the temperature range 10-37 C (18). The Arrhenius plots appeared to be linear, with both membrane preparations, over the temperature range 10-30 C; no clear-cut evidence of a change in slope or phase transition was observed with this membrane-bound enzyme. The apparent lack of any phase transitions was not due to the presence of Triton X-100 in the reaction mixture, since identical slopes were obtained in the absence of this detergent.

The effect of temperature on the oleoyl-CoA desaturase activity of the membrane preparations from 20 C and 25 C grown cells was also determined. In contrast to the results obtained with the phospholipid desaturase, the oleoyl-CoA desaturase was found to be more active in cells grown at 10 C than in those grown at 25 C. Arrhenius plots of oleoyl-CoA desaturase activity showed that the activity of the 10C membrane was higher than that of the 25C membrane at all temperatures tested from 10-37 C. These Arrhenius plots also appeared to

be linear with both membrane preparations. These data suggest that in *C. lipolytica* the acyl-CoA desaturase is involved in temperature control of membrane fatty acid composition to a greater extent than the phospholipid desaturase.

Evidence presented here has shown that the yeast *C. lipolytica* contains a membrane-bound desaturase system capable of the direct desaturation of oleoyl lecithin to α-linoleoyl lecithin. Conversion of oleate to α-linoleate does not occur in animal systems. However, we undertook studies designed to show whether the more highly unsaturated fatty acids found in animals, e.g., homo-γ-linolenic and arachidonic acids, could arise by desaturation of phospholipid-linked fatty acids as well as acyl-CoA derivatives.

After incubation of 1-acyl-2-[^{14}C] eicosatrienoyl-glycerophosphorylcholine with microsomes from normal rats in the presence of oxygen and reduced pyridine nucleotides under conditions described earlier (19), conversion of esterified eicosatrienoate to [^{14}C] arachidonate occurred to the extent of 9%. The conversion was increased to 22% when microsomes from rats that had been starved and refed a "fat-free" diet (19) were used. In the rat microsomal system, the desaturation was not accompanied by breakdown of the lecithin substrate. About 98 and 99% of the initial [^{14}C] lecithin was recovered after incubation with microsomes from normal and starved-refed rats, respectively.

The product of desaturation of 1-acyl-2-[^{14}C] eicosatrienoyl-glycerophosphorylcholine was identified as [^{14}C] arachidonic acid by conversion to [^{14}C] methyl esters (22) and analysis by radio-gas liquid chromatography. A radioactive fraction corresponding to the methyl ester of [^{14}C] arachidonate was present in the reisolated products but not in the initial substrate. The amount of arachidonate in the products as determined by radio-gas liquid chromatography agreed with that found by $AgNO_3$-TLC within ± 3%.

The liver microsomal system from normal rats was capable of desaturating both the biosynthetically prepared 1-acyl-2-[^{14}C] eicosatrienoyl-glycerophosphorylcholine (19) and the chemically synthesized 1,2-di-[^{14}C]-eicosatrienoyl-glycerophosphorylcholine (20). Under optimal conditions the biosynthetic substrate was desaturated more rapidly than the synthetic substrate (Table I); [1-^{14}C] eicosatrienoyl-CoA was also desaturated by normal rat liver microsomes at a rate more than twice that of the lecithin substrate. Free eicosatrienoic acid, in the absence of the cofactors ATP, Mg^{++} and CoA, was not desaturated (19).

When microsomes from starved-refed rats were used, the rate of desaturation of 1-acyl-2-[^{14}C] eicosatrienoyl lecithin increased 2- to 3-fold (Table I). A similar increase was observed in the rate of desaturation of eicosatirenoyl-CoA; the rate of desaturation of stearoyl-CoA, used as a control in these experiments, increased about 6-fold.

Several lecithin species other than eicosatrienoyl lecithin were tested as substrates for the phospholipid desaturase from rat liver. Although dioleoyl lecithin was desaturated in this system (presumably to the *cis*-6,9-octadecadienoyl lecithin), 1-[^{14}C] stearoyl-2-acyl- and 1-acyl-2-[^{14}C] linoleoyl-lecithin did not serve as substrates (Table I). Previous studies with hen liver microsomes (25) had shown that stearic acid desaturation occurs only with the CoA ester without prior incorporation into phospholipids.

The conversion of eicosatrienoyl lecithin to arachidonoyl lecithin required molecular oxygen and a reduced pyridine nucleotide (Table II). Either NADH or NADPH could serve as electron donor in this system although NADH was the preferred donor. The phospholipid desaturase was inhibited by potassium cyanide, and the presence of carbon monoxide in the reaction mixture had no effect on the desaturation. These properties suggest the involvement of the microsomal electron transport system that requires cytochrome b_5 instead of P-450 in the desaturation of 1-acyl-2-[^{14}C] eicosatrienoyl lecithin, as was found for desaturation of dioleoyl lecithin by *C. lipolytica* (18).

Desaturation of the lecithin substrate in the rat liver system, as in the yeast system proceeded without a lag period, was linear with time for about 15 min, and was proportional to microsomal protein concentration up to 2 mg/ml. The desaturation appeared to follow Michaelis-Menten kinetics and an apparent K_m for lecithin was calculated to be about 4×10^{-4} M and 3×10^{-4} M for rat and yeast systems, respectively (Table III).

The lecithin desaturase activity was stimulated by addition of detergents such as deoxycholate or Triton X-100. Under optimal conditions (0.1% deoxycholate or 0.2% Triton X-100), the presence of detergent increased desaturation of the lecithin substrate about 3- to 8-fold, respectively. By contrast, desaturation of eicosatrienoyl-CoA was only slightly stimulated by low concentrations of detergent and was partially inhibited by concentrations which were optimal for lecithin desaturation (19).

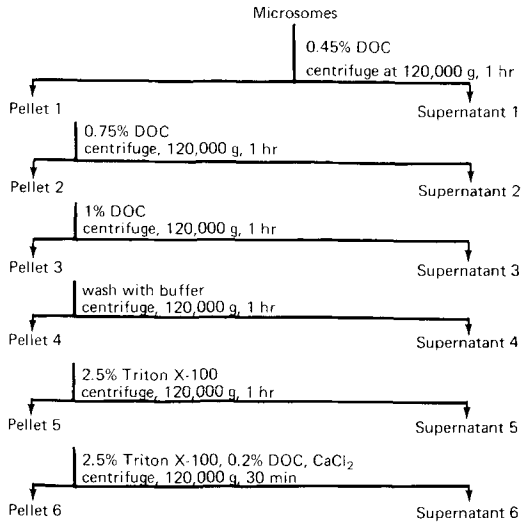


FIG 3. Purification procedure for desaturases of rat liver (12).

The eicosatrienoyl lecithin desaturase was solubilized (26) from liver microsomes of starved-refed rats by the procedure described by Strittmatter et al. (12). Microsomes prepared by the method used previously in this laboratory (19) were extracted by a combination of treatments with Triton X-100, sodium deoxycholate and $CaCl_2$ (see Fig. 3). Each of the solubilized supernatant fractions and the pellets were assayed for eicosatrienoyl lecithin desaturase activity in a reaction mixture containing the components of the microsomal transport chain (O_2, NADH, NADH-cytochrome b_5; Table IV).

The eicosatrienoyl lecithin desaturase was obtained in a solubilized form in the fraction designated "supernatant 6" (Fig. 3; Table IV). This solubilized microsomal fraction showed a 7-fold purification of lecithin desaturase activity over the microsomal pellet. Supernatant 6 was free of cytochrome b_5 and contained greatly reduced amounts of cytochrome b_5 reductase activity but was active in catalyzing the desaturation of both eicosatrienoyl-CoA and stearoyl-CoA in the incubation system containing added cytochrome b_5 and cytochrome b_5 reductase (Table IV).

The reconstituted eicosatrienoyl lecithin and eicosatrienoyl-CoA desaturase systems both required detergent in addition to the NADH-cytochrome b_5 reductase and cytochrome b_5; under conditions of optimal detergent concentrations, the desaturation was further stimulated by the addition of lecithin. Both egg lecithin and dimyristoyl lecithin were found to stimulate desaturation in the reconstituted system. Desaturation of eicosatrienoyl lecithin in the reconstituted system was proportional to desaturase protein concentration up to a concentration of 200 μg/ml.

The evidence presented in this paper has demonstrated that both yeast and rat liver contain desaturase systems capable of the direct desaturation of phospholipid substrates. The alternative possibility is that the lecithin used as

TABLE IV

Relative Desaturase Specific Activities[a]

Cell fraction	Desaturase Activities[b] pmol/min/mg					
	18:0-CoA		20:3-CoA		20:3-PC	
	S.A. x 10^{-3}	Rel. purif.	S.A.	Rel. purif.	S.A.	Rel. purif
Microsomes	2.4	1.0	50	1.0	20	1.0
Pellet 1	3.5	1.5	100	2.0	17	0.8
Pellet 2	8.5	3.5	120	2.4	19	0.9
Pellet 3	14.5	6.0	160	3.2	20	1.0
Pellet 4	16.0	6.7	200	4.0	23	1.1
Pellet 5	18.0	7.5	250	5.0	38	1.9
Supernatant 6	50.0	21.0	400	8.0	146	7.3

[a]Data of Pugh and Kates (26).

[b]Stearoyl-CoA desaturase was determined by the spectrophometric assay of Strittmatter et al. (12). Eicosatrienoyl-CoA and eicosatrienoyl lecithin desaturase were measured in a reaction mixture (1 ml) containing either 30 μM [1-^{14}C]eicosatrienoyl CoA (0.03 μCi) or 1-acyl-2-[^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphorylcholine (0.1 μCi), plus the following components: 200 μg desaturase, 33 μM cytochrome b_5 prepared by the method of Ozols (27), 1.6 μM NADH-cytochrome b_5 reductase prepared by the method of Spatz and Strittmatter (28), 30 μM egg lecithin 0.1% sodium deoxycholate, 10 mM NADH, and 100 mM Tris-acetate buffer, pH 8.1. Incubations were at 37 C for 30 min and reactions were terminated by extraction of the lipids with methanol/chloroform (2:1, v/v). The recovered lipids were methanolyzed (22) and the resulting fatty acid methyl esters analyzed by $AgNO_3$-TLC as described previously (19).

a substrate in these studies is first hydrolyzed by a phospholipase A_2 and the free fatty acid activated by a thiokinase reaction. The resulting CoA ester could then be desaturated by the acyl-CoA desaturase and the desaturation product used to resynthesize lecithin. This possibility is unlikely for several reasons. First, no significant release of free fatty acids from the lecithin substrate was observed under desaturating conditions in either system. Also, CoA and ATP were not required for lipid desaturation as would be expected if a thiokinase were involved at some intermediary step. The chain of events pictured here also seems unlikely for kinetic reasons, since the desaturation of the lipid proceeds without a lag period. Finally, the lecithin desaturase of rat liver has been obtained in a solubilized form and the activity reconstituted in a system containing phospholipid and the other components of the cytochrome b_5 electron transport chain. It has thus become increasingly unlikely that the desaturation of the phospholipid is the result of several enzyme activities. We conclude, therefore, that the desaturation observed in these studies is the direct desaturation of the membrane phospholipid.

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Linolenic Acid Deficiency

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ABSTRACT

Linolenic acid deficiency has not been demonstrated clearly in warm blooded animals, yet circumstantial evidence suggests that n-3 fatty acids may have functions in these animals. The fact that several species of fish definitely require dietary n-3 fatty acids indicates that n-3 fatty acids have important and specific functions in these animals and suggests that such functions may also be present in warm blooded animals. It is also true that n-3 fatty acid distribution in tissues of birds and mammals appears to be under strict metabolic control, and that this complex metabolic control mechanism apparently has survived evolutionary pressure for a very long time. So far, attempts to produce linolenic acid deficiency in mammals have not revealed an absolute requirement for n-3 fatty acids. If functions for n-3 fatty acids do exist in warm blooded animals, it seems probable that they may be located in the cerebral cortex or in the retina, because these tissues normally contain high concentrations of n-3 fatty acids.

INTRODUCTION

Recently, committees of the WHO-FAO and of the American National Research Council have recommended that the human diet contain linolenic acid, in addition to specific recommendations for amounts of linoleic acid, which is known to be required in human beings. There is little direct evidence that linolenic acid is required in man or other warm blooded animals. This lack of evidence suggests that a requirement for linolenic acid, if any, is so low that almost any diet can provide the needed amount, at least in the species of animals that have been investigated in detail. It is certainly true that symptoms of linolenic acid deficiency in warm blooded species of animals are not well known or widely recognized. Despite the lack of evidence for essentiality of linolenic acid in the diets of human beings and other animals, many researchers feel that there is a function for this acid and its metabolites. Therefore, we have reviewed some of the observations that suggest an essential role for n-3 fatty acids in mammals.

METABOLISM OF LONG CHAIN POLYUNSATURATED FATTY ACIDS

Linolenic acid (9,12,15-octadecatrienoic acid, 18:3n-3, or 18:3ω3) and linoleic acid (9,12-octadecadienoic acid, 18:2n-6 or 18:2ω6) are synthesized by plants but not by higher animals. In plants, 18:3n-3 is commonly found in the membrane lipids of the chloroplast, the plant photoreceptor. 18:2n-6 is usually concentrated in the lipids of seeds. Herbivores will, therefore, obtain both of these fatty acids in their diets. These fatty acids are absorbed during digestion and metabolized by the animal body (most species) according to the major paths of elongation and desaturation:

18:3n-3→18:4n-3→20:4n-3→20:5n-3→22:5n-3→22:6n-3

18:2n-6→18:3n-6→20:3n-6→20:4n-6→22:4n-6→22:5n-6.

The endogenously formed oleic acid (9-octadecenoic acid, 18:1n-9 or 18:1ω9) is also elongated and desaturated:

18:1n-9→18:2n-9→20:2n-9→20:3n-9→22:3n-9→22:4n-9.

All of these families of fatty acids are processed by the same enzyme system, which prefers the n-3 family to the n-6 family and has least preference for the n-9 family. Hence, n-9 fatty acids are desaturated and elongated to a significant extent only when the concentrations of n-3 and n-6 fatty acids are relatively low, as happens when animals are fed a fat-free diet.

In the n-3 family, the member found most abundantly in animal lipids is usually 22:6n-3 (4, 7, 10, 13, 16, 19-docosahexaenoic acid), especially in land animals. In marine animals or fish, 20:5n-3 concentrations may also be quite high. 22:6n-3 usually occurs in highest proportions in phospholipids, especially in ethanolamine phosphoglycerides (EPG) of warm blooded animals, but also is found in neutral lipids, especially of fish and shellfish. These long chain polyunsaturated n-3 fatty acids in animal lipids may be obtained preformed in the diet, or they may be formed by the animal itself from dietary 18:3n-3.

DISTRIBUTION AND DIETARY REQUIREMENT OF N-3 FATTY ACIDS IN SHELLFISH

The lipids of many fish and shellfish, including commercially important species, have been analyzed. In shellfish, the proportion of 20:5n-3 is usually greater than that of 22:6n-3. This is true of Alaska king crab (1), the North

Carolina blue crab (2), a South African marine crab (3), and a freshwater crab from Lesotho (3). The proportion of 20:5n-3 is greater than that of 22:6n-3 in a European and an American species of oyster (4) as well as in an oyster from New Zealand (5). Four species of scallops, however, have proportions of 22:6n-3 higher than those of 20:5n-3 (6,7). In several varieties of prawns, the lipids have higher proportions of 22:5n-3 than of 22:6n-3 (8-13). Shrimp, like prawns, often have more 20:5n-3 than 22:6n-3 (12,14). It is not clear whether these shellfish control metabolically the amounts of 20:5n-3 and 22:6n-3 in their tissue lipids, or whether the amounts found are due to the composition of their dietary fatty acids. The fact that the growth of prawns is improved by the addition of long chain polyunsaturated oils to the diet (10,11) suggests that prawns may require preformed long chain polyunsaturated fatty acids. Kanazawa et al. (15) have shown that either 18:2n-6 or 18:3n-3 in the diet of prawns produces far better growth than dietary 18:1n-9 does, and that the effect of 18:3n-3 on growth is greater than that of 18:2n-6. Pollack residual oil, which contains 11% 20:5n-3 and 2% 22:6n-3 (11) produces far better growth than do either 18:2n-6 or 18:3n-3 (15). These data suggest that, in prawns, both n-3 and n-6 fatty acids are required, and that prawns have a limited ability to elongate and desaturate 18:2n-6 and 18:3n-3. We are not aware of any conclusive evidence that proves a requirement for n-3 fatty acids in shellfish. The occurrence of these n-3 fatty acids in specific lipids implies that the placement of these fatty acids is under metabolic control and that this process is advantageous to the shellfish.

DISTRIBUTION AND DIETARY REQUIREMENT OF N-3 FATTY ACIDS IN FISH

Fatty acids in the lipids of many species of fish have been analyzed, and the lipids of freshwater fish often contain higher proportions of n-6 fatty acids than are found in marine fish (16-18). Most fish contain large proportions of both 20:5n-3 and 22:6n-3. Channel catfish (19) and four varieties of murrels (20), all of which are freshwater fish, have higher proportions of 22:6n-3 than of 20:5n-3. Of marine fish, menhaden (21), pilchard (22), mackerel (23), slender tuna (24), horse mackerel (25), herring (26), cod (27-29), sole, halibut and dogfish (29) all have higher proportions of 22:6n-3 than of 20:5n-3, either in total lipids or in phospholipids. In Chinook and Coho salmon, 22:6n-3 is higher than 20:5n-3 (30,31). In juvenile pink salmon captured at sea, there is more 22:6n-3 than 20:5n-3 (32). The sand launce (33), two species of hake (34), and the Chilean anchovy (35) all have higher levels of 22:6n-3 than of 20:5n-3. In contrast to these fish, the lipids of the capelin have more 20:5n-3 than 22:6n-3, and also contain large proportions of 22:1 and 20:1 (36,37). This fish is said to be an important source of food for fish harvested commercially in the North Atlantic. In the species mentioned above, it is not known whether fatty acids are incorporated unchanged from the dietary lipids or whether the fish modify the dietary fatty acids by elongation and desaturation.

The dietary requirements for essential fatty acids have been investigated in several species of fish. In rainbow trout a dietary n-3 fatty acid is definitely required (38-44), and n-6 fatty acids (18:2n-6) are of little value for normal growth (38,39,41). Long chain polyunsaturated fatty acids or 22:6n-3 are more effective than 18:3n-3 in stimulating growth (39,43,44). These data indicate that rainbow trout require n-3 fatty acids and can elongate and desaturate 18:3n-3 to 22:6n-3, but suggest that preformed 22:6n-3 is more effective in stimulating growth. Possibly, the rate of elongation and desaturation of 18:3n-3 to 22:6n-3 limits growth in these fish.

The turbot cannot elongate or desaturate either 18:3n-3 or 18:2n-6 (45), so that it is not surprising that dietary long chain n-3 fatty acids are required for good growth (46-48). The carp grows well if fed either 18:3n-3 or 18:2n-6, but cod-liver oil stimulates growth more than either 18:3n-3 or 18:2n-6 does (49,50). Red sea bream apparently can elongate n-3 or n-6 fatty acids, but grow best when fed oils containing long chain n-3 fatty acids (51-53). Thus, at least four species of fish require n-3 fatty acids, and at present it appears that 22:6n-3 will satisfy the requirement. The ability to elongate and desaturate 18:3n-3 varies with the species. This ability may depend upon the fatty acid available in the natural food supply of the fish. If the natural food supply contains an abundance of 22:6n-3, it is unnecessary for the predator to be able to elongate and desaturate 18:3n-3.

DISTRIBUTION OF N-3 FATTY ACIDS IN TISSUES OF WARM BLOODED ANIMALS

In warm blooded animals, 22:6n-3 is the most abundant member of the n-3 family, and it usually is most concentrated in the ethanolamine phosphoglycerides (EPG) and serine phosphoglycerides (SPG) of a given tissue. The lipids of warm blooded animals usually contain

relatively little 22:6n-3, except for marine mammals and birds whose diet of fish contains much n-3 fatty acid. The total fatty acids or phospholipids of most tissues contain less than 5% of this fatty acid. Thus, it is particularly noteworthy that certain organs or organelles do contain high proportions of 22:6n-3. The best known examples are the lipids of brain or cerebral cortex, retina, spermatozoa, and testis.

In chicks 22:6n-3 is the major polyunsaturated fatty acid in brain phospholipids (54), and 22:6n-3 accounts for 14% of brain total fatty acids in the house sparrow (55). In total fatty acid of mink brain, 22:6n-3 is the most abundant polyunsaturated fatty acid (56). In synaptosomal membranes of mouse brain, 22:6n-3 accounts for 14% of phospholipid fatty acids (57) and in mouse brain microsomes, the proportion of 22:6n-3 increases with the age of the mouse (58). In EPG and SPG from rat brain synaptic vesicles, 22:6n-3 accounts for 31% and 37%, respectively, of the fatty acids (59). The amount of 22:6n-3 in mitochondria of rat brain increases with the age of the rat and becomes the major polyunsaturated fatty acid in 90-day-old rats (60). In a study of lipids in many bovine organs, Gonzato and Toffano reported that the content of 22:6n-3 in phospholipids of cerebral cortex was second only to that in retina (61). In the cerebral cortex of two species of whale and one species of dolphin, 22:6n-3 is 17 to 19% of the total fatty acids in EPG (62-64). The concentration of 22:6n-3 in the cerebral cortex is higher in adult whales and dolphins than in fetuses of these species (65). In the cerebral cortex of the human brain, 22:6n-3 of EPG increases with age and at the age of about 80 years can be 34% of fatty acids in this lipid fraction (66). These data indicate that 22:6n-3 is located with great specificity in particular parts of the brain, and is concentrated in the ethanolamine phosphoglycerides rather than being evenly distributed among lipid classes.

The retina is another organ into which 22:6n-3 is selectively incorporated in many species of animals. Anderson and colleagues (67,68) have reported the fatty acid patterns of phospholipids in whole retinas of dog, pig, human, sheep, bovine and rabbit. In these species, 22 to 23% of the total fatty acid in EPG is 22:6n-3, with lesser proportions of 22:6n-3 in serine, choline (CPG), or inositol phosphoglycerides (IPG). Weiss and Graf (69,70) have shown that the 22:6n-3 content of rabbit retinal phospholipids is highest in rabbits born in the summer, and that the percentage of 22:6n-3 increases with the age of the rabbit, up to 60 days. In the bovine retina, the highest proportions of 22:6n-3 are located in phospholipids, especially EPG and SPG, of retinal rod outer segments (ROS) (71-76) with lower proportions in the phospholipids of mitochondria, microsomes, or nuclei (77). In the rat the total retina or retinal rod outer segments contain large proportions of 22:6n-3 (78-81). If rats are fed a fat-free diet or essential fatty acid-deficient diet for 10 weeks to 11 months (79,80), there is little change in the proportion of retinal 22:6n-3, although the content of 20:3n-9 increases. Evidently, the 22:6n-3 molecules are tenaciously retained in the rat, a species in which the outer segment is normally renewed ca. every 14 days. If rats are raised for two generations on diets that contain little n-3 fatty acid, the content of 22:6n-3 can be reduced to less than half the normal percentage (81,82). In rats fed a fat-free diet, the normal renewal of retinal rod outer segments is impaired (83), although electron micrographs of the rod outer segments show no abnormalities (84). In rats made diabetic with alloxan, the percentage of 22:6n-3 in the retina drops from an initial value of 35% of the total fatty acid to 25% after 116 days (78). The retinal rod outer segments of the frog *Rana pipiens* contain high proportions of 22:6n-3, i.e., 46% of total fatty acids in SPG and 51% in EPG (85). These data indicate that the retina, and especially the photoreceptor or ROS, is particularly enriched with 22:6n-3 in the vertebrate species so far analyzed.

Another tissue in which 22:6n-3 is often very high is the testis. In this organ the lipids usually contain a 22-carbon fatty acid as a major polyunsaturate, but the structure of the chain varies with the species of the animal. In humans (86-89) or bulls (90,91), 22:6n-3 is a major polyunsaturate in the phospholipid of the testis. In boar testis proportions of 22:5n-6 and 22:6n-3 are both high (90,92). In mouse, guinea pig, and hamster testis, there is somewhat more 22:5n-6 than 22:6n-3 (86). The testes of rats, dogs and rabbits contain mainly 22:5n-6 with little 22:6n-3 (86,93). In chicken testis the major polyunsaturated fatty acid is 22:4n-6 (86). Lipids of spermatozoa normally contain the same major polyunsaturate present in the testis of the same species, and the concentration of this polyunsaturate is usually higher in the spermatozoa. Bovine spermatozoa contain remarkably large proportions of 22:6n-3, especially in choline phosphoglycerides (94-96). 22:6n-3 is also the major polyunsaturated fatty acid in spermatozoa of the rhesus monkey (97), ram (98,99), and man (96,99). In the boar both 22:5n-6

and 22:6n-3 are very high in spermatozoa (96,100), while in the dog (101) and rabbit (96), 22:5n-6 is the main polyunsaturated fatty acid. In the chicken the main polyunsaturated fatty acid in spermatozoa is 22:4n-6 (101). These data indicate that 22:6n-3 is selectively incorporated into the lipids of the testis and spermatozoa of certain mammalian species, particularly those of man, monkey, bull, and ram. Considerable amounts of 22:6n-3 are also present in the boar, mouse, guinea pig, and hamster, but 22:6n-3 is a minor component in rat, rabbit, dog, and chicken.

EVIDENCE FOR FUNCTIONS OF N-3 FATTY ACIDS

In fish and shellfish, the presence of large amounts of n-3 fatty acid in the tissues suggests that n-3 fatty acid may serve metabolic functions in these species. The facts that rainbow trout definitely require either 18:3n-3 or 22:6n-3 for good growth, and that n-6 fatty acids are inadequate, indicate that n-3 fatty acids do have some function in this species. Very probably, n-3 fatty acids also have definite functions in carp, red sea bream, and turbot, because the growth of these species is increased by dietary polyunsaturated fatty acids. It is possible that prawns may also require n-3 fatty acids.

In warm blooded animals, 22:6n-3 is selectively incorporated into EPG or SPG, and these particular phospholipids are selectively concentrated in the outer segment of the retinal rod, the cerebral cortex and sometimes in the testis and spermatozoa of many animal species. The 22:6n-3 must be derived from the diet, either directly or indirectly via 18:3n-3, and must be transported, incorporated into particular phospholipids, and installed in a specific location in a particular organ. All these steps must have been subjected to evolutionary pressure for vast periods of time, and have survived to the present. These facts suggest that 22:6n-3 or other n-3 fatty acids may have metabolic functions in warm blooded animals as well as in fish.

Is there any evidence that n-3 fatty acids have specific functions in warm blooded animals? The evidence is scanty, partly because most of the attention has been devoted to the n-6 fatty acids which are easier to work with in many ways. Nevertheless, there is evidence that n-3 fatty acids may have unique functions in warm blooded animals. It is known that dietary linolenic acid will improve growth in essential fatty acid (EFA)-deficient rats, although it will not cure the other symptoms of EFA deficiency, particularly infertility and dermatitis. Bernsohn and Spitz (102) have reported that dietary 18:3n-3 but not 18:2n-6 will restore to normal the activity of 5′-nucleotidase in brain homogenates from EFA-deficient rats. Capuchin monkeys fed a purified diet containing corn oil as a source of EFA developed dermatitis and fatty livers, which were cured by dietary linseed oil (103). This report is puzzling because the lipids of the deficient animals still contained considerable n-3 fatty acid. Electroretinograms were obtained from rats given, for 40 days, fat-free diets or the same diets supplemented with 2% ethyl oleate, ethyl linoleate, or ethyl linolenate. Rats given linoleate had greater amplitudes of both the a and b waves than were found with those given oleate or fat-free diets, and those given linolenate had the highest amplitudes of all (104).

Perhaps the most convincing evidence for a possible function of n-3 fatty acids in mammals was reported by Lamptey and Walker (105). They fed rats, for two generations, purified adequate diets containing either 10% safflower oil (low linolenate, about 300 mg/kg diet) or 10% soy oil (high linolenate, about 8400 mg/kg diet). In these rats the difference between diets had no effect on food intake, growth, litter size, brain size, distribution of lipid phosphorus in brain lipids, or several other parameters. However, in male rats of the second generation, differences in physical activity were associated with the different diets. Probably the most interesting observation was the fact that the rats fed the high linolenate diet performed much better on a Y-maze discrimination test than the low linolenate rats did. For the first three consecutive days of testing, both groups of rats performed equally, but for the following four days, the high linolenate rats increased their percentages of correct responses, whereas those given the low linolenate diet did not improve. In the brain phospholipids of the low linolenate rats, the content of 22:6n-3 was only 10 to 20% of the control (high linolenate) value. The authors noted that this learning impairment may not have been due solely to changes in brain composition because visual function may also have been influenced by changes in retinal lipid composition, which was not measured in this experiment.

FATTY ACID COMPOSITIONS IN TISSUES OF LINOLENIC ACID-DEFICIENT RATS

We decided to produce a dietary linolenic acid deficiency in rats, in order to locate the organs in which the deficiency would have the

TABLE I

Polyunsaturated Fatty Acids in Tissues from Linolenic Acid-Deficient and Control Rats

Tissue	Lipid fraction	Weight percentages of total methyl esters									
	Fatty acid:	20:4n-6		22:4n-6		22:5n-6		22:5n-3		22:6n-3	
	Diet:	C[a]	D[a]	C	D	C	D	C	D	C	D
Muscle	EPG[a]	13.0	21.6[b]	1.3	5.2	1.6	24.6	4.9	0.23	36.1	1.5
Brain	EPG	11.8	13.8	5.5	7.7	0.97	20.4	0.33	0.02	23.7	1.8
Heart	EPG	23.3	27.3	1.8	3.8	2.1	21.1	2.7	0.04	23.3	0.72
Liver	EPG	25.0	31.8	0.58	1.6	1.2	14.1	1.4	0.04	15.6	0.71
Lung	EPG	33.3	36.2	8.6	11.8	1.2	7.1	3.8	0.11	6.1	0.26
Spleen	EPG	38.7	41.3	6.5	9.7	1.2	6.9	3.6	0.08	6.0	0.25
Gastrointestinal tract	EPG	26.7	30.0	4.0	6.0	0.83	5.4	1.7	<0.01	5.5	0.34
Kidney	EPG	42.8	45.2	0.92	1.5	0.59	4.5	0.5	0.03	4.4	0.37
Testis	EPG	23.0	24.7	3.2	3.7	27.9	31.9	0.23	0.10	2.5	0.13
Retina	TFA[a]	9.6	10.3	1.5	2.8	0.7	26.4	0.45	<0.01	33.7	5.1
Plasma	TFA	15.8	22.0	0.25	0.44	0.30	2.5	0.35	0.02	3.2	0.18
Erythrocyte	TFA	15.3	21.3	0.82	2.0	0.79	2.6	1.1	0.06	2.7	0.22
Adrenal	TFA	19.5	17.0	6.4	5.9	0.76	2.7	1.2	0.07	1.9	0.10
Ovary	TFA	4.5	4.7	2.2	3.6	0.27	2.0	0.70	0.03	1.6	0.17
Adipose	TFA	0.43	0.50	0.08	0.08	<0.01	0.25	<0.01	<0.01	0.07	<0.01

[a]Abbreviations: C, control diet; D, linolenic acid-deficient diet; EPG, ethanolamine phosphoglycerides; TFA, total fatty acids.

[b]Underlined values are significantly different from controls, $P < 0.05$ or less. Controls were paired with equal numbers of deficient rats of same age and sex, n=3 to 9.

most severe effect upon lipid composition. Rats were fed for two generations a diet in which the only source of lipid was methyl linoleate, 1.25% by weight. Control rats were given 1.0% methyl linoleate and 0.25% methyl linolenate. We analyzed lipids in animals from both generations, of both sexes, and of different ages. The organs analyzed included brain, heart, lung, kidney, liver, testis, gastrointestinal tract, spleen, muscle, retina, adrenal, ovary, adipose tissue, erythrocytes, and plasma. The lipids of the first nine organs were fractionated by thin layer chromatography, and fatty acids of EPG, CPG, and IPG + SPG were analyzed by gas liquid chromatography. In the other tissues, fatty acid distributions of total lipid extracts were measured. Procedures for handling of rats, preparation of diets, and analysis of lipids have been described earlier (106,107).

We found no effect of diet composition upon growth rates, litter sizes, organ weights, or distributions of lipid phosphorus. The main effect of the deficiency was to lower the proportion of 22:6n-3 in all of the tissues examined. In control rats the highest proportion of 22:6n-3 were found in phospholipids, especially EPG, of muscle, brain, heart, and liver, and in the total fatty acids of retina (Table I). In other tissues proportions of 22:6n-3 were much lower. In the deficient rats, 22:6n-3 was mainly replaced by 22:5n-6 and to a lesser extent by 22:4n-6 or 20:4n-6 (Table I). In liver, kidney, heart, muscle, and gastrointestinal tract of deficient rats, the proportions of 22:5n-6 were higher in females than in males (data not shown).

Retina, brain, and muscle seemed to retain 22:6n-3 more strongly than other tissues did, because first generation deficient rats had higher proportions of 22:6n-3 in lipids of these tissues than were found in second generation rats (data not shown). In other tissues there was little difference between the generations in proportions of 22:6n-3.

The quantity of dietary 18:3n-3 needed for survival in rats is obviously very low, and was ca. 40 mg/kg diet in our experiments. This is far less than the requirement for n-6 fatty acid, which is about 10 g/kg diet or more for most mammalian species. Fish (trout) require roughly 1 or 2% of n-3 fatty acid in the diet, which is similar to the mammalian requirement for n-6 fatty acid. If n-3 fatty acids do have essential functions in mammals, or in rats in particular, only small amounts of n-3 fatty acids are needed to fulfill these functions. One of the functions of n-6 fatty acids is to furnish precursors for the formation of prostaglandins, endoperoxides, thromboxanes and related compounds which have powerful biological activity. There is evidence that n-3 fatty acids also can give rise to prostaglandin derivatives that have activity in mammalian tissues (108-110). The physiological effects of prostaglandins and related compounds are produced by very low concentrations of these substances.

CONCLUSION

Are n-3 fatty acids required in the human diet? There appears to be no direct evidence related to this question. Human beings, in common with many other species of mammals, have high proportions of 22:6n-3 concentrated in certain phospholipids of the cerebral cortex, retina, and spermatozoa. The proportion of 22:6n-3 in human cerebral cortex increases with age, as is the case in rats, mice, and whales. No one knows what the optimal level of 22:6n-3 in any tissue may be, or if there is an optimal level.

Before the question of the essentiality of n-3 fatty acids in man can be answered, a function for these molecules must be detected and demonstrated, probably in experimental animals. The use of linolenic acid-deficient diets should allow changes in function to occur and to be measured. If functions can be detected and measured in experimental animals, then procedures can be developed for measuring these functions in human beings. At present, it seems most probable that the functions of n-3 fatty acids will be located in retina or brain, and possibly in muscle or spermatozoa of certain species.

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The Metabolism of Dihomo-γ-Linolenic Acid in Man

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ABSTRACT

Orally administered dihomo-γ-linolenic acid (DHLA) is well absorbed in man; it appears in blood after ca. 4 hr first as triglyceride ester and later as phospholipid. After sustained-dosing, DHLA penetrated membrane pools and all phospholipid components but, depending on the dosage, reached a metabolic equilibrium in 4-16 days. Intact platelets do not accumulate arachidonate following DHLA administration, and species differences occur in the capacity of animals to metabolize DHLA to arachidonic acid (AA). The rat appears to be unusual in having a very active hepatic Δ^5-desaturase enzyme system. Potentially antithrombotic changes in platelet function which followed the administration of DHLA to man were accompanied by a significant increase in the capacity of platelets to synthesize PGE_1. Concomitant increases in PGE_2 synthesis do not apparently result from an increased production of AA and suggest that DHLA, or a DHLA metabolite, interferes with the metabolism of AA. Effects on thromboxane and prostacyclin synthesis are being studied.

INTRODUCTION

Dihomo-γ-linolenic acid (DHLA) is a naturally occurring member of the ω-6 series of long chain essential fatty acids. It cannot be synthesized de novo by higher animals, including man, but can be synthesized by alternate desaturation and chain elongation of linoleic acid (1), particularly in the liver (2).

In most tissues the content of arachidonic acid (AA) predominates over that of DHLA (3). As components of phospholipids, these fatty acids play an important role in determining the structural and functional properties of membranes (4).

The precursor relationship of DHLA and AA to the prostaglandins ensures that these essential fatty acids are not functionally equivalent: DHLA is a precursor of the monoenoic prostaglandins; AA is a precursor of the dienoic prostaglandins, thromboxane, and prostacyclin.

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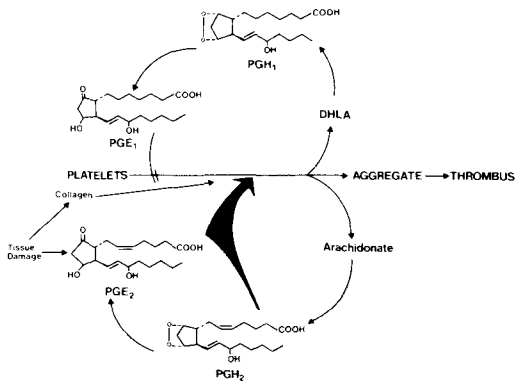

FIG. 1. Mechanism for limiting thrombus formation by redirection of platelet prostaglandin synthesis.

Metabolism of Dihomo-γ-linolenic and Arachidonic Acids to Prostaglandins: Relationship to Platelet Function

Our particular interest in DHLA developed from an assumption that platelet aggregation is a contributory factor to the initiation and development of thrombosis, and from the observation that the prostaglandin metabolites of DHLA and arachidonic acid, PGE_1 and PGE_2 respectively, have opposite effects on platelets (5). In this respect, platelets are quite unusual. In most tissues, PGE_1 and PGE_2 exert qualitatively similar pharmacological effects (5,6).

Platelets have a particularly high content of arachidonic acid (7). When platelets are stimulated to aggregate, arachidonic acid is released

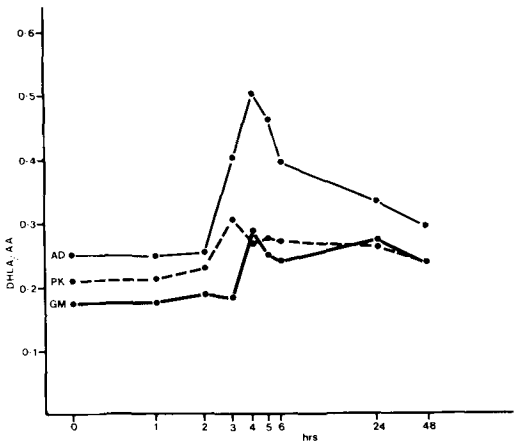

FIG. 2. Plasma DHLA/AA ratio after a single 1 g dose of DHLA. The fatty acid ratio was determined by gas liquid chromatography of the fatty acid methyl esters on a SCOT capilliary column. Information on the volunteers taking part in these studies is contained in ref. 12.

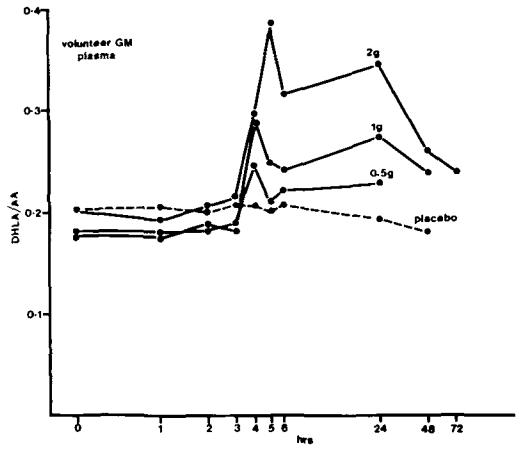

FIG. 3. Dose-response relationships to single oral doses of DHLA in one volunteer.

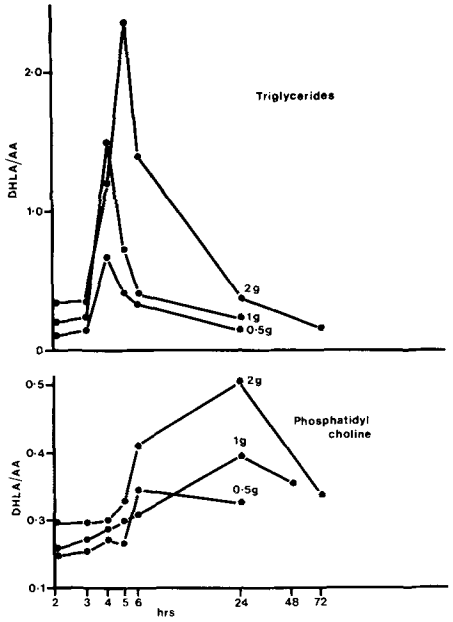

FIG. 4. Incorporation of DHLA into triglycerides and phospholipids of plasma after single oral doses of DHLA. Plasma lipids were extracted using the Bligh and Dyer procedure (13). Different lipid classes were separated by silicic acid chromatography of the extract and resolved into their constituents using thin layer chromatography.

from membrane phospholipids by phospholipase A_2 (8) and metabolized via the endoperoxide PGH_2 into the more stable end-products PGE_2 and thromboxane B_2. The intermediates, PGH_2 and thromboxane A_2 promote further platelet aggregation, leading to thrombus formation (9). Conversely, prostaglandin metabolites of DHLA, PGH_1 and PGE_1, do not induce aggregation (10) and PGE_1 is a potent inhibitor (11).

The high platelet content of AA normally ensures that, during platelet aggregation, PGH_2 and PGE_2 synthesis is dominant. Our objective in studying DHLA as an antothrombotic agent was to increase the proportion of DHLA available to the prostaglandin synthesizing system, so that more DHLA would be released during aggregation to act as a biological brake to the thrombotic process.

The schematic representation in Figure 1 is a simplified view which does not take into account possible effects on the synthesis of thromboxane (proaggregatory) or PGD_2 and prostacyclin (antiaggregatory). These arachidonate metabolites could either increase or decrease the efficacy of DHLA. An objective of the present work was to determine whether antithrombotic activity could be correlated with increased levels of PGE_1.

Some of the effects of orally ingested DHLA on platelet function in man have already been published (12). In this paper the biochemistry of this fatty acid in man is considered particularly in relation to what is known of its behavior in other animals. Clearly the degree to which DHLA exerts an antithrombotic effect in vivo will depend on its subsequent metabolism and especially on (a) its availability to the prostaglandin synthesizing system, (b) the degree to which it is desaturated to arachidonic acid, and (c) the spectrum of prostaglandin and related metabolic products which are formed by prostaglandin synthetase.

Incorporation of Dihomo-γ-linolenic Acid into Blood Lipids and Its Availability to Prostaglandin Synthetase

The relative availability of DHLA and AA presumably determines which prostaglandins (PGE_1 or PGE_2) are synthesized. We have, therefore, primarily been interested in determining the DHLA/AA ratio in blood fractions. Gas liquid chromatography has been used for analysis and, although an internal standard was not incorporated for technical reasons, some assessment of absolute changes in the concentrations of DHLA and AA were obtained by measuring the ratios of DHLA/stearic and AA/stearic. In all volunteer studies, base-line measurements were made before dosing, so that each subject acted as its own control.

Incorporation of DHLA into Circulating Triglycerides and Phospholipids

Changes in the ratio DHLA/AA which

TABLE I

Distribution of Exogenous DHLA between Blood Lipid Fractions after Dosing a Guinea Pig with [^{14}C]DHLA

Time (hr)	% of total radioactivity recovered		
	Free fatty acids	Triglycerides	Phospholipids
2	16	45	
3	13	69	
4	17	56	19
5	17	36	17
6	9	60	25
7	7	44	45

occurred in blood from subjects who received a single 1 g oral dose of DHLA are shown in Figure 2. Some variability in the starting ratios is seen, but the pattern of changes is constant – an early sharp peak which appears at ca. 4 hr and a later peak which extends beyond 24 hr.

When different doses were administered to a single donor, with periods of several weeks between doses, this two peak pattern was again reproduced and an excellent dose/response relationship was evident (Fig. 3).

By fractionating whole blood into its different lipid components, it was apparent that the first peak corresponds to the appearance of DHLA in triglyceride and the second peak to the appearance of DHLA in phospholipid, predominantly in phosphatidylcholine (Fig. 4). Little change was observed in the composition of phosphatidylethanolamine lipids, possibly reflecting their greater inaccessibility on the inside of membranes.

We have undertaken similar time course studies in experimental animals in which we were able to follow the metabolism by measuring the appearance of radiolabel from [1-^{14}C] DHLA and [^{3}H]DHLA in blood lipids. A similar pattern of phospholipids becoming labeled after triglycerides was seen (Table I).

Incorporation of DHLA into Circulating Lipids and Membrane Lipids Following Sustained Administration

In the single dose studies described above, small increases in DHLA/AA were observed in platelet lipids. Lipids in red blood cell membranes were, however, unchanged following single doses as would be expected for cells with a very slow turnover rate. It has been argued that the prostaglandin precursor pool consists of phospholipid in membranes and that, as membranes, have a relatively slow turnover rate, significant effects on PGE_1 synthesis are only seen after chronic administration of DHLA. We have studied several volunteers who received multiple doses of DHLA for various periods of time. Blood sampling was usually at 4 hr after the morning dose and analysis of blood fractions was carried out as before. The effects on blood lipids following sustained oral administration of DHLA to one of these volunteers is shown in Figure 5.

Unlike in the single dose studies, DHLA was incorporated into membranes, e.g., of red blood cells. DHLA was also incorporated into all lipid classes, i.e., triglycerides, cholesteryl esters and all phospholipids.

Relationships between Dose of Dihomo-γ-linolenic Acid and Changes in Fatty Acid Composition

In multiple dosing studies, a plateau was reached in plasma at a DHLA/AA ratio of ca. 0.6. The ratio in cell fractions, e.g., in red blood cell membranes and platelets, also plateaued, and the higher the initial dose, the faster this plateau was achieved (Fig. 6). A similar effect has been observed by us in rats and by Oelz et al. (14), who administered the ethyl ester of DHLA to rabbits for 25 days.

A linear relationship between dose and plasma level has been observed following the administration of DHLA to rats (Fig. 7). After 15 days of dosing with 2 g/kg/day, DHLA levels in plasma were similar to those obtained after a single oral dose of the same amount of DHLA (Fig. 7). The maximum level of DHLA reached in plasma and other tissues thus appears to reflect a metabolic equilibrium, rather than a failure of absorption.

Tissue Distribution of Orally Administered Dihomo-γ-linolenic Acid

Danon et al. (15) have measured the relative amounts of DHLA in a variety of rat tissues following the administration of ethyl ester of DHLA. Greatest accumulation was observed in the triglycerides of the renal inner medulla where the content of DHLA rose to 19.4% of the fatty acids. The ratio of DHLA/AA in the

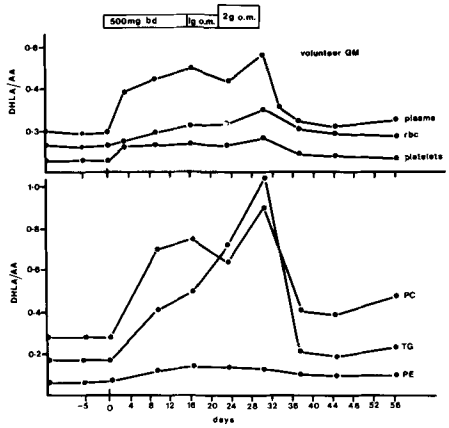

FIG. 5. Metabolism of DHLA following sustained dosing to man. The DHLA/AA ratio in total lipids is shown in the upper figure; the lower figure relates to the change in composition of plasma lipids. PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triglyceride; r.b.c., red blood cell membranes.

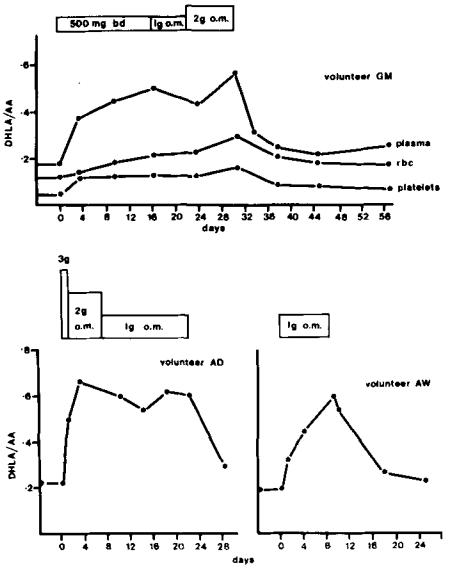

FIG. 6. Rate of attainment of maximum changes in DHLA levels in blood fractions after various dosing schedules.

triglycerides of the adrenals, renal inner medulla, adipose tissue, stomach and platelets all increased above unity. In phospholipids, DHLA rose several-fold in most tissues, but in no case did it exceed the amount of AA.

In a guinea pig study we found, using radiolabeled DHLA, that most exogenous DHLA accumulates in the liver (Table II). The liver is believed to be the most important site of

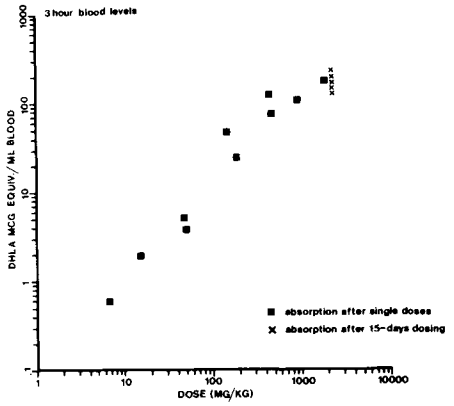

FIG. 7. Absorption of DHLA in rats.

TABLE II

Distribution of DHLA in Guinea Pig
7 Hr after 5 mg Oral Dose

Tissue	μg DHLA/g tissue	μg/organ
Liver	5.8	103.5
Spleen	4.5	3.5
Skeletal muscle	1.1	
Heart	1.0	1.6
Blood	0.6	
Kidney	0.5	2.0
Adipose tissue	0.2	

fatty acid metabolism, and many studies have demonstrated the presence in liver of enzyme systems which are capable of desaturating and chain-elongating DHLA.

Desaturation of Dihomo-γ-linolenic Acid

Rat liver has been the most widely used tissue for studying the metabolic interconversions of long chain unsaturated fatty acids. We undertook a comparative study of the ability of liver from different species to metabolize DHLA to AA and found that the rat (and to a lesser extent the mouse) was unusual in having large amounts of the Δ^5-desaturase enzyme system. Little activity could be detected in homogenates from rabbit, guinea pig or man (Fig. 8). We were also able to confirm the suggestion by Rivers et al. (16) that the cat lacks the ability to carry out this reaction.

It is possible that the low in vitro Δ^5-desaturase activity found in rabbit and guinea pig liver is a consequence of suboptimal reaction conditions. However, these results are in agreement with in vivo data from these species. Following administration of DHLA, arachidonate levels in rabbit plasma were virtually unchanged, and only a slight increase was seen

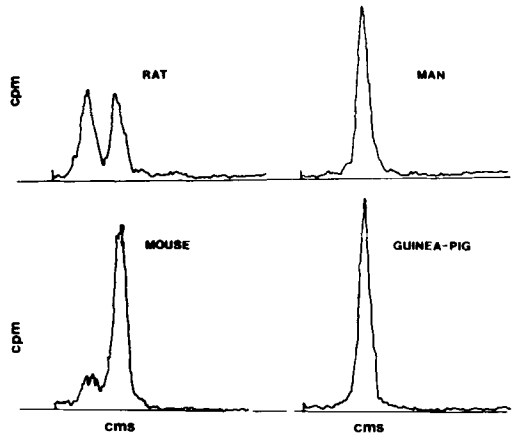

FIG. 8. Radiochromatograms of the esterified products of incubation of [^{14}C]DHLA with liver homogenates illustrating desaturation of DHLA to AA. Experimental details were essentially as described by Marcel et al. (20).

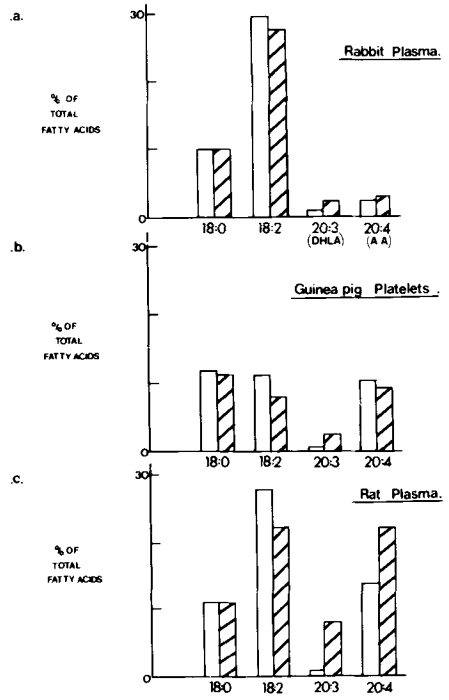

FIG. 9. Modification of the composition of rabbit plasma (a), guinea pig platelets (b), and rat plasma (c). DHLEthyl ester (100 mg/kg/day) was orally administered for 4-8 days to male New Zealand white rabbits (n=10) and male guinea pigs (n=12). DHLA was given to rats (n=7) for 8 days at 400 mg/kg/day. Control animals, rabbits (n=9), guinea pigs (n=12) and rats (n=6), received standard laboratory diets only. Histograms show fatty acids of control (open) and treated animals (shaded area).

in guinea pig plasma. In a study by Oelz et al. (14), who administered much larger amounts of DHLA (as the ethyl ester) to rabbits (1 g/kg/day for 25 days), arachidonate levels in plasma were somewhat elevated (5.8% vs. 10%), but levels in platelets were decreased as also shown in Figure 9 for guinea pig platelets.

This differs from the situation in rats where the increase in plasma DHLA is accompanied by a marked rise in arachidonate. However, even at the relatively high doses (1.3-1.5 g/kg/day) used in Danon's studies, this increase in arachidonate did not occur in platelet phospholipids (15).

Turning to the situation in man, Schoene and Iacono (17) have reported that lyzed, i.e., broken platelets, are able to metabolize [^{14}C]-linoleic acid to arachidonate. This would suggest that platelets possess an active Δ^5-desaturase enzyme system which metabolizes DHLA to AA. However, other studies by Bills et al. (18) showed that labeled linoleic acid was incorporated into intact platelets but not apparently metabolized to [^{14}C]arachidonate. We also have shown that intact platelets incorporate [^{14}C]-DHLA in good yield into phospholipids but do not generate [^{14}C]AA even after prolonged incubation (2-3 hr).

It seems, therefore, that the level of Δ^5-desaturase activity is very much lower in man than in rats, but the high arachidonate content of normal platelets in neither species appears to be a consequence of platelet DHLA metabolism. This is in agreement with results from multiple-dosing volunteer studies. Data shown in Table III were obtained following the administration of at least 1 g DHLA/day. Although significant increases occurred in the platelet content of DHLA and in the plasma content of AA, in no case was a significant increase in the AA content of platelet lipids apparent.

Conversion of Orally Administered DHLA to Prostaglandins and Effects on Platelet Function

One of the key objectives of our studies was to determine whether this increase in platelet DHLA redirects prostaglandin synthesis in vivo towards the antiaggregatory monoenoic series. The effects on prostaglandin synthesis of administering DHLA at 1 g and 2 g daily over a 28-day period to an adult male volunteer are shown in Figure 10. The capacity of platelets to produce prostaglandins was assessed by maximal stimulation with bovine thrombin. Base-line data during the pre-dose period are also shown. Potentially antithrombotic changes in platelet activity occurred during the dosing period, which was accompanied by a significant

TABLE III

Increase in Arachidonate Following the Administration of DHLA to Man

Donor	Time (days)		DHLA or AA/stearic acid					
			Plasma		Red blood cells		Platelets	
			AA	DHLA	AA	DHLA	AA	DHLA
GM	pre-dose	(-4)	0.97	0.19	0.82	0.13		
	16 days		1.11	0.55	0.79	0.23		
	last dose	(30)	1.28	0.73	0.85	0.30		
	post-dosing	(44)	0.73	0.16	0.79	0.19		
AD	pre-dose	(-4)	0.58	0.13	0.86	0.10	1.01	0.06
	10 days		0.80	0.48	0.86	0.18	1.01	0.16
	last dose	(22)	0.74	0.44	0.86	0.19	0.97	0.15
	post-dosing	(28)	0.82	0.24	0.97	0.18	0.95	0.09
AW	pre-dose		0.61	0.12	0.72	0.07	0.97	0.06
	4 days		0.64	0.28			1.02	0.12
	last dose	(9)	0.68	0.40	0.88	0.14	0.95	0.16
	post-dosing	(18)	0.76	0.20			1.05	0.09

increase in the capacity of the platelets to produce PGE_1. However, there was also a marked increase in PGE_2 production, which was greater than could be accounted for by the increased levels of plasma AA.

Malondialdehyde (MDA) release from washed platelets was unchanged, and this is significant, as MDA is an example of the nonprostanoate metabolites of arachidonate endoperoxides and is a measure of arachidonate metabolism (19). Thus, an increase in the formation of PGE_2, but not MDA caused by DHLA, suggests that some other explanation other than a rapid metabolism of DHLA to AA must be responsible. Increases in platelet production of PGE_2 following ingestion of DHLA have been observed in all but one of the donors examined and, in that particular donor, effects on platelet function tests were not observed and the synthesis of all prostaglandins was greatly reduced.

The large increase in the capacity of platelets to generate PGE_2 following the administration of DHLA to man is difficult to interpret, as there are neither corresponding increases in AA levels in platelets nor apparently a sufficient capacity of platelets to desaturate DHLA to AA. These observations might be reconciled if either DHLA, or a DHLA metabolite, selectively inhibited a particular metabolic pathway for AA. Some preliminary evidence for a concomitant increase in the capacity of platelets to generate PGD_2 following DHLA administration has been obtained; the effects on thromboxane synthesis and on prostacyclin synthesis are presently being studied.

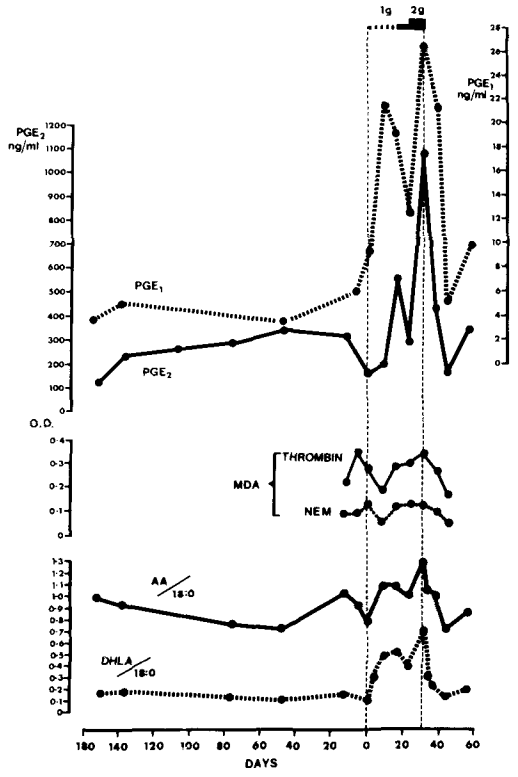

FIG. 10. Prostaglandin products generated following the administration of DHLA. Prostaglandins produced by platelets after maximal stimulation with thrombin were measured as described by Denton et al. (21). Malondialdehyde was measured by a modification of published methods (Zuzel, M., personal communication).

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The Effect of 5,8,11,14-Eicosatetraynoic Acid on Lipid Metabolism

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ABSTRACT

The purpose of this presentation is to review the current state of knowledge regarding 5,8,11,14-eicosatetraynoic acid (ETYA, Ro 3-1428) and its effects on lipid metabolism. Accordingly, the topics discussed include hypocholesterolemic and dermatological studies involving ETYA in both animals and man, as well as the effects of ETYA on desaturase enzymes. Metabolic studies involving ETYA are also noted. Primary interest is focused on the effects of ETYA on selected processes of arachidonate metabolism, and the effect of ETYA on inflammation, platelet aggregation and tumor growth are discussed, keeping in mind the relevance of arachidonate metabolism to these processes.

CHEMICAL SYNTHESIS

The total synthesis of 5,8,11,14-eicosatetraynoic acid (ETYA, Ro 3-1428) was first reported by Osbond et al. (1,2). Alternate syntheses of ETYA have also been reported (3-(5).

HYPOCHOLESTEROLEMIC STUDIES

At the time of the genesis of ETYA, the administration of polyunsaturated fatty acids (PUFA) was known to result in reduced serum cholesterol levels, and the consensus of opinion was that the hypocholesterolemic effect of PUFA should increase with the degree of unsaturation (6). On this basis, ETYA appeared to be an ideal candidate for a hypocholesterolemic drug.

Studies at Hoffmann-La Roche Inc., Nutley, New Jersey (Roche, Nutley), indicated that ETYA in doses of 50 and 100 mg/kg, p.o., lowered serum cholesterol in rats 19 and 36%, respectively (7). Shaw et al. (8) found that ETYA administration to rats (5 mg/day, i.p., for 16 days) resulted in a reduction in serum cholesterol (Table I).

It appeared initially that PUFA, in general, reduced serum cholesterol via increased bile acid excretion (6). However, in studies conducted at Roche, Nutley, no association was found between lowered serum cholesterol values and increased fecal excretion of cholesterol metabolities in ETYA-treated rats; in fact, fecal excretion was decreased (Schwartz and Carbone, Roche, Nutley, unpublished data).

Another possible mechanism for the hypocholesterolemic effect of ETYA involved the inhibition of cholesterol biosynthesis. Investigators at Roche, Nutley, (Schwartz and Carbone, unpublished data) found ETYA in rats (100 to 600 mg/kg, p.o., for 10 days) to be an effective inhibitor of in vivo liver cholesterol biosynthesis from [^{14}C]sodium acetate (i.p.); this inhibition was associated with a depression in serum cholesterol levels (Table II). At the 600 mg/kg dose, liver cholesterol biosynthesis was reduced to a tenth of the control value.

Further studies at Roche, Nutley, using rats, indicated that ETYA administration (400 mg/kg, p.o.) resulted in a decrease in serum cholesterol values after 12 hrs.; liver cholesterol biosynthesis was not affected until 24 hrs after administration (Table III). Thus, inhibition of liver cholesterol biosynthesis by ETYA may play a role in the maintenance of reduced serum cholesterol levels without contributing to the initial reduction (Schwartz, Carbone and Zavatsky, Roche, Nutley, unpublished data).

It was also determined (Schwartz and Carbone, Roche, Nutley, unpublished data), using a rat liver homogenate, that ETYA inhibited the in vitro conversion of ^{14}C-acetate to cholesterol. The lack of inhibition noted when ^{14}C-mevalonic acid was used as the precursor suggested that ETYA was inhibiting cholesterol synthesis at some state between acetate and mevalonate (9).

Sullivan, Triscari, and Hamilton (Roche, Nutley, unpublished data) studied the effect of ETYA or arachidonic acid on lipogenesis in isolated rat hepatocytes (Table IV). Both ETYA and arachidonate inhibited fatty acid synthesis, cholesterol synthesis, and CO_2 production in this system.

In contrast to these results, Abraham et al. (11) found that liver slices from mice fasted for one day and refed a 15% corn oil diet for three days exhibited a two-fold stimulation in fatty acid and cholesterol synthesis when ETYA (0.033%) was included in the diet. Lipogenic capacity in these studies was determined using either [1-^{14}C]acetate or [^{3}H]H_2O. ETYA administration was found to partially overcome the ability of linoleate administration to decrease fatty acid synthesis. Using livers from

TABLE I

Effect of ETYA Administration on Serum Cholesterol[a]

Group	Cholesterol (mg %) Control	ETYA	% change due to treatment	P
I, II, III ETYA for 16 days IV Fasted for 48 hr ETYA for 33 days	78.5 ± 4.2 (8)[b]	55.4 ± 3.8 (7)	-29.4%	0.01-0.001
	42.4 ± 1.4 (8)	33.9 ± 1.5 (11)	-20.0%	0.01-0.001

[a]Reproduced from Shaw et al. (8) with the permission of Raven Press.
[b]Figures in Parentheses indicate number of animals within each group.

TABLE II

Depression of Serum Cholesterol and Inhibition of in vivo Cholesterol Biosynthesis in Rats by ETYA (Schwartz and Carbone, unpublished data)

Dose (mg/kg p.o.)	% serum cholesterol depression 10th day[a]	% inhibition of in vivo liver cholesterol biosynthesis[b]
50	18	42
100	22	44
200	10	55
600	37	90

[a]These data were taken from a separate experiment (Randall and Zavatsky, Roche, Nuttey, unpublished data).
[b]Rats were administered [1-^{14}C]sodium acetate, i.p.; four rat livers for each ETYA dosage were pooled and the cholesterol extracted into acetone/ethanol (1:1). Cholesterol was precipitated as the digitonide, dissociated from digitonin by pyridine, extracted into ether, cystallized as the dibromide and converted by means of zinc-acetic acid back to cholesterol which was washed and crystallized from methanol.

TABLE III

Lowering of Rat Serum Cholesterol and Inhibition of Liver Cholesterol Synthesis by ETYA 400 mg/kg p.o. (Schwartz, Carbone and Zaratsky, unpublished data)

Interval between dosing and sacrifice (hr)	Serum cholesterol % lowering	Liver cholesterol % inhibition of in vivo synthesis
4	-7[a] (0.2)[b]	-42 (0.3)
12	17 (0.01)	-20 (0.4)
12	30 (0.001)	-41 (0.2)
24	31 (0.001)	58 (0.02)

[a]Negative values were obtained when treatment resulted in an elevation of serum cholesterol or a stimulation of cholesterol synthesis rather than an inhibition.
[b]P values in parentheses were determined using the student T test, based on the mg% of cholesterol, and liver cholesterol, respectively.

mice which were fasted for one day and refed a fat-free diet for three days, the inclusion of ETYA in the diet had no effect on either fatty acid or cholesterol biosynthesis. Although the dosage used in these studies represents a several-fold reduction from that used in rats by Schwartz and Carbone (unpublished data), the stimulation of hepatic lipogenesis in mice did persist for as long as four weeks at the lower dose.

Shaw et al. (8) studied isolated fat cells prepared from rats to which ETYA was administered (5 mg/day, i.p., for 16 or 33 days) and found a highly significant ($p < 0.001$) increase in the basal cyclic AMP (cAMP) levels as compared to untreated controls. Addition of epinephrine in the presence of caffeine produced a four- to five-fold increase in cAMP levels in cells prepared from the untreated controls; it produced no further increase above

TABLE IV

Effect of ETYA and Arachidonate on Fatty Acid Synthesis, Cholesterol Synthesis and CO_2 Production in Isolated Rat Hepatocytes[a]

Compound	Concentration	Fatty acid synthesis		Cholesterol synthesis		CO_2 Production
			^{14}C Alanine		^{14}C Alanine	^{14}C Alanine
	Micromolar	^3H_2O Converted	Converted	^3H_2O Converted % of control	converted	converted
ETYA	5	28[b]	9[b]	58[b]	47[b]	79[b]
	3	33[b]	4[b]	56[b]	30[b]	74[b]
	1	26[b]	5[b]	67[b]	42[b]	81[b]
	0.5	40[b]	15[b]	82	66[b]	99
	0.1	58[b]	41[b]	96	95	101
Arachidonate	5	82	84	56	44[b]	98
	1	96	97	83	65	103

[a]The Methodology reference is Sullivan et al. (10).
[b]Indicates statistical significance when compared to control. ($P<0.05$).

the augmented basal values found in the ETYA treated rats. ETYA was found to have no effect in vitro on basal cAMP levels.

Using fat cells isolated from rat epididymal tissue, Dalton and Hope (12) found that neither ETYA nor indomethacin, a known prostaglandin (PG) synthetase inhibitor (13), added in vitro, had an effect on either basal or norepinephrine stimulated release of free fatty acids or glycerol. Dalton and Hope were unable to confirm the results of Illiano and Cuatrecasas (14) who found that indomethacin when added to an isolated rat fat cell preparation enhanced the norepinephrine or adrenocorticotropic hormone-stimulated release of glycerol.

In brief, it is conceivable that ETYA administered in vivo, by raising cAMP levels, possibly due to inhibition of PG biosynthesis, could attenuate the lipolytic response of fat cells to various hormones as suggested previously (15,16). The possibility also exists, however, that ETYA may be directly affecting the adenyl cyclase, the phosphodiesterase, or some other process, to produce an increase in cAMP in the fat cell (8).

Based on the encouraging results obtained with the animal studies, ETYA was evaluated at Roche, Nutley, as a hypocholesterolemic agent in man. The administration of ETYA to nineteen subjects was associated with a modest reduction in the serum cholesterol levels (Fig. 1A) which, however, was not dose related (Fig. 1B) (7). In a study involving 32 subjects for up to 48 weeks, Bender confirmed the cholesterol-lowering properties of ETYA, although a significant incidence of minor gastrointestinal and dermatological side effects was noted (7).

No accumulation of desmosterol could be demonstrated in the serum of human subjects treated with ETYA, or in the serum and livers of rats treated with ETYA (Schwartz, Carbone and Zavatsky, unpublished data). Clinical studies involving the use of ETYA as a hypocholesterolemic agent were discontinued when the compound was judged to exhibit inadequate activity at high dosage levels (2 g/day for as long as 48 weeks).

DERMATOLOGICAL STUDIES

Although ETYA was found to be unsuitable as a hypocholesterolemic agent, it was tolerated well clinically. Dry skin was noted as one of the side effects of oral ETYA administration, and, accordingly, ETYA was evaluated at Roche, Nutley, as a sebum-lowering agent.

Oral administration of ETYA was found to produce a decrease in sebum production in all 16 male subjects studied, with an average re-

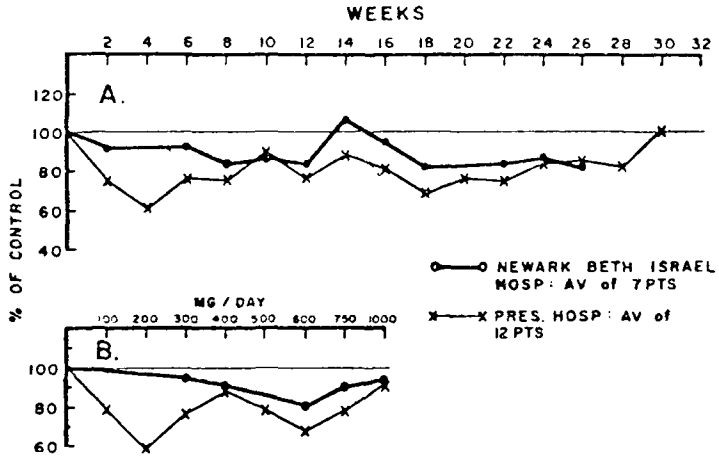

FIG. 1. The effect of ETYA on serum cholesterol levels of human subjects (reproduced from the Journal of Newark City Hospital (7) with the permission of the authors): (a) average serum cholesterol values in 19 subjects treated with ETYA, percent of control; (b) the average cholesterol values in these same subjects during treatment with doses ranging from 100 to 1000 mg/day, percent of control. The heavy lines represent seven subjects treated in the Heart Clinic at the Newark Beth Israel Hospital and the lighter lines represent twelve subjects treated in the Newark Presbyterian Hospital. It can be seen that the administration of ETYA was associated with a modest overall reduction in the serum cholesterol levels (A), which was not dose related (B).

duction in sebum production of 42% ($p < .001$) (9). Further studies, in connection with Roche, Nutley, indicated that ETYA actually reduced the size of the sebaceous glands (Fig. 2) (Strauss, unpublished data).

Ahern and Downing (17) postulated that the anti-sebum activity of ETYA may be due to reduced adrenal steroid production. Recent studies by Laychock and Rubin (18,19), using cat adrenocortical cells, demonstrated that ETYA exhibited a dose-dependent inhibition of adrenocorticotropic hormone (ACTH)-induced steroid release, but had little effect on PGE_2-induced steroid release. At the highest concentration tested (30 μM), the situation was reversed; ETYA had no effect on ACTH-induced steroid release, but inhibited PGE_2-induced steroid release.

Further tests involving an oral dosage form of ETYA were discontinued in favor of a topical form when it was found that ETYA was embryotoxic in rabbits (Banzinger and Woo, Roche, Nutley, unpublished data). Topical applications of ETYA (2% ethylene glycol in ethanol) were shown to have no effect on sebum excretion in subjects with acne (20,21); however, a slight but definite decrease in sebum excretion rate was shown to be produced by 2% ETYA in ethanol in the absence of propylene glycol (20).

Evidence which suggests that the sebum-lowering activity of ETYA may be due to a direct effect on the lipid-synthesizing components of skin has been provided by Summerly et al. (22). ETYA was shown to reduce in vitro the total incorporation of ^{14}C-acetate into the various lipid classes in rat dorsum skin. This occurred with a percentage reduction in the amount incorporated into the wax ester/sterol ester fraction with a corresponding percentage increase in the amount incorporated into the free fatty acid fraction. Although ETYA was found to have no effect on the incorporation of ^{14}C-acetate into the various lipid classes from human sebaceous glands and rat tail, the authors postulated that the lack of effect may have been due to the instability of the dosage form used.

Further clinical studies with ETYA were discontinued when it was determined that the topical formulation had no significant effect on sebum production in man.

Effects on Desaturase Enzymes

It has been demonstrated that polyenoic acids interfere with each other in desaturation reactions (23).

Coniglio et al. (24) administered orally either ETYA or arachidonate to rats maintained on a fat-free diet and then determined the fatty acid composition of the total lipids from the liver, heart, kidney, testis, brain and adrenals. ETYA administration resulted in a decrease in the amount of arachidonate, 22:4ω6 and 22:5ω6 (as percent of total fatty acids), in all of the organs studied except the adrenals, with the most significant changes occurring in the liver. The authors suggest that ETYA may be

interfering with the conversion of linoleate to arachidonate and the subsequent conversion of arachidonate to 22-carbon polyenes.

Rao and Abraham (25) studied mammary tumor-bearing mice, maintained on a 15% corn oil diet, in the presence or absence of ETYA. Their data suggest that ETYA interferes with the desturation of linoleate to arachidonate. These results have also been confirmed in normal mice fed a high linoleate diet (11).

Wood and Chumbler (26) studied normal and hepatoma-bearing rats maintained on a fat-free diet supplemented with 0.5% safflower oil (basic). The inclusion of ETYA (0.5%) in the diet, for four weeks after tumor transplant, resulted in an increase in oleic acid in all lipid classes of hepatoma, and also in normal and host liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE). In addition, the percentage of arachidonic acid decreased in normal and host liver PC, but not in PE. Normal liver, host liver, and hepatoma of ETYA-fed animals contained reduced amounts of triglycerides, relative to animals fed the basic diet. The animals fed the ETYA diet exhibited a decrease in carbon number 50 and a marked increase in carbon number 52 for plasma and liver triglycerides. Thus, ETYA administration appeared in this study to affect both the desaturation of fatty acids, and triglyceride levels, in rat liver and to a lesser degree in rat hepatoma.

Rao et al. (27) studied the effect of ETYA administration on male Sprague-Dawley rats which were maintained for 8 weeks on either a corn oil diet or a hydrogenated coconut oil (HCNO) diet. The inclusion of ETYA in the HCNO diet reduced the levels of 12:0 and 14:0 in the total fatty acids of liver and plasma. A decrease in the ratios of 16:1/16:0, 18:1/18:0 and 20:4/18:2 were noted when ETYA was included in either diet. They concluded that dietary ETYA can influence the hepatic metabolism of medium chain fatty acids and that it may inhibit the desaturase enzyme involved in the synthesis of monoenoic as well as polyenoic acids.

METABOLISM OF ETYA

Following intravenous administration in the rat, [5,6-^{14}C]ETYA was rapidly removed from the blood with a half life of less than 1 min. The majority of the administered radioactivity was found to be associated with the liver (38% after 15 min); in the bile duct cannulated rat, 91% of the administered radioactivity was found in the bile after 24 hr (28).

In the bile duct cannulated rat, four metabolites, but not ETYA, were excreted into the bile. These were equally distributed between phosphatidylethanolamine and phosphatidylcholine with little of the activity associated with the free fatty acid fraction. Three of the metabolites were assigned structures: octadeca-3,6,9,12-tetraynoic acid, octadeca-3,6,9,12-tetrayn-1,18-dioic acid and hexadeca-3,6,9,12-tetrayn-1,16-dioic acid, indicating that metabolism occurs via both the β and ω oxidative pathways (29).

Extensive enterohepatic recirculation of metabolites was evident. During a period of 5 days following intraperitoneal administration (using [5,6-^{14}C]ETYA), 62% of the administered radioactivity was recovered in feces, 12.6% in urine and 2.8% was respired as $^{14}CO_2$ (28). Five days after intravenous injection, the activity remaining in the body was associated mainly with skeletal muscle (7.7%), skin (4.3%) and fat (3.8%) (30).

Effects of ETYA on the Metabolism of Arachidonate

The mammalian metabolic pathway for arachidonic acid is summarized in Figure 3. Because of the structural similarities between arachidonate and ETYA, it would be expected a *priori* that ETYA would have an effect on one or several aspects of arachidonate metabolism, and, indeed, this has been shown to be the case. Topics of interest, in this regard, include: (a.) the inhibition of lipoxygenase and cyclooxygenase enzymes by ETYA; (b.) the mechanism of inhibition; (c.) time-dependent and time-independent inhibition; and (d) the effect of ETYA on prostacyclin and thromboxane synthetase.

Lipoxygenase and Cyclooxygenase Enzymes. Polyunsaturated fatty acids can be metabolized in plants via lipoxygenase enzymes to their respective hydroxy fatty acids. Soybean lipoxygenase, the most intensely studied plant lipoxygenase, has been found to have certain features common to both the mammalian lipoxygenase, and cyclooxygenase enzymes.

The gorgonian coral, *Plexaura homomalla,* has been found to be a rich source of prostaglandin A_2 (PGA_2) and its acetoxy methyl ester (31). Apparently the biosynthetic machinery in *P. homomalla* does not involve a typical cyclooxygenase enzyme, in that PGG_2, PGH_2 and PGE_2 are not formed (32). ETYA and a known cyclooxygenase inhibitor, indomethacin, have no effect on the PGA_2 synthetase enzyme from *P. homomalla* (33).

ETYA was found to be a competitive inhibitor of soybean lipoxygenase by Blain and Shearer (34), and was later reported by Downing et al. (35) to be an irreversible

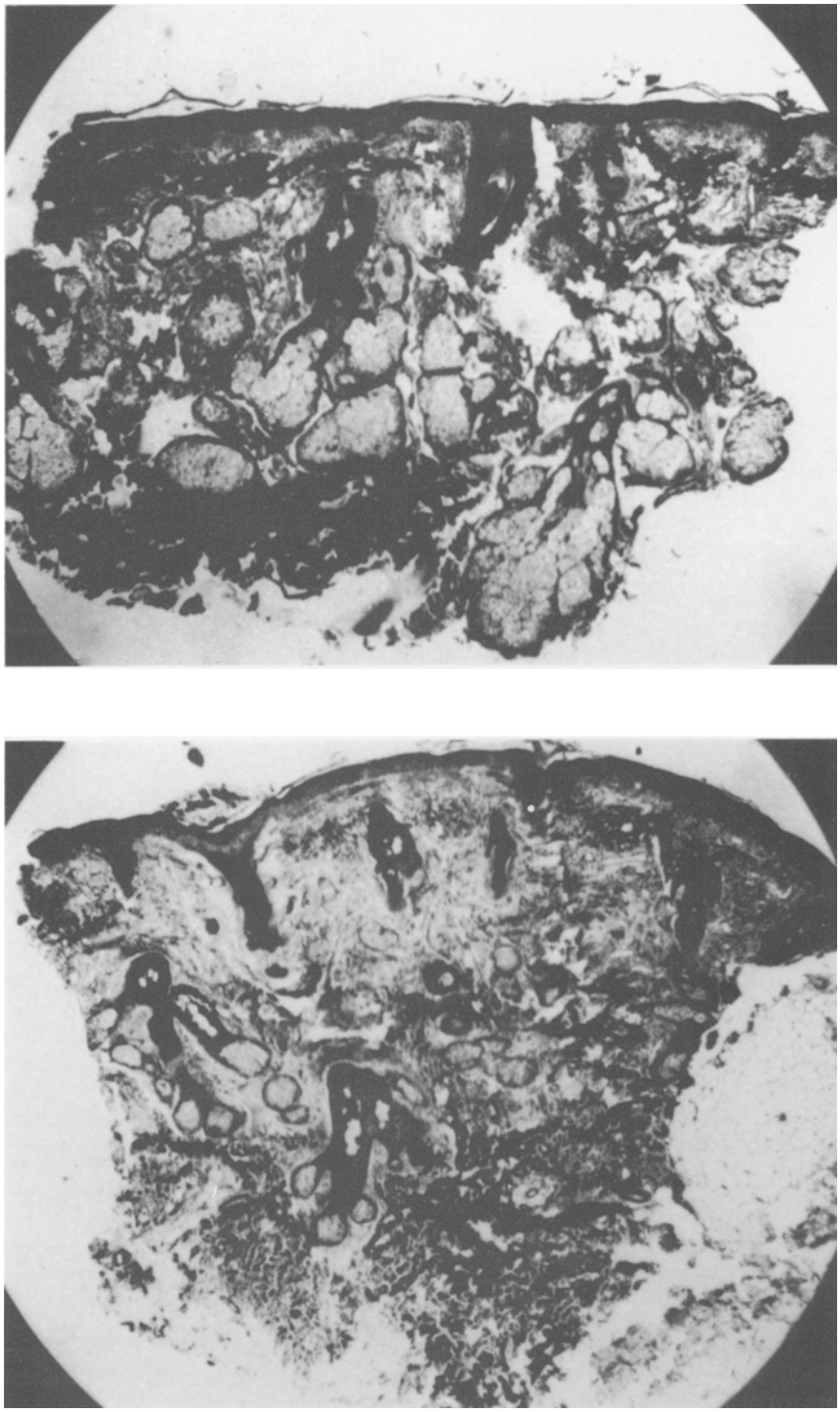

FIG. 2. Photomicrographs of skin biopsies taken from human volunteers before (top) and after (bottom) ETYA administration, illustrating the reduction in sebaceous gland size after ETYA treatment. (Published with the permission of J.S. Strauss).

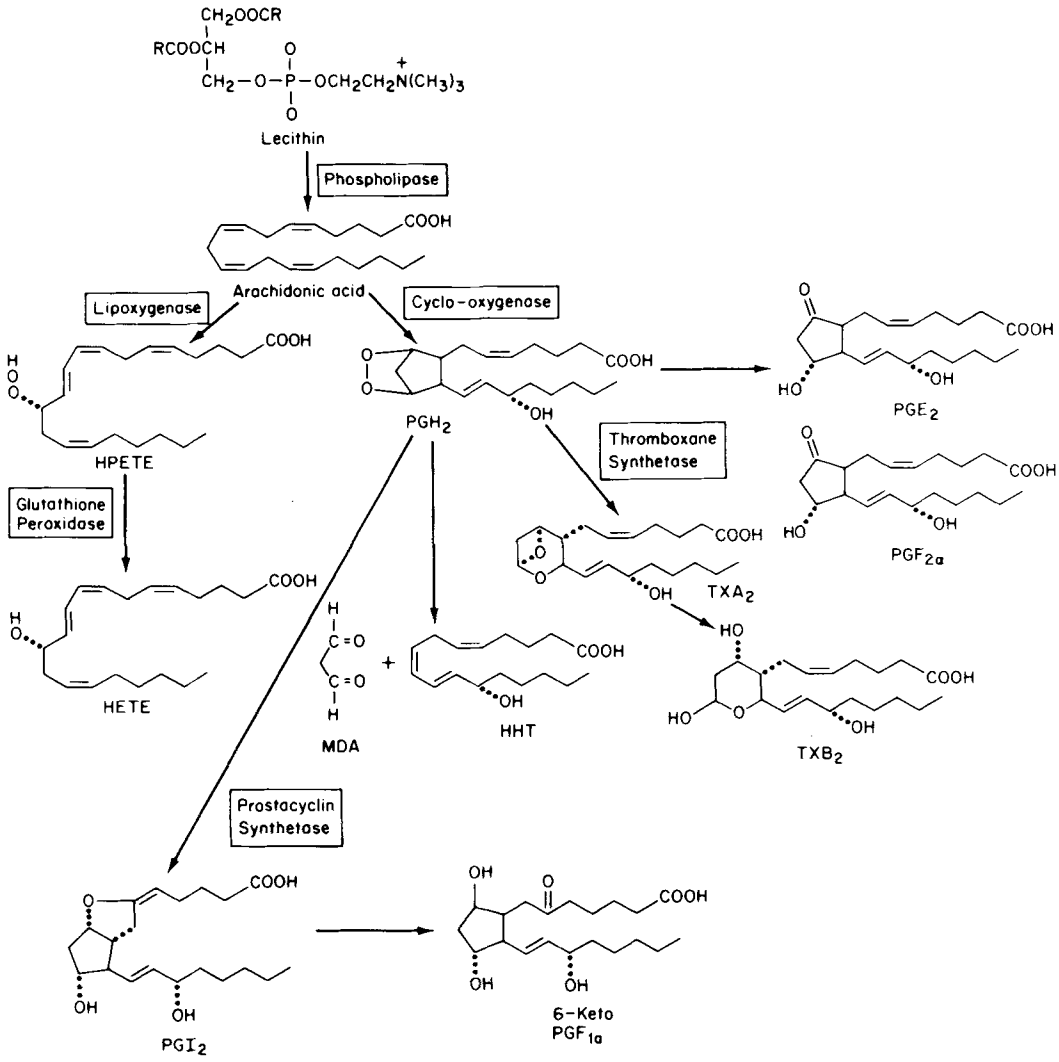

FIG. 3. Mammalian arachidonate metabolic pathways.

inhibitor of the same enzyme. Hamberg and Samuelsson (36) found that ETYA was a potent inhibitor of 12-1-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) formation by human platelets. The compound, however, was reported to have no effect on 5-1-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) formation by the lipoxygenase found in rabbit polymorphonuclear leukocytes (PMN's) (37). ETYA has also been reported to inhibit 12-HETE formation but not 5–HETE formation when arachidonate was incubated with human neutrophils which had been stimulated by phagocytosis, sodium fluoride, or the calcium ionophore A23187 (38). The various plant and mammalian lipoxygenase pathways are summarized in Figure 4 using arachidonic acid and 8,11,14-eicosatrienoic acid as substrates. Conversion to the respective hydroxy fatty acids is presumed to occur via glutathione peroxidase.

ETYA has been reported to inhibit the hydroxylation of linoleic and linolenic acid by acetone powders of sheep seminal vesicular glands (17), and to inhibit the conversion of arachidonic acid to PGE_2 (17,35) and adrenic acid to dihomo-PGE_2 (39) by this same preparation. Inhibitory potencies for ETYA against PG-generating systems from several sources are given in Table V. Thus, the cyclooxygenase enzyme system, as described here for sheep seminal vesicular gland microsomes, can be seen as possessing features common to

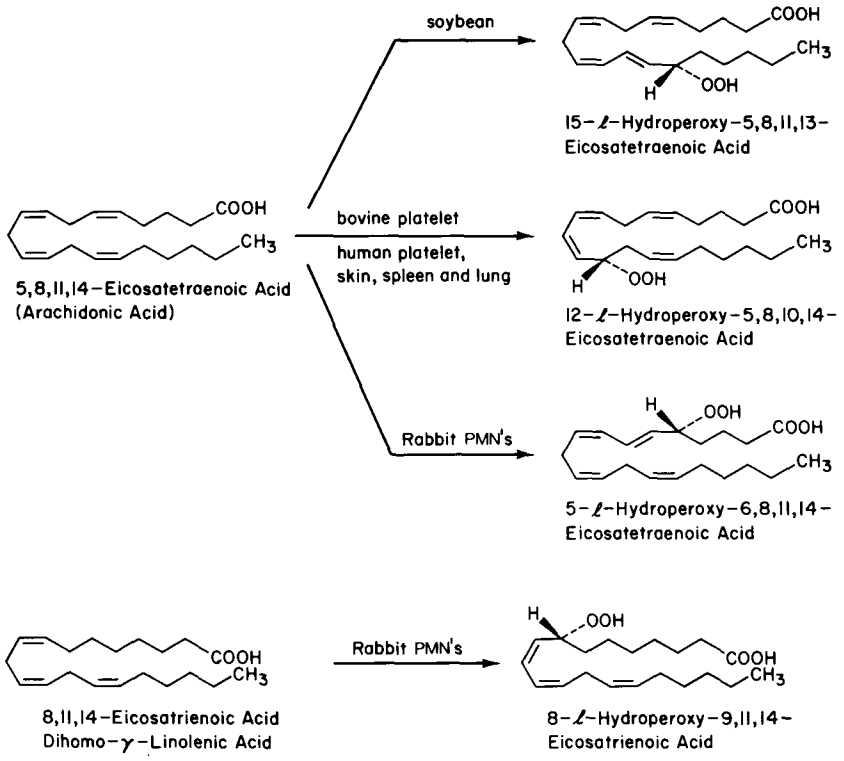

FIG. 4. Selected plant and mammalian lipoxygenase pathways.

lipoxygenase enzymes, with, interestingly, both the lipoxygenase and cyclooxygenase activities inhibited by ETYA. It is of interest to note that recently one of the isoenzymes of soybean lipoxygenase, lipoxygenase-2, after incubation with arachidonic acid and subsequent reduction with dithionite, gave rise to $PGF_{2\alpha}$, lending further credence to the similarity of the plant lipoxygenase and mammalian cycloxoygenase enzymes (46).

Linoleic and linolenic acid were found to be inhibitors of the mammalian cyclooxygenase enzyme, but in their hydroxylated forms did not function as inhibitors (17). Studies which involved the incubation of labelled ETYA with the enzyme indicated that ETYA was recovered unchanged from the incubation medium (17).

In summary, ETYA was found to inhibit mammalian cyclooxygenase and several plant and mammalian lipoxygenase systems, does not appear to be chemically modified as a result of its inhibition, and may maintain the inhibitory potency of endogenous unsaturated fatty acids by preventing their conversion to their inactive hydroxy fatty acid analogues.

Mechanism of Action. It was Downing et al. (35) who described a mechanism for ETYA which involved conversion to a reactive allene, which then would react irreversibly with a histidine moiety of the cyclooxygenase or soybean lipoxygenase enzymes. Formation of such an allene was envisioned as proceeding via enzymic abstraction of the ω_8 hydrogen from ETYA. Removal of the ω_8 hydrogen from the appropriate substrate is the requisite initial step for PG biosynthesis (47) and/or hydroxy fatty acid formation using sheep seminal vesicular gland preparations, (48-51) and also for hydroperoxide formation via soybean lipoxygenase (52).

Hammarstrom (45) has determined that ETYA has an IC_{50} of 4 μM for the lipoxygenase and 8 μM for the cyclooxygenase, both prepared from human platelets. In contrast, the triynoic analogue of ETYA, 5,8,11-eicosatriynoic acid, selectively inhibited platelet lipoxygenase (IC_{50} = 24 μM) in comparison to the cyclooxygenase (IC_{50} = 340 μM) (42). Studies in our laboratory have indicated that another triynoic analogue of ETYA, 8,11,14-eicosatriynoic acid, inhibits both arachidonic acid-dependent oxygen uptake by sheep seminal vesicular gland microsomes, and

TABLE V

Inhibition of Prostaglandin Synthetase from Various Sources by ETYA[a]

Aspect measured	Concentration (micromolar)	Inhibition (%)	Reference
E_2 synthesis by a sheep seminal vesicular gland preparation	4.0	75	17,35
E_1 synthesis by a bovine seminal vesicular gland preparation	6.6	50	41,42
E_2 synthesis by frozen powders of guinea pig ileum	5.0	50	43
PG release from isolated perfused rabbit heart in response to nerve stimulation	1.0-5.0	100	44
PG synthesis by human platelet cyclooxygenase	8.0	50	45

[a]Some data were taken from flower. (40).

hydroxy fatty acid formation from arachidonate by the 10,000 g supernatant particulate fraction prepared from human platelets. All diynoic fatty acids tested were inactive against platelet lipoxygenase (Tobias, Hamilton and Paulsrud, unpublished data).

Time-Dependent and Time-Independent Inhibition of Cyclooxygenase by ETYA. Vanderhoek and Lands (53) have shown using the cyclooxygenase enzyme that ETYA and other alkynoic inhibitors exhibit two types of inhibition: the instantaneous, concentration-dependent inhibition described previously (17,35), and a time-dependent inhibition of the enzyme. The latter appears to be related to the phenomena of self-catalyzed destruction reported for soybean lipoxygenase (54) and cyclooxygenase (55) by fatty acid substrates.

In agreement with the results of Downing et al. (35), Vanderhoek and Lands (53) did not observe any significant oxygen consumption when ETYA was incubated with the cyclooxygenase. They also found that both oxygen and hydroperoxide were required for time-dependent inhibition to occur with ETYA. Time-dependent inhibition of the enzyme was prevented by performing the incubation under anaerobic conditions, or by the inclusion of glutathione (GSH) peroxidase in the aerobic incubate. Upon oxygenating the solution and adding substrate, or, in the latter case, after the destruction of GSH peroxidase with N-ethyl maleimide, the cyclooxygenase activity was restored to a level which reflected only instantaneous, concentration-dependent inhibition by ETYA.

Smith and Lands (55) found that when a dried preparation of vesicular gland microsomes was homogenized in buffer and incubated with arachidonic acid, a low level of cyclooxygenase activity was discernible. The addition of GSH and GSH peroxidase to the homogenate resulted in an increase with time in the activity of this enzyme form. Further additions of GSH and GSH peroxidase did not appreciably diminish enzyme activity.

In contrast, when phenol alone is added to the vesicular gland homogenate, there is a proportionally larger increase in cyclooxygenase activity than that seen for the homogenate in the presence of GSH and GSH peroxidase. Also, the subsequent addition of GSH and GSH peroxidase results in inhibition of the phenol-activated cyclooxygenase activity. Thus, there appear to be two forms of cyclooxygenase enzyme, a basal form, (E_b), which is stimulated by GSH and GSH peroxidase, and a latent form, (E_a), which must be activated by phenol, and which is inhibited by GSH and GSH peroxidase.

The two enzymatic forms are remarkably similar with regard to the product spectrum one obtains, and most studies to date have been done with the E_a system, primarily because of its greater overall activity.

A time-dependent destruction of both E_a and E_b by ETYA has been noted; for E_a, however, this can be prevented by all agents used to block reversibly the normal oxygenation of substrate. In contrast, for the E_b form, addition of GSH and GSH peroxidase prevented neither oxygenation of the substrate, nor time-dependent destruction of E_b by ETYA. The effect of ETYA and other acetylenic fatty acids on E_a and E_b have been reviewed by Lands et al.(56).

Thromboxane and Prostacyclin Synthetase. ETYA has been reported to be a weak inhibitor of thromboxane synthesis with an IC_{50} of ca. 100 μM (57). Results from our laboratory (Tobias and Hamilton, unpublished data), summarized in Table VI, indicate that ETYA, arachidonic acid, dihomo-γ-linolenic acid and linoleic acid at 1 mM are weak inhibitors of

TABLE VI

The Effect of Selected Fatty Acids, and Imidazole on the Thromboxane Synthetase from Guinea Pig Lung Microsomes and Prostacyclin Synthetase from Bovine Aortic Microsomes

Compound (1mm)	% Inhibition Thromboxane synthetase[a]	% Inhibition Prostacyclin synthetase
Imidazole	52.7, 60.6	0, 8.6
ETYA	58.5, 60.5	31.6, 4.3
Arachidonic acid	59.9, 56.3	23.0, 28.7
Dihomo-gama-linolenic acid	21.3, 38.1	33.0, 37.4
Linoleic acid	36.7, 42.6	15.8, 35.9
Stearic acid	-1.4[b], -4.2	5.7, 0

[a]Formation of [1-^{14}C]radiolabeled products from PGH_2 was monitored by ITLC in isooctane-methyl ethyl ketone-acetic acid (100:19:1) (58).
[b]Minus values indicate stimulation of the enzyme.

thromboxane formation, using PGH_2 incubated with guinea pig lung microsomes; stearic acid was inactive. Imidazole has inhibitory efficacy comparable to ETYA in this system.

Imidazole was found to have, however, no effect on formation of 6-keto $PGF_{1\alpha}$ when PGH_2 was incubated with bovine aortic microsomes. In this system ETYA, dihomo-γ-linolenic acid and oleic acid, all at 1 mM, were weakly active in suppressing 6-keto $PGF_{1\alpha}$ formation. Stearic acid was neither an inhibitor of TXA_2 synthetase, nor of prostacyclin synthetase when tested at 1mM. PGE_1, PGE_2 and $PGF_{2\alpha}$ were all found to be very weak inhibitors of TXA_2 synthetase. The results indicate that ETYA, and unsaturated free fatty acids in general, inhibit prostacyclin and thromboxane synthetase, but at levels which are probably too high to have any physiological significance.

The Effect of ETYA on Selected Physiological Processes

ETYA, either through modification of arachidonate metabolism or other lipid metabolic pathways, can affect various physiological processes and pathophysiological states, including (a.) inflammation, (b.) platelet aggregation and (c.) tumor growth.

Inflammation. ETYA administered to rats (100 to 300 mg/kg, i.p.) has been reported by Willis (59) to have antipyretic and anti-inflammatory properties. ETYA was found to reduce both the carrageenan reaction and prostaglandin content of rat paw, but at doses which were much higher than those which are effective in vitro against PG biosynthesis.

Smith et al. (60) have verified these results and found that ETYA administration (100 mg, i.p.) 45 min before subplantar injection of carrageenan afforded the most effective inhibition of the carrageenan-induced rat paw oedema. ETYA was also found to block the potentiating effect of arachidonic acid on the oedema.

ETYA administered simultaneously into rat foot pads along with carrageenan has been reported to be effective in reducing the resultant paw oedema (61). The oedema reduction by ETYA noted for essential fatty acid-deficient rats was less than that expected for normal rats.

Recently, ETYA, but not aspirin or indomethacin, was shown to inhibit mediator release from isolated rat peritoneal mast cells induced by concanavalin A, anti-IgE antibody, or the calcium ionophore, A 23187 (62). Interestingly, arachidonate itself was found to induce release from unstimulated cells, and also to enhance the release induced by all three stimulating agents. Whether 12-HETE formation and its inhibition by ETYA has any relevance to the mast cell release reaction remains to be determined.

The release of slow reacting substance (SRS) from rat basophilic leukemia cells (RBL-1) by the ionophore A23187 (5-10 μg/ml) was stimulated 5-fold by arachidonate and inhibited 78% by ETYA. Linoleic acid and linolenic acid both inhibited SRS formation, but indomethacin had no effect. The evidence presented supports the contention that SRS is a metabolite of arachidonate, probably derived via a lipoxygenase pathway (63).

Valone and Goetzl, using a modified Boyden microfilter assay (64), have shown that antigen challenge of the rat peritoneal cavity, after passive preparation with IgG_a-rich hyperimmune antiserum, releases lipids that are chemotactic and chemokinetic for human PMNs. Treatment of the rat peritoneal cavity with ETYA, prior to immunological challenge, was found to inhibit the release of both the chemotactic and chemokinetic factors. Indomethacin inhibited the release of only the chemotactic factor. Both factors were chromatographically distinguishable from SRS and platelet-activating

TABLE VII

The Effect of ETYA and Indomethacin on the Growth Rate of Transplantable Morris Hepatoma No.7777 in Buffalo Rats

Group[a]	No. of rats	Initial wt.[b]	Final wt.	Tumor wt.	Liver wt.	Kidney wt.
Control	19 (0)[c]	368 ± 20.9	402 ± 30.3	11.1 ± 5.1[d]	12.3 ± 1.0	2.6 ± 0.1
ETYA	20 (2)	375 ± 25.0	391 ± 30.1	10.6 ± 4.4	18.1 ± 1.2[e]	2.8 ± 0.15
Indomethacin	20 (1)	374 ± 24.0	392 ± 35.7	19.4 ± 4.6[e]	11.2 ± 0.9	2.5 ± 0.2

[a]The dosages were formulated as follows, and administered by gastric incubation daily for 5 weeks: Control; 1.6 ml. of 1% Gum Arabic. ETYA; 1.6 ml. of ETYA (75 mg/ml as the ammonium salt) in 1% Gum Arabic (reduced to 37.5 mg/ml on the 25th day of dosing). Indomethacin: 1.6 ml. of Indomethacin (0.25 mg/ml in 1% Gum Arabic).

[b]All weights are reported in grams.

[c]The number of rats that died during the dosing regimen are indicated in parentheses.

[d]Two rats from the control groups did not develop the tumor.

[e]Significantly different from the control ($p<.001$).

factor. The evidence indicated that the lipid factors are metabolites of arachidonate or some other fatty acid.

Walker et al. (65), however, reported that ETYA (150 and 500 mg/kg i.p.) caused a predictable decrease in the PG content of, but had no effect on the extent of leukocyte migration into, the exudates of inert porous sponges implanted subdermally in rats.

ETYA has been reported by Kelly et al. (66) to inhibit the DNA synthetic response in human peripheral blood lymphocytes to four mitogens: plant lectins, A23187, antithymocyte globulin and periodate. ETYA also abolished basal and lectin-stimulated cAMP levels in this system.

Platelet Aggregation. ETYA administered to rats (5 mg, i.p., for 16 days) failed to modify ADP-induced aggregation in their platelet-rich plasma (PRP). In addition, ETYA (1 to 2 μg/ml), when added directly to the PRP of untreated rats, failed to modify ADP-induced aggregation (8). In dog PRP, ETYA has been shown to inhibit in a dose-dependent fashion, a substance (TXA_2) which aggregates human and rabbit platelets (67).

When added to human PRP, ETYA was shown to inhibit both serotonin release and irreversible platelet aggregation induced by either ADP, epinephrine, connective tissue, or thrombin; this effect appeared to be related to ETYA's ability to inhibit both endoperoxide and thromboxane A_2 (TXA_2) formation (59). Similar results have been reported using platelets from diabetic patients (68). ETYA has also been reported to inhibit 5-hydroxytryptamine release in human platelets induced by either epinephrine or arachidonic acid (69) but not by thrombin (70). ETYA had no effect on ATP secretion induced by either thrombin or A23187 (71).

The burst in oxygen consumption in human platelets, induced by either thrombin (70,72) or arachidonic acid (72), has been reported to be inhibited by ETYA.

Effects on Transplantable and Chemically Induced Tumors. Rao and Abraham (25) found that the growth rate of transplantable mammary adenocarcinoma in C3H mice was reduced, relative to controls, when ETYA was included in their 15% corn oil diet.

Because aspirin, even at high doses (0.8 mg/g diet), was found to have no significant effect on tumor growth, the authors postulated that ETYA was acting by a mechanism other than inhibition of PG biosynthesis. ETYA was found to inhibit the desaturation of linoleate to more highly unsaturated polyenes, and the authors suggested that the reduction in the availability

of arachidonic acid may have had a relationship to the retardation of tumor growth.

The effect of indomethacin and ETYA on the growth rate of Morris hepatoma (strain No. 7777) to Buffalo rats has recently been studied at Roche, Nutley (Hamilton, Kuhn and Tratnyek, unpublished data). The tumor was transplanted by i.m. injection of minced aliquots of tumor tissue in isotonic saline into the rat's right hind thigh. The results are summarized in Table VII. By the 23rd day of dosing, two rats receiving 300 mg/kg of ETYA had died, and on the 25th day, the dosage was reduced to 150 mg/kg. On the 25th day, all of the rats receiving ETYA exhibited extremely dry, scaly skin, which in many cases was cracked and bleeding. The livers of the ETYA-treated rats were found to be significantly larger ($p < .001$) than those of the controls, but there was no significant difference in tumor size when this group was compared with the control group. Interestingly, the tumors in the rats receiving indomethacin (1 mg/kg) were significantly larger ($p< .001$) than those of the controls.

The effect of ETYA on chemical carcinogenesis in mouse skin has also been studied (73). Mice were maintained on either a diet rich in polyunsaturated fat (PUFA) (15% safflower oil), or a diet rich in saturated fat (SF) (15% beef tallow oil). Skin papillomas in these animals were produced using either β-propiolactone or dimethylbenzanthracene as the initiator, and phorbol myristate acetate as the tumor promoter. Under these conditions, the PUFA diet has been reported to increase the number of chemically induced papillomas, when compared to the SF diet (Machlin, Troll and Belman, unpublished data). The inclusion of ETYA (0.3 g/kg of diet for 37 days) in the PUFA diet was found to significantly reduce the number of chemically induced papillomas. Similar results were obtained when ETYA was included in the SF diet, although a longer time period (44 days) was required for the results to become significant (73).

SUMMARY

In summary, ETYA has been found to be a very useful biochemical tool for elucidating the role of PGs in various biochemical processes. As described in this paper, however, the compound possesses a varied pharmacological profile, which may include certain activities unrelated to inhibition of PG biosynthesis. To further illustrate this point, very few compounds, besides ETYA, which inhibit the formation of 12-HETE, have been mentioned in the literature; this hydroxy fatty acid has been shown to stimulate human eosinophil and neutrophil (PMN) chemotaxis and random migration (74,75), and may play a role in inflammation. Because of its varied pharmacological profile, ETYA, or one of its di- or triynoic analogues, may prove to have future clinical utility for indications other than those originally proposed at Roche, Nutley.

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Polyunsaturated Fatty Acids, Vitamin E, and the Proliferation of Aortic Smooth Muscle Cells

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ABSTRACT

Smooth muscle cell cultures were obtained from the aortas of prepubertal guinea pigs. Cell proliferation in these cultures was inhibited by 8,11,14-eicosatrienoic acid, 5,8,11,14-eicosatetraenoic acid, and their prostaglandin E derivatives, PGE_1 and PGE_2. Prostaglandin F derivatives, $PGF_{1\alpha}$ and $PGF_{2\alpha}$, stimulated cell proliferation. Cell proliferation was also inhibited by 5,8,11-eicosatrienoic acid and 11,14,17-eicosatrienoic acid. The monoene and diene precursors of the triene and tetraene acids, 9-octadecenoic acid and 9,12-octadecadienoic acid, did not inhibit cell proliferation. Indomethacin alone had no effect on cell proliferation, and indomethacin did not suppress the inhibition of cell proliferation with a triene acid. The antioxidant α-naphthol alone stimulated cell proliferation and suppressed prostaglandin E formation. α-Naphthol in the presence of either triene or tetraene acids also stimulated cell proliferation and suppressed prostaglandin E formation. The antioxidants butylated hydroxy toluene and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid either alone or in the presence of triene and tetraene acids stimulated cell proliferation and had no effect on prostaglandin E formation. Vitamin E either alone or in the presence of triene or tetraene acids stimulated cell proliferation and had no effect on prostaglandin E formation. More prostaglandin E was formed from 8,11,14-eicosatrienoic acid than from 5,8,11,14-eicosatetraenoic acid in the presence of antioxidants. Vitamin E suppressed the inhibitory effects of both PGE_2 and palmitic acid on cell proliferation. The cyclic nucleotide phosphodiesterase inhibitors, caffeine and papaverine, suppressed the stimulatory effect of vitamin E on cell proliferation and enhanced the inhibitory effect of a triene acid on cell proliferation. Substrate and inhibitor specificities are consistent with the oxidative regulation of cell proliferation through the formation of hydroperoxy fatty acids. We propose that hydroperoxy fatty acids may regulate both cyclase and cyclic nucleotide phosphodiesterase enzymes through sulfhydryl-disulfide interconversions. We suggest that this regulatory mechanism may help to explain the acculation of 5,8,11-eicosatrienoic acid in essential fatty acid deficiency, the effects of antioxidants on cell proliferation, and one of the several effects of polyunsaturated fatty acids in proliferative disorders such as cancer and atherosclerosis.

INTRODUCTION

The accumulation of cholesterol and cholesteryl esters in aortas was an early observation (1) and has been repeatedly confirmed in studies on the development of atherosclerosis (2-6). Studies relating morphology and lipid composition demonstrate that there is no simple progression in either relative lipid composition on total lipid content from normal intima to fatty streaks to fibrous plaques (7,8). However, it is clear that the relative amount of cholesteryl ester increases with the appearance of intracellular lipid in the fatty streak lesion (7-9). The physical properties and the fatty acid composition of this cholesteryl ester fraction of aortic lipid may have profound consequences on the metabolism of the aortic smooth muscle cell.

Cholesteryl esters of long chain fatty acids are only slightly soluble in other lipids (10) and are not surfactants (11,12). Thus, an increment in the relative and/or absolute cholesteryl ester content of the cell should result in the separation of cholesteryl ester droplets from other lipids. Indeed, anisotropic lipid droplets were observed in intima as early as 1902 (13), and droplets have been described in a number of subsequent studies (14,15). These lipid droplets were later found to be almost pure cholesteryl ester (16). An extensive study of lipid phase behavior has demonstrated unequivocally that the cholesteryl ester droplet is characteristic of the fatty streak lesion (8) where it forms a relatively inert lipid pool (8,17).

The fatty acid composition of cholesteryl esters in the fatty streak lesion is unique (2). The relative amounts of oleate and an eicosatrienoate are high, the relative amount of arachidonate is unchanged, and the relative amount of linoleate is low in fatty streaks (2,8,9,18-20) compared to normal intima (2,8,9,18-20) or blood (2,21). The cholesteryl esters of the fatty

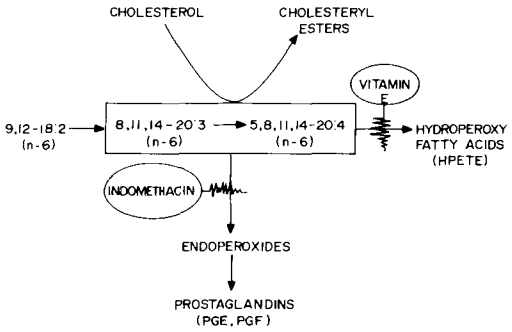

FIG. 1. Fatty acid metabolism in the microsome showing desaturation (in rectangle), cholesterol esterification, and oxidation through the endoperoxide-prostaglandin and hydroperoxide (HPETE) pathways.

streak, particularly their eicosatrienoate content, are very similar to cholesteryl esters of the adrenal and probably represent *de novo* synthesis and intracellular storage in both tissues (2). The eicosatrienoate has been identified (22) as the 8,11,14 isomer, 20:3 (n-6), a fatty acid formed during the biosynthesis of arachidonic acid, 20:4 (n-6), from linoleic acid, 18:2 (n-6), desaturation-chain elongation in the microsome (23). Both 20:3 (n-6) and 20:4 (n-6) are converted to endoperoxides by microsomal cyclooxygenase, and these endoperoxides are then converted to prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2), respectively (24,25). It is apparent that the fatty acid precursors of PGE_1 and PGE_2 accumulate in cholesteryl esters as the cholesteryl ester content increases in fatty streak lesions.

Most cells in the intimal lesion are smooth muscle (26). Proliferation of smooth muscle cells is a consistent feature of atherosclerotic lesions of all types (27-32). A number of studies have concentrated on a mitogenic factor(s) released from platelets (28) or present in serum (29,32) as agents in the proliferation of smooth muscle cells. We (2,33,34) have proposed that cell proliferation also results from cholesterol esterification in the fatty streak lesion. Cholesterol esterification (4), fatty acid desaturation-chain elongation (23), and prostaglandin biosynthesis (24,25) are all microsomal reactions (Fig. 1). It seemed highly probable that esterification shunted the precursor fatty acids from the endoperoxide pathway (Fig. 1) making prostaglandins less available as mediators (35,36) of cell proliferation.

Preliminary experiments in this laboratory showed that polyunsaturated fatty acids had highly specific effects on proliferation in both mammalian smooth muscle cell and human fibroblast cultures (37,38). The precursor fatty acids, 20:3 (n-6) and 20:4 (n-6), inhibited proliferation in cultures of aortic smooth muscle cells (37,38). Their prostaglandin derivatives are synthesized by the smooth muscle cell (39). We found that PGE_1 and PGE_2 were even more potent inhibitors of cell proliferation (37). New and more detailed experiments with specific inhibitors and other precursor fatty acids suggest that hydroperoxy trienoic and tetraenoic fatty acids (HPETE), synthesized in the microsome by an alternative lipoxygenase pathway (40,41) (Fig. 1), were mediators of cell proliferation. These experiments, which may also explain both the role of vitamin E in cell proliferation (42) and the role of 5,8,11-eicosatrienoic acid in essential fatty acid deficiency (43), are described in the present investigation.

MATERIALS AND METHODS

Materials

All fatty acids were purchased from Nu-Chek Prep (Elysian, MN) except 20:3 (n-9) which was kindly supplied by Dr. H. Sprecher. Fatty acids were monitored for the absence of peroxides by thin layer chromatography on Silica Gel G using heptane/ether/acetic acid (50:50:0.8, v/v) as the developing solvent and a potassium iodide-starch spray. Prostaglandins were kindly supplied by Dr. J. Pike (The Upjohn Co.) and Ono Pharmaceutical Co., Osaka, Japan. Indomethacin was kindly supplied by Dr. F.A. Kuehl, Jr. (Merck Institute). d-α-Tocopherol was purchased from Eastman Organic Chemicals (Rochester, NY). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C) was kindly supplied by Dr. W.E. Scott (Hoffman-La Roche). Butylated hydroxy toluene (BHT) and α-napthol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Caffeine was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) and papaverine was purchased from Sigma Chemical Co. (St. Louis, MO).

Tissues Culture for Smooth Muscle Cells (SM)

Our methodology for SM cultures is described elsewhere in detail (44). Growth medium was prepared from 1 X Eagle's minimum essential medium containing Hank's salts (Flow Labs, Rockville, MD) supplemented with 50 μg per ml gentamycin sulfate (Schering Corp, Newark, NJ), 2 mM glutamine (GIBCO, Grand Island, NY), 1 X nonessential amino acids (Microbiological Associates, Inc., Walkersville, MD) and 2.2 mg/ml of sodium bicarbonate.

Growth medium was supplemented with 5% fetal bovine serum (Biofluids, Lot 829096; Armour-Reheis, Lot M 27003). The free fatty acid level, 286 μM in both lots of fetal bovine serum (38), was similar to the mean free fatty acid level reported for commercial fetal bovine serum (45). Fetal bovine serum was checked for mycoplasma contamination (46) and for adequate growth-supporting properties (47).

SM cultures were derived as primary cultures from the aortas of male guinea pigs as previously described (44). SM cells from population doubling level two were cloned at low cell densities (200 cells/cm^2) in Falcon 4-well 60 x 15 mm cluster dishes. Growth media was supplemented with 20% fetal bovine serum when cells were cloned at low cell densities. Seeding and plating efficiencies, defined by standard cell culture terminology (48), were measured.

Water insoluble compounds were dissolved in 95% ethanol, diluted 1 to 500 with complete medium, and added as 0.1 ml aliquots to 4.9 ml of complete medium in the Falcon well. An ethanol blank was added to the control culture. The final ethanol concentration was 0.04 M.

The proliferative potential of SM cultures was measured as previously described (38). After 7-9 days in culture, plates were fixed with formalin and stained with Ehrlich's glycerin alum hematoxylin or May-Grumwald Giemsa stain. Clones that contained at least 50 cells were counted under a dissecting microscope to determine the plating efficiency. All values were corrected to 100% seeding efficiency. The data are reported as plating efficiency in control and treatment cultures or as relative plating efficiency. Relative plating efficiency is defined as the ratio of the plating efficiency obtained with a specific treatment to the plating efficiency obtained with the control in %.

Radioimmunoassay of Prostaglandins

The PGE concentration in the tissue culture medium was measured by radioimmunoassay (49,50) with antiserum to PGE kindly supplied by Dr. L. Levine and goat antibody to rabbit γ-globulin (Clinical Assays, Inc., Cambridge, MA). PGE_1 and PGE_2 showed similar cross-reactivities with the antibody (37). Fatty acid precursors gave false-positive PGE values of 1030 pg/ml and 125 pg/ml when 160 μm 20:3 (n-6) and 160 μM 20:4 (n-6), respectively, were incubated with buffer alone (37).

RESULTS

Fatty Acid Precursors of the Prostaglandins

Figure 2 describes the effects of increasing concentrations of 18:2 (n-6), 20:3 (n-6) and 20:4 (n-6) on the proliferation of SM cultures. At the lowest concentration level, 18:2 (n-6) inhibits proliferation ($p<0.03$ using the Student "t" test for paired means), while its desaturation-chain elongation products (Fig. 1) have no effect on proliferation. 18:2 (n-6) appears to stimulate proliferation at higher concentrations, but proliferation at these concentrations does not differ significantly from proliferation in control samples. 20:4 (n-6) inhibits proliferation significantly ($p<0.02$) at a 16 μM concentration. Higher concentrations of both 20:3 (n-6) and 20:4 (n-6) inhibit proliferation. These data show clearly that the immediate precursors of prostaglandin endoperoxides, 20:3 (n-6) and 20:4 (n-6), inhibit cell proliferation in SM cultures, while the parent fatty acid, 18:2 (n-6), has little effect on cell proliferation in SM cultures.

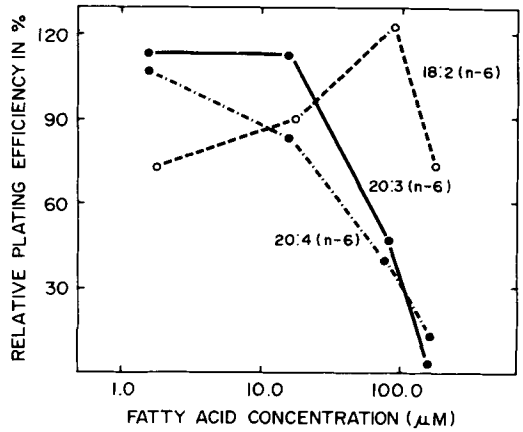

FIG. 2. Effects of increasing concentrations of unsaturated fatty acids in the n-6 series on proliferation in SM cultures. Data for 20:3 (n-6) and 20:4 (n-6) were reported previously (38).

While 20:3 (n-6) and 20:4 (n-6) always inhibit proliferation, different primary cultures are inhibited to a different extent by the same fatty acid concentration. The inhibition obtained with the specific primary culture is noted in every experiment.

Prostaglandins

Figure 3 describes the effects of increasing concentrations of PGE_1 and PGE_2 on the proliferation of SM cultures. PGE_1 inhibits proliferation significantly ($p<0.001$) at a 1.6 μM concentration. Thus, prostaglandin E derivatives are more potent inhibitors of cell proliferation in SM cultures than their fatty acid precursors (Fig. 2).

Figure 4 describes the effects of increasing

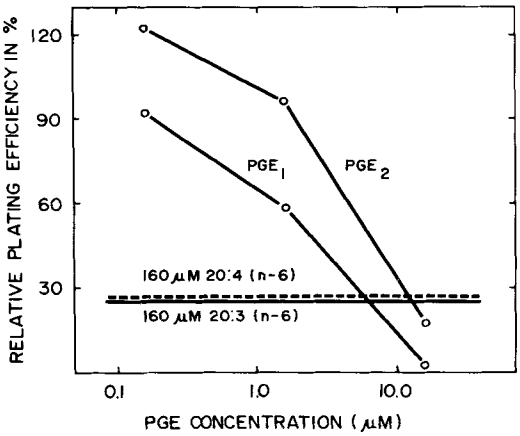

FIG. 3. Effects of increasing prostaglandin E concentrations on proliferation in SM cultures. Horizontal lines show the relative plating efficiencies obtained with the same primary cultures for precursor fatty acids at the 160 μM level. Some of the data for PGE_1 and PGE_2 were reported previously (37).

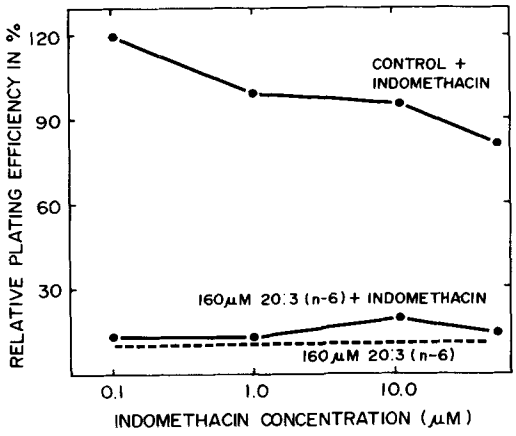

FIG. 5. Effects of increasing indomethacin concentrations on proliferation in SM cultures. Indomethacin was added either alone (control) or together with 160 μM 20:3 (n-6). The horizontal line shows the relative plating efficiency obtained with the same primary culture for 160 μM 20:3 (n-6) alone.

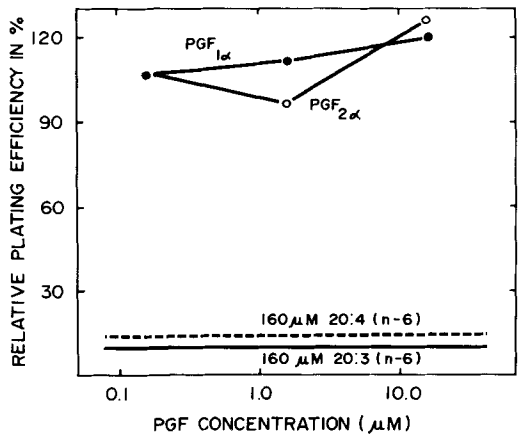

FIG. 4. Effects of increasing prostaglandin F concentrations on proliferation in SM cultures. Horizontal lines show the relative plating efficiencies obtained with the same primary cultures for precursor fatty acids as the 160 μM level.

concentrations of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ on the proliferation of SM cultures. At low concentrations, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ have no effect on proliferation. Both $PGF_{1\alpha}$ and $PGF_{2\alpha}$ stimulate proliferation significantly ($p<0.02$) at a 16 μM concentration. It is apparent that prostaglandin F derivatives stimulate cell proliferation in SM cultures at a concentration level where prostaglandin E derivatives inhibit (Fig. 3) this process.

Indomethacin

The anti-inflammatory agent indomethacin inhibits endoperoxide formation and prostaglandin biosynthesis (24,25) but has little effect on the HPETE pathway (40,51). The specificity of indomethacin is described in Figure 1. In a previous study (37), we showed that indomethacin at 5.6 μM and 11.2 μM concentrations inhibited PGE biosynthesis in SM cultures. Indomethacin in this concentration range has little effect on proliferation in control SM cultures (Fig. 5). Proliferation decreases throughout the concentration range. However, relative plating efficiencies do not vary significantly from their control values. Furthermore, indomethacin has no effect on the inhibition of cell proliferation by 160 μM 20:3 (n-6). These data strongly suggest that 20:3 (n-6) does not exert its inhibitory effect solely through the biosynthesis of PGE_1, even though PGE_1 is itself a potent inhibitor (Fig. 3) of cell proliferation.

Synthetic Antioxidants

Several investigators (52,53) have shown that antioxidants inhibit prostaglandin biosynthesis. In a recent sutdy we (54) reported that some synthetic antioxidants such as α-naphthol inhibit prostaglandin biosynthesis, while other synthetic antioxidants such as BHT and Trolox C (55) have little effect on prostaglandin biosynthesis in microsomes from the bovine vesicular gland and in human platelets. Another recent study (56) also showed that BHT did not alter PGE_2 biosynthesis in slices from the renal medulla of the rat. These observations were confirmed in the present study (Table I). α-Naphthol stimulates SM proliferation and

TABLE I

Effect of Antioxidants on the Proliferation of Smooth Muscle Cells and the Biosynthesis of Prostaglandin E_1 and E_2

	Treatment	Clones (Mean ± S.D.)	PGE (pg/ml)
A.	Control + antioxidant		
	Control	420 ± 25	2700
	Control + 10 μM α-naphthol	920 ± 76[a]	590
	Control + 50 μM α-naphthol	780 ± 19[a]	860
	Control	620 ± 84	1060
	Control + 50 μM BHT	1080 ± 16[a]	1400
	Control	510 ± 48	1030
	Control + 160 μM Trolox C	1210 ± 61[a]	2200
	Control	420 ± 30	2700
	Control + 10 μM vitamin E	1120 ± 109[a]	4400
B.	160 μM 20:3 (n-6) + antioxidant		
	20:3 (n-6)	530 ± 28	7000
	20:3 (n-6) + 50 μM α-naphthol	510 ± 79	440
	20:3 (n-6)	190 ± 33	4900
	20:3 (n-6) + 50 μM BHT	800 ± 105[a]	7000
	20:3 (n-6) + 150 μM Trolox C	1080 ± 96[a]	17,750
	20:3 (n-6)	530 ± 28	7000
	20:3 (n-6) + 10 μM vitamin E	1250 ± 167[a]	18,750
C.	160 μM 20:4 (n-6) + antioxidant		
	20:4 (n-6)	470 ± 31	2500
	20:4 (n-6) + 50 μM α-naphthol	720 ± 47[a]	725
	20:4 (n-6)	250 ± 70	2800
	20:4 (n-6) + 50 μM BHT	980 ± 117[a]	4250
	20:4 (n-6) + 160 μM Trolox C	1170 ± 217[a]	9250
	20:4 (n-6)	470 ± 31	2500
	20:4 (n-6) + 10 μM vitamin E	970 ± 63[a]	5750

[a]The Student "t" test for paired means showed that the antioxidant treatment was significantly different ($p<0.001$).

lowers the PGE levels in media from the stimulated cultures while BHT and Trolox C stimulate SM proliferation and raise the PGE levels in media from the stimulated cultures. It is important to note that all three antioxidants stimulate proliferation even though only α-naphthol inhibits prostaglandin biosynthesis

The antioxidant experiments were extended through studies with fatty acid inhibitors of cell proliferation (Table I). α-Naphthol lowers the PGE levels of the media when both 20:3 (n-6) and 20:4 (n-6) are added to the cultures and α-naphthol stimulates proliferation in the presence of 160 μM 20:4 (n-6). BHT and Trolox C raise the PGE levels of the media and stimulate cell proliferation when either 20:3 (n-6) or 20:4 (n-6) are added to the cultures. These data support the indomethacin experiments since antioxidants which inhibit and antioxidants which do not inhibit prostaglandin biosynthesis both stimulate cell proliferation. It is apparent that cell proliferation is mediated, at least in part, by a process different from the endoperoxide-prostaglandin pathway.

Vitamin E

The natural antioxidant vitamin E has little effect on prostaglandin biosynthesis in microsomes from the bovine vesicular gland (54), human platelets (54), and slices from the renal medulla of the rat (56). Vitamin E behaves very much like its soluble analog Trolox C and like BHT in these systems. Vitamin E also behaves like these antioxidants when it is added to SM cultures (Table I). Vitamin E stimulates cell proliferation and raises the PGE level both in control cultures and in cultures inhibited by 20:3 (n-6) and 20:4 (n-6). Vitamin E is apparently more potent than either Trolox C or BHT since its stimulatory effects are noted at a much lower concentration (Table I). The dramatic effects of vitamin E are shown in photographs of representative tissue cultures (Fig. 6).

The role of vitamin E in cell proliferation

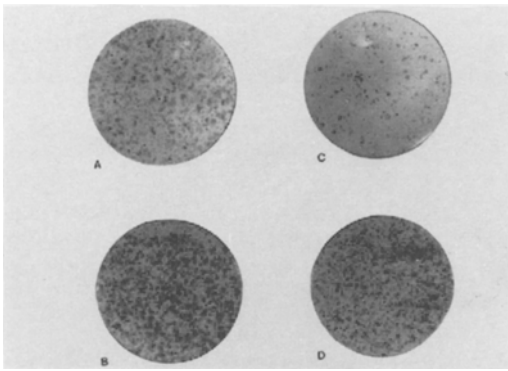

FIG. 6. Photographs of representative wells from tissue cultures treated with 20:3 (n-6) and vitamin E. A, control; B, control + 10 μM vitamin E; C, control + 160 μM 20:3 (n-6); D, control + 160 μM 20:3 (n-6) + 10 μM vitamin E.

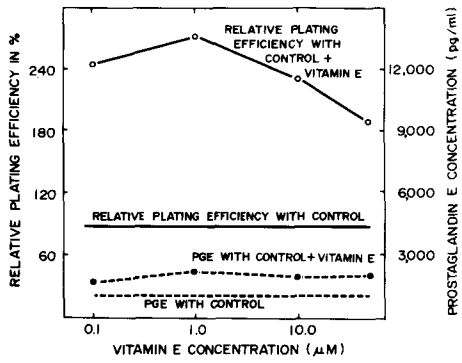

FIG. 7. Effects of increasing vitamin E concentrations on proliferation and PGE levels in SM cultures. The relative plating efficiency (100%) is noted for the control culture by one horizontal line. The PGE level in media from the control culture is noted by a second horizontal line.

and PGE biosynthesis was next studied in detail. Figure 7 describes the effects of increasing concentrations of vitamin E on cell proliferation and the PGE level in SM cultures. The relative plating efficiency is elevated dramatically ($p<0.001$) when as little as 0.1 μM vitamin E is added to the culture. The relative plating efficiency remains elevated throughout the concentraion range. However, the relative plating efficiency begins to decrease at higher vitamin E levels. In fact, all antioxidants used in this study were toxic at high concnetrations. The PGE level in the tissue culture media is elevated throughout the vitamin E concentration range (Fig. 7). These studies indicate that SM cell proliferation is controlled by oxidant stress even in the absence of exogenous polyunsaturated fatty acids.

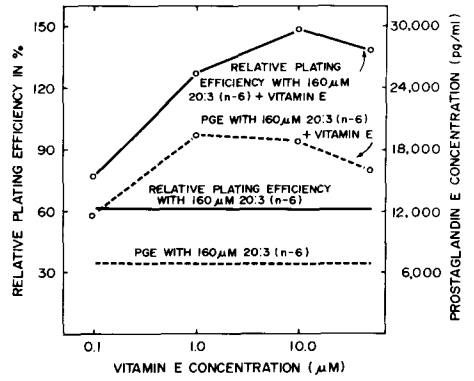

FIG. 8. Effects of increasing vitamin E concentrations on proliferation and PGE levels in SM cultures treated with 160 μM 20:3 (n-6). The relative plating efficiency nd the PGE level found with 20:3 (n-6) alone are shown by horizontal lines.

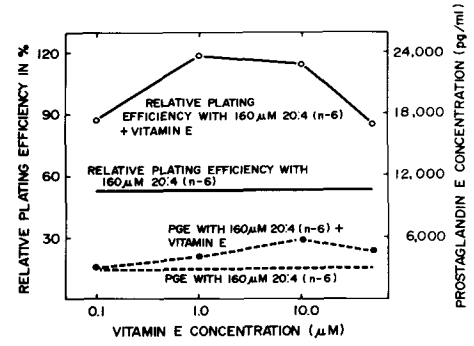

FIG. 9. Effects of increasing vitamin E concentrations on proliferation and PGE levels in SM cultures treated with 160 μM 20:4 (n-6). The relative plating efficiency and the PGE level found with 20:4 (n-6) alone are shown by horizontal lines.

Figures 8 and 9 describe the effects of increasing concentrations of vitamin E on cell proliferation and PGE levels in SM cultures inhibited by either 160 μM 20:3 (n-6) or 160 μM 20:4 (n-6). Cell proliferation (Fig. 8) in the presence of 20:3 (n-6) is not stimulated significantly by 0.1 μM vitamin E. Relative plating efficiencies are very much elevated ($p<0.001$) by higher concentrations of vitamin E. Cell proliferation (Fig. 9) in the presence of 20:4 (n-6) is stimulated significantly by vitamin E at all concentrations ($p<0.05$ at 0.1 μM vitamin E and $p<0.001$ at all other vitamin E concentrations). PGE levels are elevated throughout the vitamin E concentration range when either fatty acid precursor is added to the tissue culture (Figs. 8 and 9). It is interesting that 20:3 (n-6) generates a much higher PGE level than 20:4 (n-6), an observation

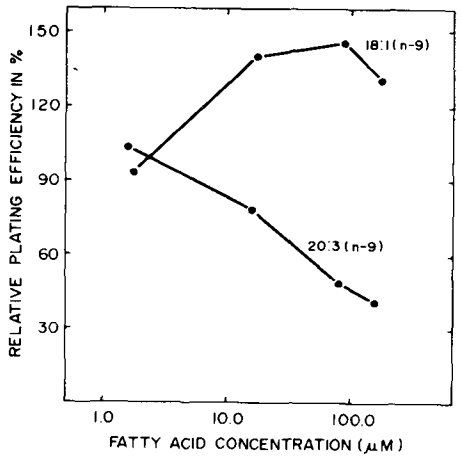

FIG. 10. Effects of increasing concentrations of unsaturated fatty acids in the n-9 series on proliferation in SM cultures. Data for 18:1 (n-9) were reported previously (38).

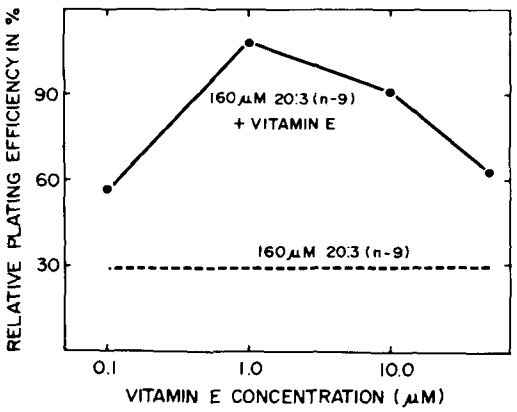

FIG. 11. Effects of increasing vitamin E concentrations on proliferation in SM cultures treated with 160 μM 20:3 (n-9). The relative plating efficiency found with 20:3 (n-9) alone is shown by the horizontal line.

suggested in our initial study (37). This difference in PGE levels with the two fatty acid substrates may reflect the preferential synthesis of prostacyclin rather than PGE from 20:4 (n-6) that was recently noted in lysed aortic smooth muscle cells (57). The data with the natural antioxidant vitamin E further support the observation that proliferation is not controlled simply by the prostaglandin level.

Fatty Acids in the 5,8,11-Eicosatrienoic Acid Pathway

5,8,11-Eicosatrienoic acid, 20:3 (n-9), is synthesized from oleic acid, 18:1 (n-9), by microsomal desaturation-chain elongation

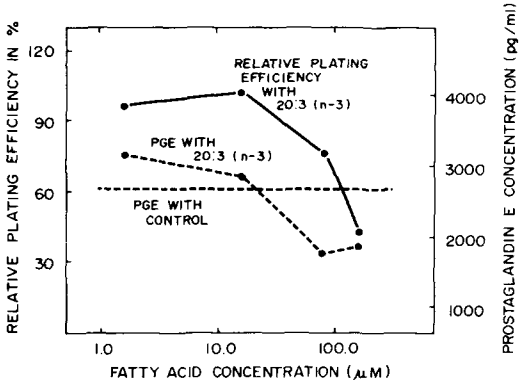

FIG. 12. Effects of increasing 20:3 (n-3) concentrations on proliferation and PGE levels in SM cultures. The PGE level in the media from the control culture is shown by the horizontal line.

(23,58). 20:3 (n-9) accumulates during an essential fatty acid deficiency (EFA), and this fatty acid is used as the biochemical index of EFA (43,58). 20:3 (n-9) does not serve as a precursor of the prostaglandins (24,25).

The effects of increasing concentrations of 18:1 (n-9) and 20:3 (n-9) on cell proliferation are described in Figure 10. The precursor fatty acid, 18:1 (n-9), stimulates proliferation significantly ($p<0.05$ at a 18 μM concentration and $p<0.01$ at a 90 μM concentration) behaving very much like 18:2 (n-6), the analogous fatty acid for the n-6 desaturation-chain elongation series (Fig. 2). 20:3 (n-9) inhibits proliferation significantly ($p<0.002$ at 80 μM and 160 μM concentrations) behaving very much like 20:3 (n-6) and 20:4 (n-6), the prostaglandin precursors formed in the n-6 desaturation-chain elongation series (Fig. 2). These data show clearly that a polyunsaturated fatty acid which does not function as a prostaglandin precursor inhibits SM cell proliferation.

Figure 11 describes the effects of increasing concentrations of vitamin E on cell proliferation in cultures inhibited by 20:3 (n-9). The relative plating efficiency is elevated significantly ($p<0.0001$) at all concentrations of vitamin E added to the culture. Thus, the natural antioxidant vitamin E overcomes the inhibitory effect of a polyunsaturated fatty acid that cannot serve as a prostaglandin precursor.

11,14,17-Eicosatrienoic Acid

11,14,17-Eicosatrienoic acid, 20:3 (n-3), may be synthesized from 18:3 (n-3) by a minor pathway (59). 20:3 (n-3) does not itself serve as a prostaglandin precursor (25) although it is partially degraded (58,60,61) to 18:3 (n-3), the

TABLE II

Effect of Antioxidants on the Inhibition of Proliferation of Smooth Muscle Cells by PGE_2 and Palmitic Acid (16:0)

	Treatment	Relative plating efficiency	
		PGE_2 (16 μM)	16:0 (180 μM)
A.	Without antioxidant	31	7
	+ 0.1 μM α-naphthol	22	17[a]
	+ 1.0 μM α-naphthol	34	37[a]
	+ 10 μM α-naphthol	48[a]	83[a]
	+ 50 μM α-naphthol	54[a]	60[a]
B.	Without antioxidant	7	6
	+ 1.6 μM Trolox C	25	7
	+ 16 μM Trolox C	40[a]	32[a]
	+ 80 μM Trolox C	61[a]	53[a]
	+ 160 μM Trolox C	13	42[a]
C.	Without antioxidant	47	
	+ 0.1 μM vitamin E	107[a]	
	+ 1.0 μM vitamin E	177[a]	
	+ 10 μM vitamin E	128[a]	
	+ 50 μM vitamin E	57	

[a]The Student "t" test for paired means showed that the antioxidant treatment was significantly different ($p<0.005$).

ultimate precursor of 20:5 (n-3) and PGE_3. 20:3 (n-3) is particularly interesting because some data (62) suggest that n-3 acids, which do not contain double bonds at the 8 and 11 positions, will not function as substrates for lipoxygeanse.

Figure 12 describes the effects of increasing concentrations of 20:3 (n-3) on both cell proliferation and PGE levels in SM cultures. 20:3 (n-3) inhibits proliferation significantly ($p<0.005$ at a 80 μM concentration and $P<0.001$ at a 160 μM concentration) behaving very much like the triene acids in n-6 (Fig. 2) and n-9 (Fig. 11) series. 20:3 (n-3) does not appear to stimulate PGE production and indeed the shape of the PGE curve mirrors the shape of the relative plating efficiency curve. These data indicate that 20:3 (n-3), like 20:3 (n-9), inhibits proliferation without stimulating prostaglandin biosynthesis.

Other Antioxidant Effects

Increased amounts of PGE are found in the media when precursor fatty acids are added to tissue cultures together with the antioxidants Trolox C (Table I) and vitamin E (Figs. 8 and 9). PGE_1 and PGE_2 are more potent (Fig. 3) than their precursor fatty acids (Fig. 2) in the inhibition of cell proliferation. Yet, antioxidants prevent the inhibition of cell proliferation in these tissue culture systems (Table 1 Figs. 8 and 9). These data suggest that antioxidants may exert an effect on the inhibition of cell proliferation by PGE. The data in Table II show that α-naphthol, an antioxidant that blocks PGE biosynthesis (Table I), and both Trolox C and vitamin E, antioxidants that do not block PGE biosynthesis (Table I, Figs. 8 and 9), all suppress inhibition with PGE_2. Thus, relative plating efficiencies increase, and in one instance surpass the control level (vitamin E), when antioxidants are added together with PGE_2.

Various saturated fatty acids inhibit cell proliferation (37), and it is interesting that antioxidants suppress the inhibitory effect of palmitic acid on cell proliferation (Table II). The PGE_2, palmitic acid, and polyunsaturated fatty acid data suggest that antioxidants may actually function at several points in the control of cell proliferation.

Cyclic Nucleotide Phosphodiesterase (PDE) Inhibitors

Many investigators (63-65) have begun to correlate prostaglandins with cyclic nucleotides and their effects on cell proliferation. These correlations prompted us to study PDE inhibitors (66) in SM cultures where proliferation was either inhibited with 20:3 (n-6) or stimulated with vitamin E. The xanthine derivative, caffeine, and the isoquinoline derivative, papaverine, were used as PDE inhibitors. The results are summarized in Table III.

Both papaverine and caffeine inhibit SM cell proliferation (Table III). The PDE inhibitors enhance the inhibition obtained with 20:3 (n-6). For example, 50 μM papaverine and 1 mM caffeine inhibit control cultures less than

TABLE III

Effect of Cyclic Nucleotide Phosphodiesterase (PDE) Inhibitors of the Proliferation of Smooth Muscle Cells

Treatment	Relative plating efficiency		
	Control	20:3 (n-6) 160 μM	Vitamin E 10 μM
A. Without PDE inhibitor	100	36	143
+ 0.1 M papaverine	92	45	147
+ 1.0 M papaverine	102	34	116[a]
+ 10 M papaverine	86	29	119[a]
+ 50 M papaverine	56[a]	0[a]	80[a]
B. Without PDE inhibitor	100	37	167
+ 0.1 mM caffeeine	123[a]	26	212
+ 1 mM caffeine	71[a]	9[a]	62[a]

[a]The Student "t" test for paired means showed that the inhibitor treatment was significantly different ($p<0.001$).

50%. Yet, these PDE inhibitors tend to eliminate proliferation in cultures treated with 160 μM 20:3 (n-6). The PDE inhibitors overcome the stimulation obtained with vitamin E. The caffeine effect is most interesting. A low concentration of this PDE inhibitor stimulates proliferation in both control- and vitamin E-treated cultures. A higher concentration of caffeine has a marked inhibitory effect on the vitamin E-treated cells. These data suggest but do not prove that polyunsaturated fatty acid and antioxidant effects are related to cyclic nucleotide levels.

DISCUSSION

Fatty acids may function as anionic detergents, and every study that identifies a fatty acid effect on a biological system must consider detergency as one explanation for the biological effect. This problem has been debated recently in relation to 20:4 (n-6) and platelet aggregation (67-69). The arguments against detergency are based on concentration, specificity and inhibition. We believe that these are compelling arguments in the interpretation of our data with SM cells in culture.

The role of concentration in generating a specific fatty acid effect or a nonspecific detergent effect has been well documented in many studies. For example, low concentrations of fatty acids in the 20 nmole/mg protein range have a very different effect on energy coupling in mitochondria than fatty acids at 10- to 100-fold higher concentrations (70,71). However, fatty acids in the higher concentration range which were added as an albumin complex, 1 μmole/100 mg albumin, do not exhibit this detergency effect (72). In the present study, we added fatty acids at concentrations below their critical micelle concentrations (73) to complete media which contained 20% fetal bovine serum (see Methods). Serum albumin has a number of strong binding sites for fatty acids (67,74) and even 20% fetal bovine serum should contain sufficient albumin (45) to prevent a detergent effect with fatty acids added in the 100 μM concentration range.

The proliferation of SM cells in culture is inhibited by specific fatty acids. All triene and tetraene acids examined in this study inhibited proliferation (Figs. 2,10,12). Monoene and diene precursors of the triene acids behaved in a very different manner by actually stimulating cell proliferation (Figs. 2 and 10). It has been suggested (67) that 20:4 (n-6) specificity in platelet aggregation may be explained by the greater solubility of 20:4 (n-6). Since the critical micelle concentration for the 18:2 (n-6) anion is 150 μM, it is unlikely that solubility is a factor in a study where the highest fatty acid concentration is 180 μM. Furthermore, 20:4 (n-6) stimulates proliferation in human fibroblasts (38) while 18:0, a less soluble fatty acid anion, inhibits proliferation in this cell line (38). These data are not easily explained by a nonspecific detergent effect related to fatty acid solubility.

Antioxidants overcome the inhibitory effect of triene and tetraene fatty acids on cell proliferation (Table I; Figs. 6-9,11). This effect which is found with a number of antioxidants such as α-naphthol, BHT, Trolox C and vitamin E is difficult to explain if the fatty acid anions are acting only as detergents. The soap might conceivably damage a lysosomal membrane and increase cellular susceptibility to oxidant stress. Detergents have been shown to damage cytoplasmic membranes in bacteria

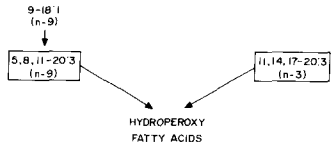

FIG. 13. Proposed biosynthesis of HPETE from 20:3 (n-9) and 20:3 (n-3).

(75). However, antioxidants also suppress the inhibitory effects of PGE_2 and a saturated fatty acid (Table II). It is difficult to imagine that a variety of inhibitory compounds such as polyunsaturated fatty acids, saturated fatty acids, and some prostaglandins all exert a nonspecific detergent effect on cytoplasmic membranes while other fatty acids and other prostaglandins have not effect on this system. Finally, $PGF_{2\alpha}$ enhances lysosomal enzyme release (76), and yet this prostaglandin stimulates cell proliferation (Fig. 4).

Since precursor fatty acids and their prostaglandin E derivatives (Figs. 2 and 3) all inhibited cell proliferation, we initially proposed (37) that 20:3 (n-6) and 20:4 (n-6) mediated cell proliferation through the prostaglandin-endoperoxide pathway. Three experimental observations do not support this hypothesis. Indomethacin inhibits prostaglandin biosynthesis (24,25,37) but has no effect on the inhibition of cell proliferation with a precursor fatty acid (Fig. 5). Two triene fatty acids, 20:3 (n-9) and 20:3 (n-3), inhibit cell proliferation (Figs. 10 and 12) even though they do not serve as prostaglandin precursors (24,25). Finally, anti-oxidants that inhibit prostaglandin biosynthesis such as α-naphthol (Table 1) and antioxidants that have little effect on prostaglandin biosynthesis, such as BHT, Trolox C, and vitamin E (Table I, Fig. 7-9,11), all suppress the inhibition of cell proliferation by triene and tetraene fatty acids. These data suggest that a different oxidation product of the polyunsaturated fatty acids is involved in the inhibition of cell proliferation.

HPETE derivatives of the polyunsaturated fatty acids are synthesized by lipoxygenases. Soybean lipoxygenase (Linoleate: O_2 Oxidoreductase EC 1.13.11.12) has been studied in great detail (77-80). Mammalian lipoxygenases have recently been isolated from platelets (40,41,62), neutrophils (81), lung (82), spleen (82) and perhaps the sheep vesicular gland (52,83). Substrate and inhibitor specificities in our tissue culture sysstem suggest that polyunsaturated fatty acids mediate cell proliferation through the lipoxygenase pathway.

20:3 (n-6) and 20:4 (n-6) are both good substrates for platelet lipoxygenase while 18:2 (n-6) is a poor substrate for this enzyme (62). These data could explain the inhibition pattern obtained with fatty acids in the n-6 series (Fig. 2). 20:3 (n-9) is a good substrate for lipoxygenase (62), and its conversion to a HPETE derivative could explain inhibition with this fatty acid (Fig. 10). 20:3 (n-3) has not been tested as a substrate for lipoxygenase (62). Although this fatty acid does not appear to have the necessary double bond configuration (62), it is a good inhibitor of cell proliferation (Fig. 12). We suggest that both 20:3 (n-9) and 20:3 (n-3) function through their HPETE derivatives (Fig. 13).

Indomethacin does not inhibit mammalian lipoxygenase (39,51,62), and indomethacin will have little effect on polyunsaturated fatty acid inhibition of cell proliferation if inhibition is mediated through the HPETE pathway. Thus, indomethacin data (Fig. 5) are consistent with the HPETE hypothesis. Antioxidants that inhibit prostaglandin biosynthesis and antioxidants that do not inhibit prostaglandin biosynthesis all inhibit soybean lipoxygenase (54). Indeed, vitamin E is one of the most effective inhibitors of this enzyme. Vitamin E does not appear to inhibit platelet lipoxygenase when vitamin E is added, in vitro, as an alcoholic solution (84,85). However, a very important recent study (85) shows that platelet HPETE production is significantly greater in platelets from vitamin E-deficient animals than platelets from vitamin E-supplemented animals. These observations are consistent with the well established role of vitamin E as an inhibitor of lipid peroxidation, in vivo (86,87). We suggest that vitamin E and other antioxidants could suppress the inhibitory effect of polyunsaturated fatty acids on cell proliferation (Table I, Figs. 6-9,11) by inhibiting the biosynthesis of HPETE. These inhibitor effects are summarized in Figure 1.

Cyclic nucleotides are important in the control of cell proliferation (63-65). Cyclic nucleotides are regulated by cyclase and cyclic nucleotide phosphodiesterase (PDE) activities. HPETE derivatives may function through the regulation of both cyclase and PDE. The oxidative regulation of guanylate cyclase has been summarized in several reports (65,88). Guanylate cyclase is apparently activated by a sulfhydryl-disulfide interconversion involving either endoperoxides (89-93) or hydroperoxides (93,94) in the generation of hydroxyl radical (95-97). Furthermore, the antioxidant butylated hydroxyanisole prevents guanylate cyclase activation by agents such as N-methyl-N′-nitro-N-nitrosoguanidine (98). 18:1 (n-9),

18:2 (n-6) and saturated fatty acids also activate guanylate cyclase (99,100), but activation with these fatty acids is found at concentrations that could suggest a detergent effect. We propose the HPETE derivatives of the polyunsatuarted fatty acids alter cyclic nucleotide levels through cyclase activation. This hypothesis is consistent with the synergism between 20:3 (n-6) and PDE inhibitors (Table III) and explains the role of vitamin E and other antioxidants. The hypothesis is described in Figure 14.

The HPETE-oxidant stress hypothesis that we propose in Figure 14 is based on the many known interactions between redox systems and membrane-bound enzymes (101-103). HPETE will initiate the formation of oxygen centered radicals and singlet oxygen (104-106). Vitamin E and other antioxidants may prevent either the formation of HPETE (54,85-87) or the biological effects of oxygen-centered radicals and singlet oxygen formed from HPETE (104-107). The exact locus of vitamin E activity is deliberately omitted from Figure 14. Indeed, we stress that our HPETE-oxidant stress hypothesis is based, at this time, on inhibitor and fatty acid substrate specificities and not the identification and quantitative estimation of HPETE and its products.

Polyunsaturated fatty acid and antioxidant effects on proliferation in SM cultures may be explained by the oxidative regulation of a cyclase. This regulatory mechanism does not explain why antioxidants suppress the inhibitory effect of PGE_2 on cell proliferation (Table II). PGE_1, $PGE_{2,}$, and prostacyclin stimulate cAMP formation. These effects have been described by many investigators and only a few representative reviews and papers are cited here (35,36,63-65,108-112). We suggest that antioxidants may control a prostaglandin-mediated cyclic nucleotide response by protecting PDE. Two exhaustive reviews of PDE (66,113) conclude from the available literature that PDE may be a sulfhydryl enzyme stabilized by agents such as dithiothreitol. We propose that antioxidants protect PDE from HPETE and other sources of oxidant stress (Fig. 14). This hypothesis is consistent with our experimental data. If vitamin E protects PDE, it will facilitate the removal of cyclic nucleotides and suppress the inhibitory effect of PGE_2 (Table II). If vitamin E protects PDE, caffeine and papaverine will suppress the stimulatory effect of vitamin E on cell proliferation (Table III). A relationship between PDE activity and vitamin E levels, in vivo, was proposed previously (114). We suggest that the higher PDE level found in a vitamin E-deficient animal may result from an

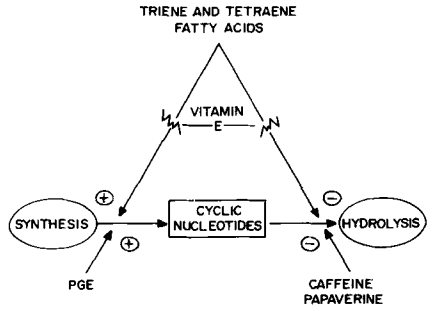

FIG. 14. Hypothetical scheme explaining the effects of polyunsaturated fatty acids and vitamin E on cyclic nucleotide levels. The scheme explains how PDE inhibitors may both enhance the inhibitory effect of triene and tetraene acids and suppress the stimulatory effect of vitamin E, and how vitamin E may suppress the inhibitory effect of PGE_2.

attempt to overcome oxidant stress. This suggestion is supported by the observations that vitamin E and other antioxidants protect cells depleted of sulfhydryl groups (115) and that both vitamin E and selenium, a component of glutathione peroxidase, stimulate growth in tissue cultures (116). A certain economy is provided in cell regulation if oxidant stress stimulates cyclase and suppresses PDE (Fig. 14). However, we are aware that the consequences of oxidant stress may be considerably more complex than our hypothetical model implies. For example, oxidant stress actually inhibits adenylate cyclase in adrenal cells (117).

The oxidant stress hypothesis may even explain why antioxidants suppress the inhibitory effect of saturated fatty acids on cell proliferation. Saturated fatty acids stimulate a cyclase (99). However, the most effective saturated acid in that system, 14:0, is the least effective saturated acid in SM cultures (38). Some PDE enzymes are activated by a Ca^{++}-protein complex (65,66,113). Saturated fatty acids such as 18:0 have unique Ca^{++} binding properties (118), and indeed saturated fatty acids suppress the effect of the Ca^{++}-protein modulator (119). The interpretation of these data is complicated by the observation that relatively high concentrations of the fatty acids themselves activate PDE in the absence of the Ca^{++}-protein modulator (119). If saturated fatty acids suppress PDE through Ca^{++} binding, antioxidants may overcome their effects by protecting PDE from oxidant stress (Table II). Ca^{++}-cyclic nucleotide interactions are complex (120) and not easily interpreted. Indeed, other attractive hypotheses have been proposed in discussions of the relationships between oxidant stress, sulfhydryl groups, cyclic nucletides, Ca^{++} and cellular control processes (121,122).

The HPETE oxidant stress hypothesis that we propose in this study has a number of implications. This hypothesis may help to explain the accumulation of 20:3 (n-9) in an EFA deficiency, the effect of antioxidants on cell proliferation, and the role of polyunsaturated fatty acids in proliferative disorders such as cancer and atherosclerosis.

20:3 (n-9) accumulation is the biochemical index of EFA deficiency (43,57). It is difficult to imagine that 20:3 (n-9) will affect membrane fluidity because a significant biological effect on fluidity occurs only between a saturated fatty acid and the introduction of one double bond to that acid (123,124). 20:3 (n-9) does not serve as a precursor of prostaglandins (24,25). However, 20:3 (n-9) inhibits the isomerase that converts prostaglandin H to PGE (125). The inhibitory effect on cell proliferation that we find is not correlated with any effect on prostaglandin levels, and it appears unlikely that 20:3 (n-9) acts in our system by inhibiting the isomerase. We propose that 20:3 (n-9) may yield a HPETE derivative that is important in metabolic regulation during an EFA deficiency.

Enhanced cell proliferation, one of the first biological effects identified with vitamin E (42), has been confirmed in recent studies (116,126). Other antioxidants such as BHT also stimulate cell proliferation (127). Indeed, rapidly proliferating cells are resistant to peroxidation (128) and may generate an antioxidant (129). We suggest that these effects may be explained, in part, by the control of oxidative regulation through HPETE. Different cells may be affected in different ways by specific polyunsaturated fatty acids (38), and it is possible that oxidative regulation through HPETE is involved in correlations between dietary fat and cancer that are suggested both by epidemiology (130,131) and by laboratory investigations (131). The HPETE hypothesis may even help to explain why agents that initiate cellular redifferentiation are also good hydroxyl radical scavengers (132). It must be noted that the effects of HPETE and vitamin E on cell proliferation are quite different from any effect that vitamin E may or may not have on the lifespan of cultured human diploid cells (133-135). Regulatory control may exist ultimately within quantitative interrelationships between prostaglandins, prostacyclin, and HPETE. Thus, we suggest that enhanced prostaglandin synthesis in proliferating tissue (136) could reflect precursor shunting from the HPETE pathway to the prostaglandin pathway just as diminished growth in tumors treated with indomethacin (137) could reflect precursor shunting from the prostaglandin pathway to the HPETE pathway.

We initially proposed (2,33,34,37) that the proliferation of smooth muscle cells was controlled, in part, by the availability of polyunsaturated fatty acids for prostaglandin biosynthesis (Fig. 1). We now believe that polyunsaturated fatty acids may regulate SM cell proliferation through the HPETE pathway (Fig. 1). Further studies on the identification of HPETE, the possible role of prostacyclin, and other aspects of this system are in progress.

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Stereospecificity of Linoleic Acid Hydroperoxide Isomerase from Corn Germ

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ABSTRACT

Linoleic acid hydroperoxide isomerase from corn germ inverted the stereoconfiguration of its substrate. 9-D(S)-Hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid was converted to 10-oxo-9-L(R)-hydroxy-*cis*-12-octadecenoic acid. Presumably, the H_2O solvent of OH^- acted as a nucleophile. In the presence of another nucleophile, linoleate, the 9-D(S)-hydroperoxide was transformed into 9-L(R)-linoleoyloxy-10-oxo-*cis*-12-octadecenoic acid. The substitution of nucleophiles from the incubation solution and the inversion of stereoconfiguration at carbon-9 are consistent with a bimolecular nucleophilic substitution (S_N2) mechanism.

INTRODUCTION

Linoleic acid hydroperoxide isomerase (LAHI), an enzyme which catalyzes the production of α-ketol fatty acids from hydroperoxylinoleic acid, was discovered in flaxseed by Zimmerman (1). Flaxseed LAHI required 13-hydroperoxylinoleic acid as a substrate (2); whereas, LAHI from corn germ converted both isomeric 9- and 13-hydroperoxylinoleic acids to the corresponding 10-oxo-9-hydroxy-*cis*-12-octadecenoic and 12-oxo-13-hydroxy-*cis*-9-octadecenoic acids, respecitvely (3). Also, corn germ LAHI produced γ-ketols; i.e., 9- and 13-hydroperoxylinoleic acids are transformed into 10-oxo-13-hydroxy-*trans*-11-octadecenoic and 12-oxo-9-hydroxy-*trans*-10-octadecenoic acids, respectively (3,4). In the formation of ketols, both flaxseed (5) and corn germ (6) LAHI transferred one of the hydroperoxide oxygens to the vicinal olefinic carbon. Thus, a 13-hydroperoxy oxygen became incorporated into a 12-oxo group, whereas a 9-hydroperoxy oxygen became a 10-oxo oxygen. The hydroxyl function of α- and γ-ketols did not arise from the hydroperoxy function, but instead, presumably originated from the solvent, H_2O (5,6). Various nucleophiles other than H_2O can participate in the LAHI reaction (7). Either linoleate (K salt), oleate, ethanethiol, or CH_3OH substituted at the carbon that normally bore the hydroxyl group, and thus, α-ketol analogues were formed that contained either linoleoyloxy, oleoyloxy, ethylthio, or methoxy substituents, respectively. Similarly, γ-ketol analogues were produced by nucleophiles other than H_2O (4).

In this communication the stereochemistry of α-ketol formation is reported.

METHODS

Isomerase products

Products were formed by the sequential action of corn germ lipoxygenase (8) and corn germ LAHI (3). The enzymes were buffer-extracted from whole germ (16 g) dissected from air-dried field corn (*Zea mays*), and the supernatant obtained from centrifugation of the extract was prepared and used as described before (3). The germ extract converted linoleic acid (0.546 g) into the products, 9-linoleoyloxy-10-oxo-*cis*-12-octadecenoic acid and 10-oxo-9-hydroxy-*cis*-12-octadecenoic acid. The products were isolated by column chromatography (3).

Derivatives

Methyl 9,10-dihydroxystearates were synthesized from the LAHI products. The sample of 10-oxo-9-hydroxy-*cis*-12-octadecenoic acid was: (a) hydrogenated with H_2-Pd (10% Pd on carbon; Matheson, Coleman and Bell, Norwood, OH) in CH_3OH at 1 atm and 25 C for 45 min, (b) reduced with $NaBH_4$ in CH_3OH at 25 C for 30 min, and (c) esterified with diazomethane in ether-CH_3OH, 9:1. 9-Linoleoyloxy-10-oxo-*cis*-12-octadecenoic acid was: (a) hydrogenated as above for 75 min, (b) $NaBH_4$-reduced as above for 50 min, (c) deacylated with 0.1 N KOH in CH_3OH for 4.5 hr at 25 C, and (d) esterified with diazomethane as above.

Methyl 9,10-bis(trimethylsilyloxy)stearate was synthesized from methyl 9,10-dihydroxystearate with hexamethyldisilazane-chlorotrimethylsilane-pyridine, 2:1:1.

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

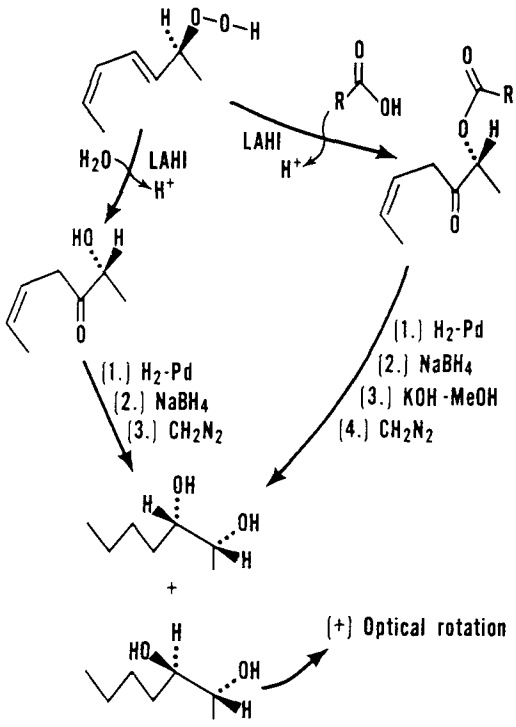

FIG. 1. Formation of products by linoleic acid hydroperoxide isomerase (LAHI) and the chemical transformations required to establish stereoconfiguration at carbon-9. Structures are abbreviated; the structure at the top represents 9-D(S)-hydroperoxy-*trans*-10, *cis*-12-octadecadienoic acid.

Chromatography and Spectral Methods

Syntheses of the methyl 9,10-dihydroxystearates from LAHI products resulted in a racemic mixture of *erythro* and *threo* isomers. This isomeric pair was separated by sodium arsenite-impregnated thin layer chromatography (TLC) plates (9), and by this method, the threo isomer was isolated from a 1-mm thick preparative plate.

IR spectra were recorded with a Perkin-Elmer Model 621 spectrometer using 10% solutions of sample in CCl_4 with a 0.1 mm NaCl cell.

Gas liquid chromatography-mass spectroscopy (GLC-MS) was used to analyze samples of methyl 9,10-bis(trimethylsilyloxy)stearate (10). The GLC column (1.8 m x 4 mm) was packed with 5% Apiezon L on Gas-Chrom Q. The column temperature was programmed from 190-250 C at 5 C/min.

Optical rotations were measured as reported before (8).

RESULTS AND DISCUSSION

Isomerase Products

Corn germ extracts oxidize linoleic acid to 10-oxo-9-hydroxy-*cis*-12-octadecenoic and 9-linoleoyloxy-10-oxo-*cis*-12-octadecenoic acids as shown previously (3). These products arise from a sequential reaction during which corn germ lipoxygenase oxidizes linoleic acid to an intermediate 9-D(S)-hydroperoxy-*trans*-10,*cis*-

TABLE I

Optical Rotations of Products Derived From the Action of Linoleic Acid Hydroperoxide Isomerase (LAHI)

Product	546.1 nm [α] (c:solvent)	Origin
10-Oxo-9-hydroxy-*cis*-12-octadecenoic acid	-4.7° (1.5:CH_3OH)	α-Ketol product of LAHI
Methyl 9,10-dihydroxystearate (mixture of erythro and threo isomers)	+8.7° (1.5:CH_3OH)	Synthesized from α-ketol
Methyl *threo*-9,10-dihydroxystearate (Partial arsenite complex)	+31.0° (0.7:CH_3OH)	From α-ketol; isolated from TLC; complexed with arsenite
Methyl *threo*-9,10-dihydroxystearate (Partial arsenite complex)	+45.4° (0.8:CH_3OH)	From acyl product[a]; isolated from TLC; complexed with arsenite
Methyl *threo*-9,10-dihydroxystearate	+13.6° (0.5:CH_3OH)	Synthesized from α-ketol; *threo* isomer isolated by TLC; arsenite removed with mannitol
Methyl *threo*-9,10-dihydroxystearate	+16.9° (0.9:CH_3OH)	Synthesized from acyl product[a]; *threo* isomer isolated by TLC; arsenite removed with mannitol
Methyl *threo*-9-L, 10-D-dihydroxystearate	+22.5° (1.2:CH_3OH)	See Morris and Crouchman (12)

[a]9-Linoleoyloxy-10-oxo-*cis*-12-octadecenoic acid.

12-octadecadienoic acid (8). Before it can accumulate, the hydroperoxide is converted by LAHI to 10-oxo-9-hydroxy-*cis*-12-octadecenoic and 9-linoleoyloxy-10-oxo-*cis*-12-octadecenoic acids. Because the substituent at carbon-9 is derived from certain nucleophiles present during the LAHI reaction (7), the 9-hydroxy product is derived from H_2O and the 9-linoleoyloxy product originates from the presence of linoleate. The sequential enzyme reaction affords the convenience of not having to isolate specific isomers of hydroperoxylinoleic acid nor accounting for possible racemization and rearrangement of an isolated hydroperoxide (11). The previous study (3) of the sequential enzyme oxidation of linoleic acid did not reveal the presence of 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid probably because 13-hydroperoxylinoleic acid did not form either by autoxidation or rearrangement of the 9-hydroperoxide.

In the present study, GLC-MS data from methyl 9,10-bis(trimethylsilyloxy)stearate derived from LAHI products did not show evidence for the corresponding isomer derived from 13-hydroperoxylinoleic acid [methyl 12,13-bis(trimethylsilyloxy)stearate]. The mass spectra showed the same fragmentation pattern as methyl 9,10-bis(trimethylsilyloxy)stearate prepared from authentic methyl *threo*-9,10-dihydroxystearate (Analabs, North Haven, CT). Fragment ions from cleavage between carbons-9 and -10 were most intense [215 m/e, 100% relative intensity (RI); 259 m/e, 83.7% RI]. Fragmentations characteristic of methyl 12,13-bis(trimethylsilyloxy)stearate were almost absent (173 m/e, 0.5% RI; 301 m/e, 0.2% RI); thus, only a trace of 12-oxo-13-hydroxy-*cis*-9-octadecenoic acid was present.

Stereoconfiguration

Since the two LAHI products, α-ketol and its fatty acyl ester, could be converted to a compound of known stereoconfiguration, i.e., methyl 9,10-dihydroxystearate (12), this derivative was synthesized (Fig. 1).

Because the 10-oxo was reduced, the resultant 10-hydroxy was optically racemic, Therefore, if the 9-hydroxy was stereochemically pure, the dihydroxystearates were a pair of diastereomers with *erythro* and *threo* configuration (Fig. 1). The *erythro* and *threo* isomers were isolated from sodium arsenite TLC plates; however, the isolates obtained from arsenite TLC plates were arsenite complexes which resisted dissociation by the alkaline hydrolysis method of Morris and Wharry (13). The complex was problematic because it could not be

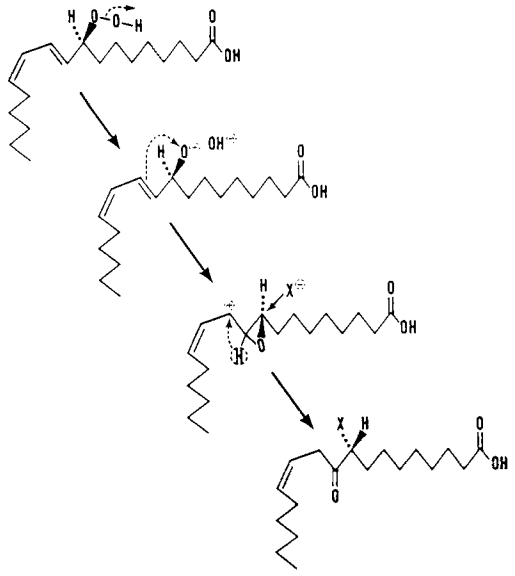

FIG. 2. Proposed mechanism for the action of linoleic acid hydroperoxide isomerase on 9-D(S)-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid. X^- represents a nucleophile.

used for optical rotations to compare with literature values. The arsenite complex gave higher optical rotation than the uncomplexed methyl *threo*-9,10-dihydroxystearate (Table I). Infrared (IR) spectroscopy was an effective method of assessing the presence of arsenite. For the complex, IR absorptions at 3420 cm^{-1} (OH) were absent and a strong absorption was noted at 655 cm^{-1} (probably arsenite).

The arsenite complex, partly dissociated by alkaline hydrolysis, was completely decomposed by an acidic mannitol solution. The sample was dissolved in 4 ml CH_3OH-H_2O, 1:1, containing 0.3 g mannitol. Two drops 1 N HCl were added, and the solution was kept at 25 C for 90 min, after which 4 ml $CHCl_3$ was added. The $CHCl_3$ layer was collected and given two H_2O washes. As a precaution to remove trace impurities, the sample was purified once again by a 0.5 mm thick TLC plate spread with Silica Gel G (devoid of sodium arsenite). The TLC plates were developed twice with $CHCl_3$-CH_3OH, 98:2, and TLC scrapings of the separated band were eluted with $CHCl_3$-CH_3OH, 2:1. Water was added to the eluted solution so that the final solvent composition was $CHCl_3$-CH_3OH-H_2O, 2:1:1. The $CHCl_3$ layer was collected to obtain pure methyl *threo*-9,10-dihydroxystearate which gave an R_f value by sodium arsenite TLC comparable with an authentic standard.

According to Morris and Crouchman (12), a

positive rotation is observed for the methyl 9-L,10-D-dihydroxystearate (*threo*) [α]546.1 nm = +22.5°; whereas, 9-D,10-L (*threo*) and 9-D,10-D (*erythro*) gave negative values, [α] 546.1 nm = -20.6° and -0.12°, respectively. In this study, the *threo* isomer was selected for measurement because it has a relatively large rotation at 546.1 nm. As shown in Table I, the methyl *threo* 9,10-dihydroxystearates were 9-L,10-D [+] regardless of which LAHI product served as a precursor. Thus, LAHI catalyzed the inversion of stereoconfiguration at carbon-9 from D(S) to L(R) regardless of whether OH^- (H_2O) or linoleate served as the nucleophile.

The inversion of stereoconfiguration is typical of a bimolecular nucleophilic substitution (S_N2). This finding and others suggest the heterolytic mechanism proposed in Figure 2. An epoxy-cation could serve as a transient intermediate which accepts a nucleophile (X^-) at carbon-9, as well as transfers hydride from carbon-10 to carbon-11. The proposed hydride shift from an epoxide carbon to a vicinal carbon is similar to the "NIH Shift" observed in many biological reactions (14). The mechanism is consistent with: (a) the observed products, (b) transfer of hydroperoxy oxygen from carbon-9 to carbon-10 (5,6), and (c) substitution by nucleophiles (7). Veldink et al. (2) already had anticipated the possible involvement of an epoxy intermediate. However, they discovered that isomeric 12,13-epoxyoleic acids were unreactive with flaxseed LAHI. The epoxides tested by them were uncharged; whereas, the proposal illustrated by Figure 2 requires a cationic species as an intermediate.

The γ-ketol also could be formed by an analogous mechanism. If the carbon-11 cation distributed its charge over carbons-11 to -13 to form an allylic hybrid, the nucleophile could substitute at carbon-13 and hydride could transfer from carbon-10 to carbon-9.

Presumably the substitution is enzymically mediated; however, an enzyme is not a prerequisite for retention of optical activity. A nonenzymic S_N2 reaction also could result in inversion and retention of optical activity. Since the values of rotation found in this study were lower than those reported by Morris and Crouchman (see Table I), one could argue for a nonenzymic reaction. However, the lower rotation values may be due to possible isomerization of the α-ketol into an enediol form as suggested by Zimmerman and Vick (15).

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Oxidative Activation of Guanylate Cyclase by Prostaglandin Endoperoxides and Fatty Acid Hydroperoxides[1,2]

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ABSTRACT

Purified prostaglandin endoperoxides (PGG_2 and PGH_2) and hydroperoxides (15-OOH-PGE_2) as well as fatty acid hydroperoxides (12-OOH-20:4, 15-OOH-20:4 and 13-OOH-18:2) were examined as effectors of soluble splenic cell guanylate cyclase activity. The procedures employed for the preparation and purification of these components circumvented the use of diethyl ether which obscured effects of lipid effectors because of contaminants presumed to be ether peroxides which were stimulatory to the cyclase. Addition of prostaglandin endoperoxides or fatty acid hydroperoxides to the reaction mixture led to a time-dependent activation of guanylate cyclase activity; 2.5- to 5-fold stimulation was seen during the first 6 min. The degree of stimulation and rate of activation were dependent on the concentration of the fatty acid effector; when initial velocities (6 min) were assessed, half maximal stimulation was achieved in the range of 2 to 3 μM. However, by extending the incubation time to 90 min, similar maximal increases in specific activity could be achieved with 3 or 10 μM PGG_2 or PGH_2. Activation of guanylate cyclase upon addition of prostaglandin endoperoxides or fatty acid hydroperoxides was prevented or reversed by the thiol reductants dithiothreitol (3 to 5 mM) or gluthathione (10 to 15 mM). $Na_2S_2O_4$, not known as an effective reducing agent of disulfieds, prevented but was relatively ineffective in reversing activation after it had been induced by PGG_2. Pretreatment of the enzyme preparation with increasing concentrations of N-ethyl-maleimide in the range of 0.01 to 1.0 mM prevented activation by PGG_2 without effecting basal guanylate cyclase activity. These observations indicate that fatty acid hydroperoxides and prostaglandin endoperoxides promote activation of the cyclase by oxidation of enzyme-related thiol functions. In contrast, PGE_2, $PGF_{2\alpha}$, hydroxy fatty acids (13-OH-18:2, 12-OH-20:4) as well as saturated (18:0), monoenoic (18:1), dienoic (18:2), and tetraenoic (20:4) fatty acids were ineffective in promoting cyclase activation in the range of 1 to 10 μM. Studies to identify the species of the rapidly metabolized prostaglandin endoperoxides that serve as effectors of the cyclase indicated that PGG_2 but not 15-OOH-PGE_2 (the major buffer-rearrangement product of PGG_2) is most likely an activator. In the case of PGH_2, a rapidly generated (30 sec) metabolite of PGH_2 was found which contained a hydroperoxy or endoperoxy functional group and was equally as effective as PGH_2 as an apparent activator of the enzyme. The combined effects of PGG_2 and dehydroascorbic acid, another class of activator, exhibited additivity with respect to the rate at which the time-dependent activation was induced. These results suggest that activation of soluble guanylate cyclase from splenic cells can be achieved by the oxidation of sulfhydryls that may be associated with specific hydrophobic sites of the enzyme or a related regulatory component.

INTRODUCTION

Cyclic GMP has been implicated as a biological regulatory component in the expression of a number of hormone actions, in mitogen action, and in a variety of altered physiological states. The evidence in support of an involvement of cGMP in these processes is indirect, deriving primarily from two lines of investigation: a) that the action of these agents or conditions is associated with enhanced cellular accumulation of this cyclic nucelotide, and b) that exogenous addition of cyclic GMP or derivatives (e.g., 8-bromo or dibutyryl) can mimic some of the cellular actions of these agents and conditions. Although this seems to coincide with the sort of evidence that has been used to define the biological importance of cyclic AMP as a mediator of hormone action and underscores

[1]This report is an abbreviated form of a manuscript to be published in the Journal of Biological Chemistry, (1978), (viz. Ref. 12).

[2]Abbreviations used in the text: PGG_2 = 15-hydroperoxy-9,11-peroxido-prosta-5,13-dienoic acid (prostaglandin G_2); PGH_2 = 15-hydroxy-9,11-peroxido-prosta-5,13-dienoic acid (prostaglandin H_2); 15-OOH-PGE_2 = 11-hydroxy-15-hydroperoxy-9-oxo-prosta-5,13-dienoic acid; PGE_2 = 11,15-dihydroxy-9-oxo-prosta-5,13-dienoic acid (prostaglandin E_2); $PGF_{2\alpha}$ = 9,11,15-trihydroxy-prosta-5,13-dienoic acid (prostaglandin $F_{2\alpha}$); 13-OOH-18:2 = 13-hydroperoxy-9,11-octadecadienoic acid; 13-OH-18:2 = 13-hydroxy-9,11-octadecadienoic acid; 15-OOH-20:4 = 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 15-OH-20:4 = 15-hydroxy-5,8,11,13-eicosatetraneoic acid; 12-OOH-20:4 = 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-OH-20:4 = 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 18:0 = octadecanoic acid (stearic acid); 18:1 = 9-octadecenoic acid (oleic acid); 18:2 = 9,12-octadecadienoic acid (linoleic acid); 20:4 = 5,8,11,14 eicosatetraenoic acid (arachidonic acid); ETY = 5,8,11,14 eicosatetraynoic acid.

a parallelism between cyclic GMP and cyclic AMP, it is becoming increasingly apparent that the differences between the two cyclic nucleotide systems are more striking than their similarities. Further, in spite of the fact that each cyclic nucleotide system is comprised of analogous metabolic components (e.g., cyclases, phosphodiesterases, and protein kinases) that catalyze seemingly similar metabolic steps, some of the evidence emerging indicates that they may be interposed at different sites in very dissimilar biological communication and regulatory systems.

How cellular guanylate cyclase-catalyzed generation of cyclic GMP is regulated remains a key process to be defined in elucidating the role played by the cyclic GMP system in biological communication and regulation. Guanylate cyclase and adenylate cyclase catalyze seemingly identical reactions with the appropriate nucleoside triphosphates, but the two cyclases exhibit strikingly different characteristics (1). Adenylate cyclase is located almost exclusively in cell membranes and can be stimulated in cell-free systems by membrane-active hormonal agents (i.e., epinephrine, glucagon, etc.) which promote cyclic AMP accumulation in the intact cell. On the other hand, guanylate cyclase is distributed in both soluble and particulate subcellular fractions and is not stimulated by agents (a number of which are thought to be membrane-active) known to promote accumulation of this cyclic nucleotide in the intact cell (1). This apparent hormonal insensitivity of guanylate cyclase in cell-free systems suggests that the regulation of this enzyme is accomplished indirectly and/or that a greater degree of cell integrity is necessary to transmit regulatory signals to guanylate cyclase. Intermediate components which arise as a result of hormone interaction at the cell plasma membrane may, therefore, be interposed between the hormone receptor and guanylate cyclase.

Several cellular constituents which could conceivably participate in an indirect mechanism of control have recently been reported to support or to alter guanylate cyclase activities from various sources. These include Ca^{2+} (2,3), lysophosphatides (4), phospholipids (5), and fatty acids (6,7). A meaningful correlation has not, however, been made between changes in the intracellular concentrations of any of these cell constituents and alterations that occur in cellular cGMP concentration or guanylate cyclase activity.

We recently suggested that cellular events involving oxidation and reduction may represent a general mechanism for regulating guanylate cyclase activity and the metabolism of cGMP (8-12). This concept was developed from observations that oxidants increase and reducing agents decrease splenic cell cGMP concentrations. Some more direct evidence that guanylate cyclase may be activated by a process involving oxidation has appeared; the soluble enzymes from lung (2,13), platelets (6), and splenic cells (9,10) can undergo a spontaneous activation in room air which can be prevented or suppressed by dithiothreitol or a N_2 atmosphere. It has also been shown that substances which activate guanylate cyclase, such as NaN_3, NH_2OH, and nitroprusside (14,15), may generate oxidizing equivalents in the form of nitric oxide (14), which is a potent activator of the cyclase. It has been suggested that free radicals generated by carcinogenic nitrosamines may also stimulate the enzyme (16) and that hydroxyl free radicals are effective activators.

We have demonstrated that soluble guanylate cyclase from splenic cells can be activated by at least one naturally occurring oxidant, dehydroascorbic (DHA) acid. In these studies evidence was presented that the cyclase could be modulated by the interconversion of sulfhydryl groups to disulfides and vice-versa in the intact cell and with a soluble preparation of the enzyme employing the mild oxidant DHA or thiol reducing agents such as dithiothreitol or glutathione.

We have also reported that polyunsaturated fatty acids with n-6 structure selectively activated guanylate cyclase (6). Since fatty acid hydroperoxides and prostaglandin endoperoxides exhibit both hydrophobic and oxidant properties, these substances should be reasonably effective activators of this cyclase.

In this report we describe the effectiveness of fatty acid hydroperoxides and prostaglandin endoperoxides to stimulate the soluble form of guinea pig splenic cell guanylate cyclase and some of the characteristics of the activation inducible by these lipid components.

METHODS AND MATERIALS

Materials

ATP, GTP, cAMP, cGMP, dithiothreitol, bovine serum albumin, sodium borohydride, N-methyl-N-nitroso-p-toluene sulfonamide, N-ethylmaleimide, cysteine and glutathione were obtained from Sigma Chem. Co. (St. Louis, MO). Dehydroascorbic acid was purchased from ICN, (Plainview, NY) and Chemical Procurement Laboratories. Dehydroascorbic acid solutions were dissolved in distilled water immediately before use. Creatine phosphate and creatine phosphokinase were obtained

from Boehringer-Mannheim (Indianapolis, IN), 3-isobutyl-1-methylxanthine was from Aldrich Chem. Co. (Milwaukee, WI), RPMI 1640 media was from Grand Island Biological Co., $Na^{125}I$ was purchased from New England Nuclear (Boston, MA). Antibodies against cGMP were prepared in this laboratory from goats. Succinyl cGMP tyrosine methyl ester from Sigma was iodinated according to the method of Steiner et al. (17).

Guanylate Cyclase Preparation

Spleens were removed from decapitated 200-250 g male Hartley guinea pigs and homogenized in 10 ml of phosphate (100 mM) buffered saline, pH 7.4 (PBS). Connective tissue was removed by sedimentation and the red blood cells lysed by incubation for 10 min at 37 C in 10 mM Tris-HCl, pH 7.5, containing 0.83% ammonium chloride. All solutions were equilibrated with argon. The white cells were sedimented and washed 3 times by successive centrifugation and resuspension in PBS. The cells were finally suspended at a density of 200 x 10^6 cells per ml PBS and lysed by sonication (under argon or nitrogen). Cell disruption was determined to be ca. 95%. The cell lysate was centrifuged at 105,000 x g for 60 min to separate the soluble and particulate enzyme fractions. The soluble fraction was removed and maintained for no longer than 60 min at 4 C under argon or nitrogen until assayed.

Guanylate Cyclase Assay

Activity of the enzyme was measured by a modification of the method of Kimura and Murad (18). Unless otherwise noted, final concentrations of the components in the cyclase assay (30 μl) were 1 mM GTP, 2 mM $MnCl_2$, 5 mM 3-isobutyl-l-methylxanthine, 15 mM creatine phosphate, and 0.2 mg/ml of creatine phosphokinase in 50 mM Tris·HCl, pH 7.5. Fatty acids and prostaglandins tested as possible effectors of guanylate cyclase were added prior to addition of the reaction mixture and enzyme to reaction tubes which were stoppered and maintained on dry ice. Solvent, containing the lipid component, was evaporated under vacuum (10-20 sec) before addition of reaction mixture followed by the enzyme extract (10 μl containing 10-12 μg protein); addition of the reaction mixture preceded the enzyme by 5 sec. Appropriate volumes of the solvent, alone representative of those used to transfer the lipid effector to the reaction tube, were employed for control reactions. The cyclase reaction was conducted at 30 C for the times designated and terminated by the addition of 30 μl of 110 mM sodium acetate pH 4.0 containing 11 mM EDTA followed by heating at 90 C for 3 min. Blank reactions were represented by enzyme extract added to the sodium acetate/EDTA solution heated at 90 C for 3 min followed by the addition of the reaction mixture.

When the cyclase velocities were determined under anaerobic conditions, in addition to employing deaerated solutions equilibrated with nitrogen or argon as described above, the cyclase reactions were conducted in rubber stoppered tubes which were evacuated then gassed with nitrogen or argon. The reaction mixture and enzyme were added separately to these reaction vessels with a Hamilton syringe and samples withdrawn from stoppered vessels by syringe and transferred to tubes in which reactions were terminated as described above.

The protein concentration of the extract was determined by the method of Bradford (19) and adjusted to 1.0-1.2 mg per ml prior to assay. The cGMP formed was converted to the 2'-0-acetyl derivative (20) and assayed by radioimmuno-assay as previously described (6). The binding reaction (300 μl) was conducted in 50 mM sodium acetate, pH 4.0, containing 20 mM $CaCl_2$, 0.5 mg gamma globulin, 0.75 mg bovine serum albumin, 25,000 cpm of ^{125}I succinyl cGMP tyrosine methyl ester, and antibody sufficient to bind 40% of the total CPM added. Incubation at 4 C was terminated after 14 or more hr by ethanol precipitation and the precipitated antigen-antibody complexes counted in a gamma spectrometer. Standard curves were prepared in glass-distilled water and in the presence of all the reaction components (including boiled enzyme) to control for any possible interference by the reactants in the acetylation or binding reactions.

Isolation and Characterization of Fatty Acid and Prostaglandin Metabolites

A detailed description of the methods used for the isolation and purification of the fatty acid and prostaglandin components examined in these studies as well as the evidence obtained to document their chemical structure has been described elsewhere (12).

RESULTS

Soluble guinea pig splenic cell guanylate cyclyase, like the soluble form of the enzyme from lung (2,13) and platelets (6,21), undergoes a spontaneous increase in activity upon incubation of tissue extracts in air (Fig. 1). Spontaneous activation was suppressed when reactions were conducted in an argon atmosphere (and deaerated argon saturated reagent) (viz. Fig. 3). It has been suggested that this

spontaneous activation in air results from an oxidative process (6,13,21); in the case of the splenic cell enzyme, it has been reported to involve the oxidation of thiol functions of the enzyme or a closely associated component (9,10). This is consistent with the effect that the thiol reductant, dithiothreitol, has to prevent (Fig. 1) or to reverse (viz., Fig. 8) this activation of the splenic cell enzyme; 3 mM dithiothreitol was required to completely prevent activation while lower concentrations (i.e., 0.1 and 1.0 mM) were only partially effective (Fig. 1). Qualitatively similar results were obtained with glutathione, but the effective concentration range was 10 to 15 mM (viz., Fig. 2). In addition to preventing spontaneous activation, dithiothreitol, at the highest concentration (3 mM) employed, also suppressed basal activity. This inhibition is apparent from the decreased velocities during the early (i.e., 2 to 6 min) course of the reaction when the rates of cGMP production are linear with respect to time (Fig. 1) with or without dithiothreitol. Because of this apparent inhibition of basal activity by the thiol reductant, velocities obtained in the presence of thiol reducing agents could not be used as a valid representation of control rates to compare with those obtained in the presence of lipid peroxides with which reductants could obviously not be used. Such a comparison would overestimate any stimulatory action of a lipid effector. As demonstrated by White et al. (13), with the enzyme in soluble lung extracts, the reaction rates with the splenic cell cyclase at 30 C are relatively linear for the first 5 to 6 min before activation becomes apparent. The early linear portion of the reaction velocity

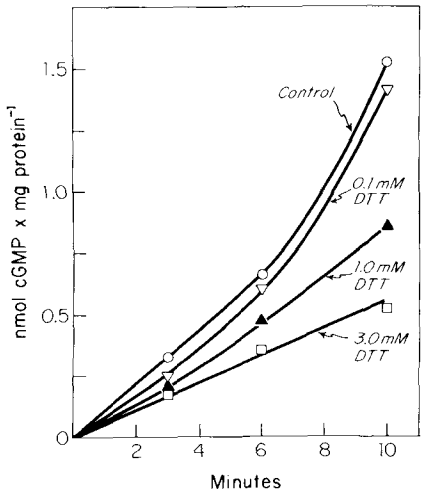

FIG. 1. Prevention of spontaneous activation in air of soluble splenic cell guanylate cyclase by increasing concentrations of dithiothreitol. The activity of the soluble fraction of guanylate cyclase from splenic cell lysates was assayed as described in METHODS in the absence or presence of dithiothreitol at the concentrations indicated. The values represent the mean of duplicate determinations.

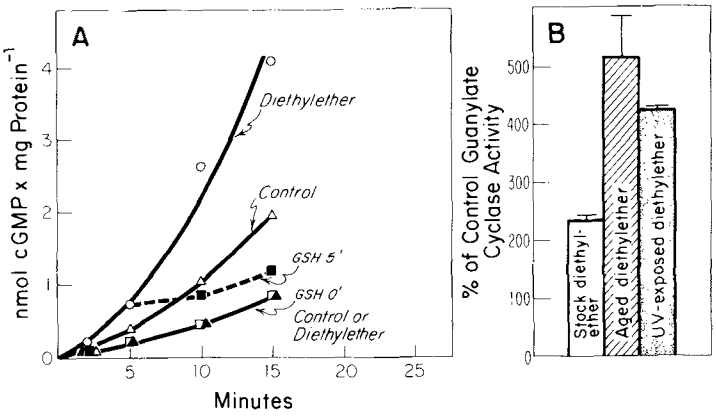

FIG. 2. Activation of guanylate cyclase by a nonvolatile component from diethyl ether. Panel A: Diethyl ether (60 μl) from a freshly opened can was evaporated under vacuum (-70 C) and guanylate cyclase reaction mixture without or with glutathione (GSH) (10 mM) followed by enzyme extract was added. In reactions not containing glutathione at the onset, the reducing agent (10 mM) was added where indicated 5 min (5') after initiation of the reaction and the cGMP formed thereafter was determined (dashed line). Panel B: Diethyl ether (60 μl) from either a freshly opened can (designated STOCK), after storage for 7 days at room temperature in a clear glass container (designated AGED) or after exposure to a short wave ultraviolet (Mineralite) hand lamp for 24 hr (designated UV-EXPOSED) was evaporated to dryness under vacuum. Guanylate cyclase reaction mixture was added followed by the addition of cell extract to initiate the reaction and the cGMP formed after 10 min determined as described in METHODS. Each value represents the mean of duplicate determinations; the range is indicated by the vertical bars.

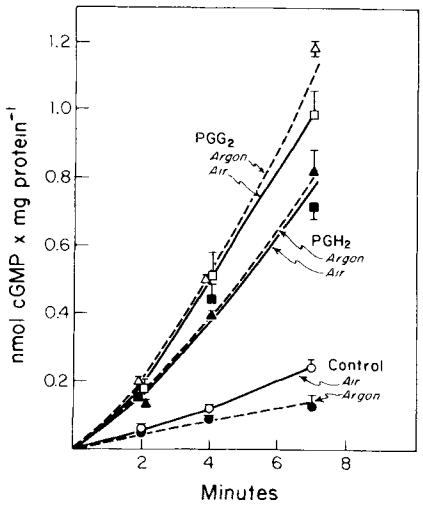

FIG. 3. Time course of PGG_2 and PGH_2 activation of soluble splenic cell guanylate cyclase in air or argon. The reactions were conducted as described in METHODS. The concentration of PGG_2 and PGH_2 was 10 μM. The values represent the means of duplicate determinations and the vertical bars represent the range.

(i.e., at or before 6 min) was, therefore, used when assessing alterations inducible by fatty acid derivatives in enzyme activity at a fixed time of reaction.

Another potential source of error that was identified during the initial phase of this work related to the conventional procedures used to extract, purify, and store fatty acid metabolites (22,23) in which diethyl ether is employed as the solvent. A visibly undetectable, apparently nonvolatile substance(s) remaining after evaporation of the solvent (i.e., diethyl ether), was found to be stimulatory to guanylate cyclase (Fig. 2). The residue after evaporation of aged (i.e., stored in clear glass container for seven days), ultraviolet-exposed (i.e., for 24 hr) of freshly opened diethyl ether all stimulated (200 to 500%) the cyclase (Fig. 2B). The stimulatory effect of the nonvolatile diethyl ether component could be prevented and reversed with glutathione (10 mM) (Fig. 2A) or dithiothreitol (3 mM) (not sown), indicating that the alteration in enzyme activity involved oxidation. Although the nonvolatile constituent remaining after evaporation of diethyl ether was not characterized, the properties exhibited correspond to those of nonvolatile ether peroxides which are readily formed from diethyl ether upon light exposure or prolonged storage (24). Because there was no assurance that diethyl ether employed for fatty acid metabolite purification, even if initially distilled over lithium aluminum hydride to remove ether peroxides, did not undergo subsequent oxidation due to unavoidable light exposure, or perhaps, as a result of ether radical formation initiated by the fatty acid hydroperoxides and endoperoxides, diethyl ether was eliminated as the solvent from all purification procedures. This necessitated the development of new procedures for extracting, purifying, and stabilizing the labile prostaglandin endoperoxides and the fatty acid hydroperoxides. The basic modification in the method devised was the use of a solvent comprised of ethyl acetate and purified petroleum ether, both of which are not known to form oxidation products. The basic solvent, which was a mixture of 20% ethyl acetate in petroleum ether, was determined in 20 separate experiments to have no significant (i.e., 117% ± 3.5) effect on guanylate cyclase velocity.

Because of earlier reports (6) that soluble guanylate cyclases (i.e., from platelets) can be stimulated by (n-6) polyunsaturated fatty acids, it was judged essential to eliminate endogenous lipid components (i.e., nonradiolabeled) and possible oxidized lipid products deriving from freshly prepared microsomes or acetone powder preparations of microsomes which are ordinarily used to generate the prostaglandin endoperoxides. The precaution taken to eliminate this potential source of error was to lipid deplete (25) the sheep vesicular gland microsomes used for the enzymic generation of PGG_2 and PGH_2. From 300 mg of microsomes prepared by this procedure (i.e., the amount used in a standard reaction mixture to generate prostaglandin endoperoxides), there was no lipid detectable upon thin layer chromatography of pooled extracts obtained from three successive extractions with chloroform-methanol (2:1). It was also determined that fractions from silicic acid chromatography corresponding to those containing PGG_2 and PGH_2 had no detectable effect on guanylate cyclase activity when extracts of lipid-depleted microsomes without added arachidonic acid were chromatographed (not shown).

Activation by Prostaglandin Endoperoxides and Fatty Acid Hydroperoxides

Micromolar concentrations of prostaglandin endoperoxides PGG_2 and PGH_2 were found to be effective activators of the soluble form of splenic cell guanylate cyclase (Fig. 3). Guanylate cyclase velocity was increased over four-fold upon the addition of PGG_2 (10 μM), and over three-fold when PGH_2 (10 μM) was added to the reaction. Activation was apparent with the inclusion of either prostaglandin endo-

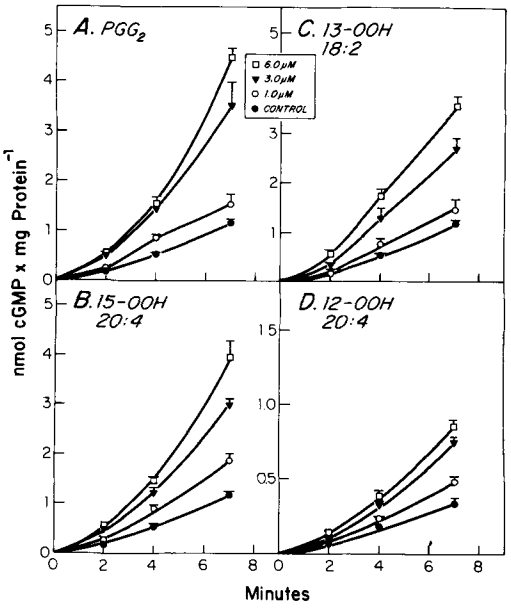

FIG. 4. Time course of the activation of soluble splenic cell guanylate cyclase by various concentrations of PGG_2, 13-OOH-18:2, 15-OOH-20:4 and 12-OOH-20:4. Conditions and procedures are described in METHODS. The values represent average of triplicate determinations±SEM.

peroxide when the reaction was conducted in room air or in an argon atmosphere with argon equilibrated reagent. Because spontaneous activation was suppressed in reactions conducted in argon, the relative increase in activity attributable to PGG_2 or PGH_2 under these conditions was slightly greater (Fig. 3). The stimulatory effectiveness of these prostaglandin endoperoxides in an argon atmosphere indicates that they are not merely accelerating the process by which the undefined spontaneous activation is promoted and that their stimulatory effect is not dependent upon the presence of molecular oxygen.

The fatty acid hydroperoxides, 15-OOH-20:4, 12-OOH-20:4 and 13-OOH-18:2 (Fig. 4B,C,D) also serve as effective enhancers of the cyclase activity; the stimulatory effect is concentration dependent with respect to the lipid effector. The time course of the reaction (in room air) in the presence of 1, 3, or 6 μM PGG_2 (Fig. 4A), or the fatty acid hydroperoxides (Fig. 4B,C,D), indicates that the stimulatory effect of these lipid metabolites is time-dependent. This progressive increase in enzyme activity is apparent with all concentrations of the activators tested. With the lowest concentration (1 μM), little or no increase in activity is detectable until some time between 2 and 4 min of the reaction. With higher concentrations of the fatty acid effectors [3 and 6 μM (Fig. 4) or 10 μM (Fig. 3)], activation is apparent at the earliest time (i.e., 2 min) examined and velocities become progressively greater with time. A more extensive examination of the characteristics of the time-dependent activation is shown in Figure 5 where the changes in guanylate cyclase specific activity are plotted with respect to time over a period of 90 min; this extended incubation period permitted apparent completion of the activation that occurred spontaneously or in the presence of PGG_2 or PGH_2. The specific activity increased over 3.5-fold from 163 to 600 pmole/min/mg protein as a result of spontaneous activation and ca. 8.5-fold to values between 1,300 to 1,500 pmole/min/mg protein in the presence of PGG_2 or PGH_2. The maximal increase in

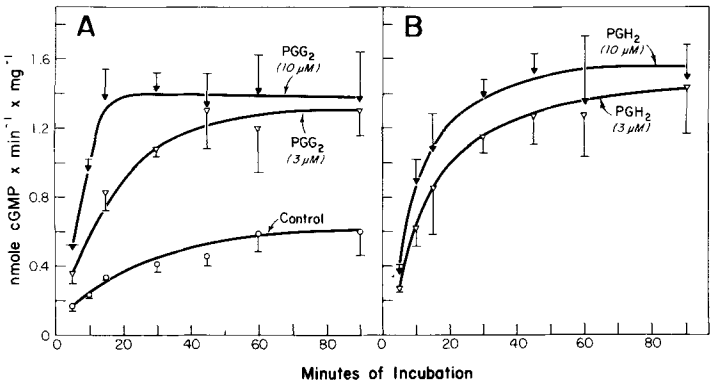

FIG. 5. Changes in guanylate cyclase specific activity during an extended incubation with different concentrations of PGG_2 and PGH_2. The enzyme reactions were conducted as described in METHODS in the presence or absence of 3 or 10 μM PGG_2 or PGH_2 as indicated and the mean specific activities during the time intervals shown determined. The values shown represent the means of quadruplicate determinations ± SEM.

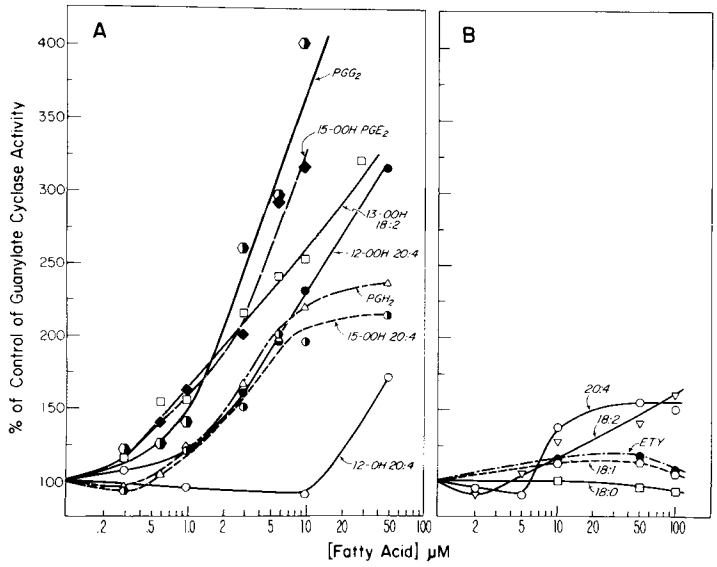

FIG. 6. Concentration dependence of the activation of soluble splenic cell guanylate cyclase by prostaglandin endoperoxides, fatty acid hydroperoxides and hydroxy fatty acids. cGMP generation after a 6 min incubation with the designated concentration of the lipid component was compared with that in control reactions containing equivalent volumes of the solvent which was evaporated before initiating the reaction as described in METHODS. The values represent averages of 2 to 10 determinations.

specific activity was comparable with either of the prostaglandin endoperoxides, but the time required for maximal activation in the presence of 10μM PGG_2 was considerably shorter (ca. 15 min) than with 10 μM PGH_2 (ca. 45 min). Furthermore, virtually the same maximum specific activity was achieved with 10 μM as with 3 μM concentrations of either prostaglandin endoperoxide, but the rate at which activation proceeded was faster with the higher concentration of each.

The concentration dependence of guanylate cyclase activation by the various fatty acid hydroperoxides and prostaglandin endoperoxides determined after a 6 min reaction in room air is shown in Fig. 6A. PGG_2 and 15-OOH-PGE_2 appear to be among the most potent of the components exhibiting stimulatory activity; half maximal activation is achieved at 2 to 3 μM and increases in activity of 300 to 400% at 10 μM under the experimental conditions employed. The hydroperoxy fatty acids, 13-OOH-18:2, 12-OOH-20:4, 15-OOH-20:4, and PGH_2 produced more than a doubling of activity at concentrations between 5 and 10 μM in these experiments when rates were determined at 6 min. The extent to which enzyme activity is enhanced by these stimulatory fatty acids varies among experiments and can range from 250% as shown here for PGH_2 and three of the hydroperoxy fatty acids, to 300 to 500% (viz., Figs. 3 and 4). However, under the conditions employed, the concentration dependence of the effect of the stimulatory lipids remains relatively constant between 2 and 3 μM for half maximal activation. Considering the time-dependent nature of the activation, additional complications of spontaneous activation and the presence of other components in the crude cell extract with which these effectors probably interact, these values should be considered only as first approximations of effective activator concentrations.

The relative ineffectiveness of 12-OH-20:4 compared to its hydroperoxy-containing counterpart (i.e., 12-OOH-20:4) is also apparent (Fig. 6A): the hydroxy fatty acid was ineffective until a concentration of 50 μM was achieved. Similarly, the 13-OH analogue of the stimulatory 13-OOH-18:2 was totally ineffective at concentrations of 1, 3 and 10 μM (not shown). As shown in Fig. 6B, 18:1, and 18:0 and 5,8,11,14-eicosatetraynoic acid (ETY) are relatively ineffective in the 1 to 10 μM range; only 20:4 and 18:2 produce any stimulatory effect, but maximum activation by these two fatty acids is no more than 50% at concentrations between 50 and 100 μM. The splenic cell soluble guanylate cyclase, therefore, differs from the soluble platelet enzyme with respect to eicosatetraenoic acid stimulability since the

TABLE I

Effect of Aqueous Rearrangement Products of PGG_2 and PGH_2 on Guanylate Cyclase Activity[a]

Prostaglandin endoperoxide (10 μM)	Time of rearrangement in buffer (min)	Guanylate cyclase activity (pmole cGMP/min/mg protein)
---	---	78 ± 0
PGG_2	0	344 ± 19
	3	322 ± 4
	5	286 ± 2
	10	370 ± 5
	15	330 ± 3
	30	332 ± 30
	60	343 ± 18
---	---	103 ± 6
PGH_2	0	259 ± 24
	2	205 ± 4
	5	188 ± 11
	10	131 ± 9
	30	123 ± 3

[a]Three hundred nmoles of PGG_2 or PGH_2 was incubated at 30 C for the times indicated in 1.8 μl of 50 mM Tris·HCl, pH 7.5. Guanylate cyclase reagent (20 μl) followed by soluble cell extract (10 μl) was then added and the cyclase reaction conducted for 6 min. The values represent the mean of triplicate determinations ± SEM.

TABLE II

Prevention and Reversal by Dithiothreitol of Guanylate Cyclase Activation in the Presence of PGH_2 or PG (R_f 0.71)

Additions	pmole cGMP/min/mg protein[a]		
	without DTT	DTT* 0 min	DTT[b] 5 min[c]
Control	62 ± 4	42 ± 7	38 ± 7
PG (R_f 0.71) 10 μM	199 ± 47	52 ± 12	68 ± 34
PGH_2 10 μM	183 ± 22	34 ± 20	60 ± 11

[a]Specific activity calculated during a 3 min interval representing the 5 to 8 min period of the incubation.

[b] Dithiothreitol concentration was 2 mM.

[c]Dithiothreitol was added at 5 min and the velocity during the subsequent 3 min period determined.

activity of the platelet enzyme is enhanced significantly by eicosatetraenoic acid at concentrations in the range of 1 to 10 μM (6). It is noteworthy that H_2O_2 at concentrations as high as 100 μM had no effect on splenic cell cyclase, while the more hydrophobic benzoyl peroxide stimulated activity 70% at a concentration as low as 2 μM and as much as 300% with increasing concentrations from 10 to 100 μM (not shown).

The Effector Species of Prostaglandin Endoperoxides

A characteristic of the prostaglandin endoperoxides PGG_2 and PGH_2 is their lability in aqueous solution (i.e., T½ of 4.5 to 5.5 min) (22,26). Both the PGG_2 and PGH_2 used in these experiments were found to undergo degradation in buffer with a half-time of 4 to 4.5 min determined by the loss of the characteristic biological action each has to promote aggregation of washed human platelets (not shown). To aid in establishing that the activation of guanylate cyclase observed with PGG_2 or PGH_2 added to the reaction mixture results from an action of the prostaglandin endoperoxides rather than from a product deriving from aqueous rearrangement, the effect of preincubating the endoperoxides in the reaction mixture before addition of the enzyme-containing extract was examined (Table I). The stimulatory effectiveness of PGH_2 disappeared (T½ of 5.5 min) upon incubation in the aqueous solution. The major aqueous rearrange-

ment product of PGH_2 was shown to be PGE_2 (12) in confirmation to previous reports (22); PGE_2 at concentrations up to 10μM has no effect on soluble splenic cell guanylate cyclase (Table II). In contrast to the loss of PGH_2 effectiveness upon its rearrangement in buffer, incubation of PGG_2 for as long as 60 min before addition of the enzyme did not result in any loss of stimulatory activity (Table I). The basis for the result was investigated by isolating the major products of PGG_2 rearrangement and examining their effect on guanylate cyclase activity. Four ^{14}C-labeled rearrangement products were detectable by thin layer chromatography (TLC) (12). Of these four components isolated by chromatography on silicic acid, one of the major products was found to be as potent as PGG_2 with regard to activating guanylate cyclase. This component was conclusively identified as 15-OOH PGE_2 (12). This agrees with the findings of Hamberg et al. (22) who reported 15-OOH PGE_2 to be the major product of PGG_2 degradation in buffer. The characteristics of the stimulatory action of 15-OOH-PGE_2 with respect to the time course of enhancing enzyme activity (time-dependent, progressive enhancement of activity) were found to be identical to those exhibited by PGG_2 (10 μM) (not shown); their relative potencies were comparable (viz., Fig. 6A). Guanylate cyclase stimulatory activity was also found with one of the minor components generated that migrated similarly to PGA_2; it was similar in potency to PGG_2. The component was not characterized. The demonstration that two of the hydrolytic rearrangment products of PGG_2 can stimulate the cyclase explains the persistence of stimulatory activity after PGG_2 degradation in aqueous solution and the presence of a hydroperoxy function on the major product formed (i.e., 15-OOH-PGE_2) provides a basis for explaining the stimulatory effectiveness of this component.

The possibility that the stimulation of guanylate cyclase activity seen upon addition of PGG_2 to the enzyme reaction mixture may derive from a rearrangement of PGG_2 to 15-OOH-PGE_2 (or other hydroperoxy-containing products) was further examined by determining the steady state level of 15-OOH-PGE_2 achieved in the cell extract-containing reaction mixture.

As shown in Figure 7 (Panels A-D), some of the products generated from PGG_2 upon incubation (1 and 6 min) in the cell extract-containing reaction mixture (Panels B and C) differ from those formed upon incubation in buffer alone (Fig. 7, Panel D). [The products obtained in buffer alone were identical to those found to be generated in reaction mixture devoid of enzyme (not shown)]. One of the marked differences in the products generated in the presence or absence of cell extract is that the 15-OOH-PGE_2 (R_f 0.39) is not detectable in the enzyme-containing reaction mixture at 1 or 6 min (Fig. 7B and C) or at 15, 30, or 120 sec (not shown). In the presence of the soluble cell extract, the major product generated (16% at 1 min and 32% at 6 min) chromatographed with authentic PGE_2. Also apparent is that PGG_2 is the major component present in the extract-containing reaction mixture at 1 and 6 min of incubation (64 and 34%, respectively) [i.e., accounted for by the chromatographic degradation product of PGG_2 plus undegraded PGG_2 (see Legend Fig. 7)]. These findings help to establish that activation of guanylate cyclase by PGG_2 is not due to the formation of 15-OOH-PGE_2. Also, since PGE_2, the major metabolic product, is ineffective as an activator in the 1 to 10μM concentration range, a reasonable conclusion is that PGG_2 probably serves as an activator of the cyclase. The possibility that other still unidentified metabolic products of PGG_2, which constitute no more than 35% of the total lipid present at 6 min, may be stimulatory to guanylate cyclase has, however, not been eliminated.

A similar assessment was made of the products generated from PGH_2 during incubations in reagent with and without cell extract. As shown in Fig. 7F-H, some additional components are generated from PGH_2 in the cell extract-containing reaction. It is also apparent that PGH_2 (R_f 0.60) undergoes a much more rapid metabolic conversion than PGG_2 in reaction mixture containing splenic cell extract; by 0.5 min, the small amount of PGH_2 remaining (ca. 10 to 15%) appeared as small shoulder of a major component generated with an R_f value of 0.71. The different chromatographic behavior of PGH_2 and this PGH_2-derived component could be established in two additional chromatographic systems (not shown). After 1 min (not shown), as in the example shown in 6 min (Fig. 7, Panel G), no detectable PGH_2 remained. It was pointed out above that the aqueous rearrangement products of PGH_2 are ineffective in stimulating guanylate cyclase (viz., Table I). This is consistent with the demonstration that the major product formed (40%) from PGH_2 in buffer is PGE_2 (Fig. 7H) and indicates that the minor product (19.3%) (shoulder of PGE_2 with R_f 0.43), which migrated identically to authentic PGD_2 in this system (not shown), is also not a stimulatory product of PGH_2. Therefore, the additional

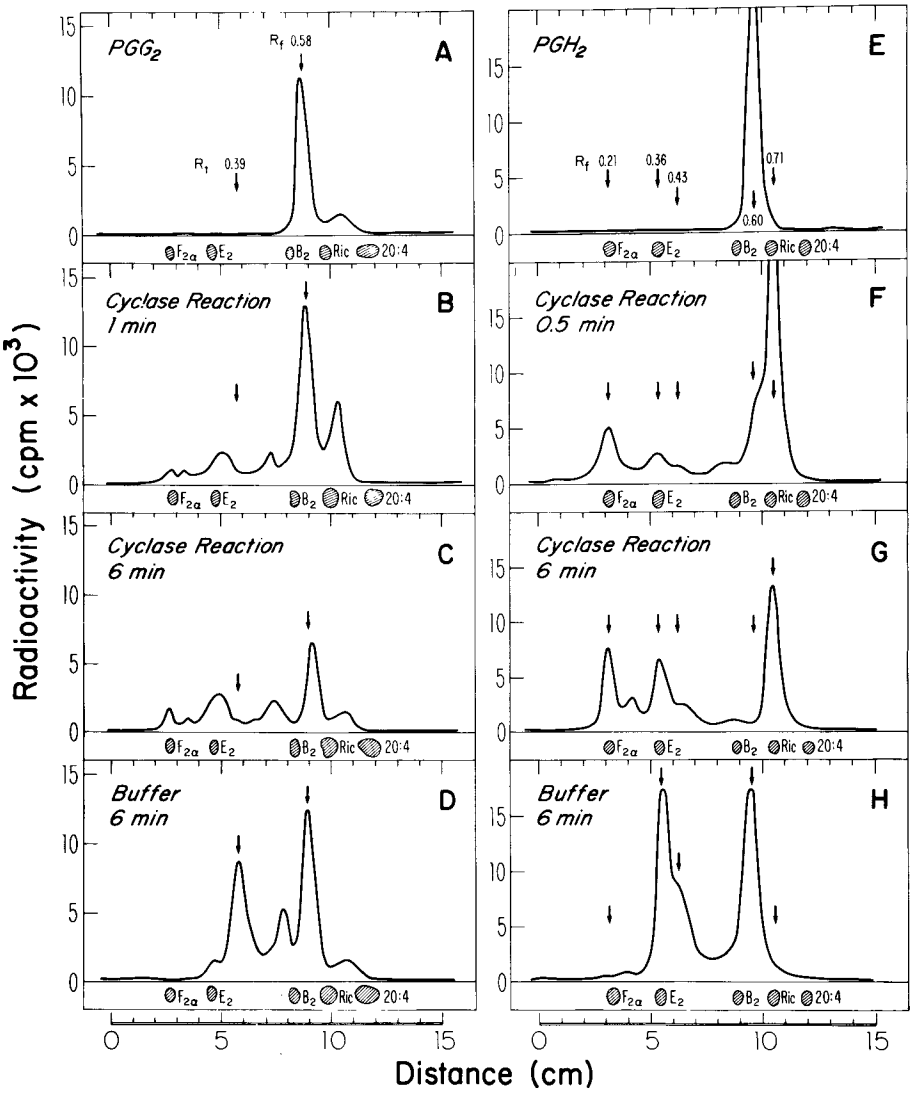

FIG. 7. Thin layer chromatographic analysis of organic extracts obtained from incubations of [1-^{14}C]PGG_2 and [1-^{14}C]PGH_2 with buffer or reaction mixture containing the soluble enzyme fraction from guinea pig splenic cells. Panels A and E: chromatography of unreacted PGG_2 (A) and PGH_2 (E). Panels B and C or F and G: 10 μM [1-^{14}C]PGG_2 (B,C) or 10 μM [1-^{14}C]PGH_2 (F,G) was incubated at 30 C with 0.75 ml of complete guanylate cyclase reaction mixture as described in METHODS containing 0.27 mg/ml of soluble enzyme fraction from guinea pig splenic cells. Panels D and H: reaction products isolated from incubation of 10 μM PGG_2 (D) or 10 μM PGH_2 (H) in 0.75 ml of 100 mM phosphate buffer, pH 7.4. Incubations were conducted for the times indicated. Reactions were terminated by addition of 3 ml of -20 C ethyl acetate-petroleum ether (1:1, v/v) followed by the addition of 2 ml 0.2 M citric acid. The combined extract of two sequential extractions resulted in an overall isotope recovery of 92.9 ± 5.8% (n=26) with 84.5 ± 8.1% distributed in the organic extract and 8.4 ± 0.5% in the aqueous fractions. Thin layer chromatography was carried out with a solvent system consisting of chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v). Chromatography (Panel A) in the above solvent system at room temperature caused a small but detectable degradation of PGG_2 which did not occur when chromatography was carried out at 4 C with iso-octane/petroleum ether/ethyl acetate/acetic acid (50:20:50:0.3, v/v) as the eluting solvent.

major products of PGH_2 formed in extract-containing media are represented by $PGF_{2\alpha}$ (22.6% at 6 min) and the component with R_f 0.71. Since $PGF_{2\alpha}$ was shown to be ineffective as a stimulator of the cyclase at a concentration of 10 μM (viz., Table III), the only remaining candidate of the major components generated, besides PGH_2, that might serve as an

TABLE III

Ineffectiveness of PGE_2 or $PGF_{2\alpha}$ to Activate or to Prevent Activation of Soluble Splenic Cell Guanylate Cyclase by PGG_2 or 15-OOH-PGE_2[a]

Prostaglandin μM		Guanylate cyclase activity pmol cGMP/min/mg prot.
PGG_2	$PGF_{2\alpha}$	
---	---	139 ± 16
4	6	311 ± 7
6	---	355 ± 7
---	10	140 ± 10
4	10	269 ± 15
6	10	340 ± 5
15-OOH-PGE_2	$PGF_{2\alpha}$	
---	---	67 ± 5
---	10	75 ± 3
10	---	291 ± 28
10	3	259 ± 4
10	10	242 ± 33
15-OOH-PGE_2	PGE_2	
---	---	67 ± 5
---	10	86 ± 6
10	---	291 ± 28
10	3	306 ± 2
10	10	319 ± 22

[a]PGE_2 or $PGF_{2\alpha}$ in absolute ethanol or absolute ethanol alone was transferred to a tube from which the ethanol was evaporated under vacuum and to which reagent and soluble enzyme extract was added. A 30 μl aliquot was immediately transferred to a tube with PGG_2 or 15-OOH-PGE_2 present or absent and the reaction conducted for 6 min at 30 C. The values represent the mean of the triplicate determinations ± SEM.

activator of the enzyme is the component with R_f 0.71 which represents 49% and 23.7% of the degraded PGH_2 at 0.5 and 6 min, respectively.

This PGH_2-derived component was isolated by silicic acid chromatography upon elution with 20% ethyl acetate in petroleum ether. When tested as a possible effector of guanylate cyclase activity, it was found that this metabolite at a concentration of 10 μM stimulated cyclase activity 3.2-fold (Table II) compared to 10 μM PGH_2, which enhanced enzyme activity 2.95-fold in this experiment. The activation induced by the PGH_2-derived metabolite (PG-R_f 0.71) as well as PGH_2 could be prevented and reversed by dithiothreitol (Table II). This rapidly generated metabolite of PGH_2 does not correspond to any metabolite of PGH_2 that has been reported to date (Fig. 7F and G). Although the characterization of this component has not been accomplished, one characteristic of it that has been uncovered is a positive reactivity with N,N-dimethyl-p-phenylenediamine, which indicates the presence of a peroxy functional group. This is a characteristic of all of the other fatty acid and prostaglandin metabolites which have been found to be stimulatory to soluble splenic cell guanylate cyclase.

The structural similarities of the prostaglandins E_2 and $F_{2\alpha}$ with PGG_2 and especially with 15-OOH PGE_2 prompted an examination of the effectiveness of these prostaglandins to serve as modifiers of guanylate cyclase activity, particularly with respect to an interaction with the enzyme that would interfere with PGG_2 of 15-OOH-PGE_2 promoted activation. It was found (Table III), as pointed out earlier, that neither $PGF_{2\alpha}$ or PGE_2 alone had any significant influence on the cyclase activity at concentrations of 3 or 10 μM or at concentrations as high as 50 μM (not shown). $PGF_{2\alpha}$ (10 μM) also did not interfere significantly with activation induced by PGG_2 (2 or 6 μM) or by 15-OOH-PGE_2 (10 μM), nor did PGE_2 (3 or 10 μM) diminish the stimulatory effectiveness of 15-OOH-PGE_2 (10 μM). The apparent lack of competition between these structurally similar substances with contrasting stimulatory effectiveness supports the conclusion that an oxidizing function (i.e., endoperoxy or hydroperoxy groups) is required for activation of this enzyme by fatty acid metabolites.

Oxidative Mechanisms of Guanylate Cyclase Activation

That the activation by these endoperoxy- and/or hydroperoxy-containing fatty acids derives from their oxidizing potential is strong-

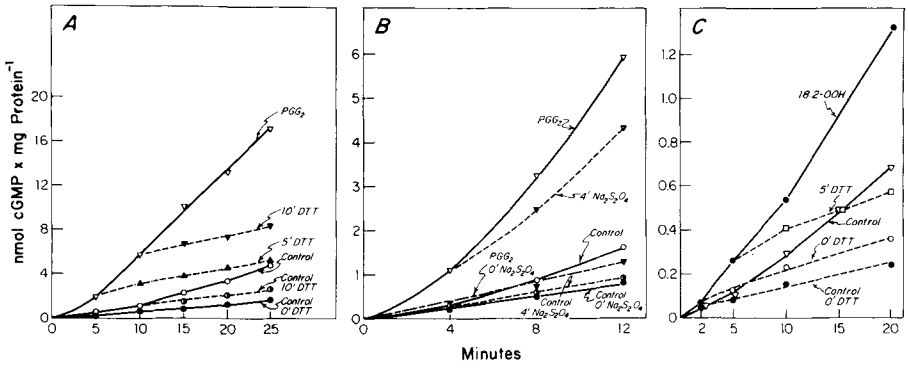

FIG. 8. Effectiveness of dithiothreitol and relative ineffectiveness of $Na_2S_2O_4$ to reverse activation of soluble guanylate cyclase promoted upon the addition of PGG_2 or 13-OOH-18:2. A: Soluble splenic cell guanylate cyclase was assayed in the presence or absence of 6 μM PGG_2. Dithiothreitol (3 mM) was present when the reaction was initiated (O') or added at 5 min (5') or 10 min (10') as indicated by the origin of the dashed lines which represent cGMP generation in the presence of the reducing agent. B: Same protocol as in Panel A except that 10 μM PGG_2 was employed and $Na_2S_2O_4$ (5 mM) was used as the reducing agent. C: Same protocol as a Panel A except that 10 μM 13-OOH-18:2 (18:2-OOH) and 1 mM dithiothreitol were used. The values shown are the means of triplicate determinations which did not differ by more than 10%. The differences in specific activity of guanylate cyclase in the three experiments reflects the variation ordinarily encountered with the enzyme activity from this source.

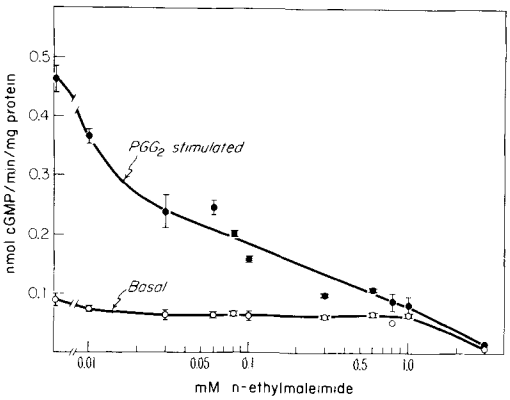

FIG. 9. Prevention of guanylate cyclase activity with PGG_2 upon pretreatment of soluble cell extract with N-ethylmaleimide. The cell extract was incubated for 5 min at 4 C with or without the concentration of N-ethylmaleimide indicated, and a 10 μl aliquot was then transferred to the cyclase reaction mixture as described in METHODS with or without 10 μM PGG_2. The guanylate cyclase reaction was conducted for 6 min at 30 C. The values represent the means of duplicate determinations, the vertical bars represent the range.

ly suggested by the demonstration that thiol reductants such as dithiothreitol or glutathione not only prevent, but also reverse, the activation they induce. In Fig. 8A and C the effect of dithiothreitol to prevent and to reverse the activation 5 or 10 min after it occurs in the presence of either PGG_2 or 13-OOH-18:2 is shown. Identical results were obtained with glutathione and with either of the reductants when activation is promoted in the presence of PGH_2, (viz., Table II) or 12-OOH-20:4 and 15-OOH-20:4, (not shown). In contrast to this action of thiol reductants to reverse cyclase activation, $Na_2S_2O_4$ was relatively ineffective in reversing PGG_2 activation once it has been induced (Fig. 8B). $Na_2S_2O_4$ could, however, prevent PGG_2 activation when present at the onset of the reaction. The contrasting results with these two classes of reducing agents with regard to reversing PGG_2-induced activation is consistent with the concept that sulfhydryl functions associated with a hydrophobic site on the enzyme (or related component) undergo oxidation promoted by the lipid oxidants. The effect that $Na_2S_2O_4$ and the thiol reductants have to prevent activation probably derives from actions both have to reduce the oxidizing component (27). The prevention of spontaneous activation by $Na_2S_2O_4$ and dithiothreitol (Fig. 8) would also be consistent with rapid removal (i.e., by chemical reduction) of the oxidizing species which is generated (e.g., spontaneously). However, the reduction of transition metals or heme iron that may be involved in the redox reactions underlying the spontaneous activation is also possible. The involvement of sulfhydryls in the activation of PGG_2 is also indicated by the effect that N-ethylmaleimide has to block activation by the prostaglandin endoperoxides (Fig. 9). Pretreatment of the soluble enzyme fraction with increasing concentrations of N-ethylmaleimide from 0.01 to 1.0 mM, which have little or no effect on basal guanylate cyclase activity, leads

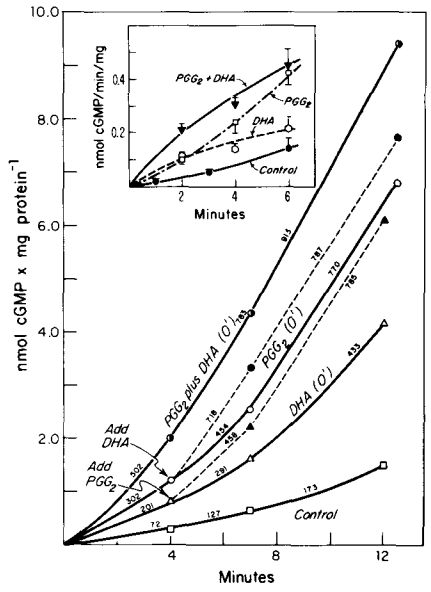

FIG. 10. Effect of PGG_2 and dehydroascorbic acid alone and in combination to activate soluble splenic cell guanylate cyclase. PGG_2 (6 μM) and/or dehydroascorbic acid (DHA) (5 mM) was present at the onset of the reaction (O'), or the second effector was added at 4 min (intercept of dashed line) to reactions which originally contained only PGG_2 or DHA. The values on the lines connecting each time point represent the average specific activities (pmole/min/mg) determined during that time interval. Inset: the changes in specific activity of soluble splenic cell guanylate cyclase plotted with respect to time of incubation. The reactions were conducted in the presence or absence of 15 μM PGG_2 and/or 5 mM dehydroascorbic acid.

to a progressive loss of PGG_2 stimulability.

The Possibility of Separate Hydrophobic and Hydrophilic Sites

It has been suggested that dehydroascorbic acid activates soluble guanylate cyclase from guinea pig splenic cells by an oxidative mechanism involving sulfhydryl-disulfide interconversion at a site distinct from the catalytic site (10). At least two classes of oxidants are, therefore, represented by the agents which have thus far been shown to serve as activators of this cyclase; the hydrophobic fatty acid hydroperoxides and endoperoxides, and the hydrophilic dehydroascorbic acid. The possibility that there are separate hydrophobic and hydrophilic ligand sites associated with the enzyme was investigated by determining if the effects of the two classes of oxidants exhibit additivity (Fig. 10). With saturating concentrations of dehydroascorbic acid (5 mM) and 6 μM PGG_2 added at the onset of the reaction, the velocity measured during the first 4 min (502 pmoles/min/mg) was equivalent to the sum of the rates

TABLE IV

Activability of Soluble Guanylate Cyclase by PGG_2 or Dehydroascorbic Acid with Mn^{2+}, Mg^{2+}, or Ca^{2+} as the Divalent Cation[a]

Additions	Enzyme activity (pmole cGMP/min/mg protein)	
A.[b]		+PGG_2
Ca^{2+}	2.3 ± 0.5	6.7 ± 0.6
Mg^{2+}	14.2 ± 1.3	88.3 ± 2.7
Mn^{2+}	76.2 ± 8.0	377.0 ± 13.0
B.[c]		+DHA
Ca^{2+}	0.5 ± 0.0	0.5 ± 0.1
Mg^{2+}	7.1 ± 0.2	13.2 ± 0.0
Mn^{2+}	45.0 ± 6.0	104.1 ± 3.8

[a]Each divalent cation was present at a concentration of 2 mM and the GTP concentration was 1 mM.
[b]Reactions were conducted for 6 min in the presence or absence of 10 μM PGG_2.
[c]A separate experiment in which reactions were conducted for 10 min in the presence or absence of 5 mM dehydroascorbic acid (DHA). Values are the means of duplicate determinations ± range.

at 4 min with dehydroascorbic acid (201 pmoles/min/mg) or PGG_2 (302 pmoles/min/mg) alone. In this experiment, PGG_2 and dehydroascorbic acid alone stimulated the control rate (72 pmole/min/mg prot.) at 4 min, 4.2- and 2.8-fold, respectively, while the activation produced by the two activators together was 7-fold by 4 min. The addition at 4 min of PGG_2 or dehydroascorbic acid to reactions initially exposed to only dehydroascorbic acid or PGG_2, respectively, promoted additional enhancement of enzyme specific activity measured at 7 min; the specific activity of the enzyme in the reaction originally containing only dehydroascorbic acid increased from 291 to 458 pmole/min/mg protein after addition of PGG_2, and the velocity of the PGG_2-containing reaction increased from 454 to 718 pmole/min/mg protein after addition of dehydroascorbic acid. In the inset of Fig. 10, the results of another experiment are shown in which the changes in specific activities of the cyclase are plotted with respect to the time of incubation with one or the other or a combination of the activators. In this experiment, a concentration of PGG_2 (15 μM) was employed which produced near maximal stimulation within 6 min [442 pmoles/min/mg (viz., Fig. 6)]. Even under these conditions, the specific activities measured during the early course (2 min) of the reaction were substantially greater than dehydroascorbic acid (5 mM) which was included with the prostaglandin endoperoxide. Although the complexities of the time-dependent enzyme activation preclude any unequiv-

ocal conclusions, these results suggest the presence of separate hydrophobic and hydrophilic regulatory sites. However, from the results obtained thus far, it can be concluded only that oxidation at these two proposed sites accelerates the rate at which the enzyme achieves a state of enhanced catalysis since it has not been determined if a greater specific activity is ultimately achieved with PGG_2 and dehydroascorbic acid together than with either alone.

Another indication that hydrophobic and hydrophilic oxidants may act at separate sites or at least promote distinctive alterations in the cyclase is the different kinetic behavior of the enzyme seen upon stimulation by the two classes of activator. It was found earlier (10) that activation of soluble splenic cell guanylate cyclase induced by dehydroascorbate or by the spontaneous process that occurs in air leads to greater measurable velocity when Mn^{2+} or Mg^{2+}, but not when Ca^{2+} is the divalent cation used to support activity (Table IV). Stimulation by PGG_2, however, leads to a greater velocity with Ca^{2+} as well as with Mg^{2+} or Mn^{2+} (Table IV), although Ca^{2+} supports only 1-3% and Mg^{2+} 10-20% of the activity achievable with optimal levels of Mn^{2+}. The degree of stimulation of PGG_2 at subsaturating concentrations of GTP (i.e., 0.1 mM with a fixed 2 mM excess of divalent cation) is comparable with Mn^{2+}, Mg^{2+}, or Ca^{2+} as the divalent cation. This suggests that the stimulatory effect of PGG_2 derives primarily from an alteration of the Vmax.

It was also found that 10 μM PGG_2 and PGH_2 have little, if any, effect (i.e., less than 25%) on the particulate fraction of splenic cell guanylate cyclase (not shown), whereas dehydroascorbic acid produces a significant activation of the particulate enzyme (10).

DISCUSSION

The results of the present experiments indicate that purified fatty acid metabolites with oxidizing functions represented by hydroperoxy or endoperoxy groups are effective stimulators of splenic cell guanylate cyclase activity. The results also indicate that activation of the cyclase by these lipid components involves oxidation of the enzyme or a closely associated regulatory component.

Of the fatty acid components tested, the fatty acid hydroperoxides represented by 12-OOH-20:4, 15-OOH-20:4, and 13-OOH-18:2, as well as the prostaglandin hydroperoxide, 15-OOH-PGE_2, activated the soluble splenic cell gluanylate cyclase 250 to 500%. Half maximal activation was achieved with concentrations of these lipid hydroperoxides in the 2 to 3 μM range. In contrast, the hydroxy-containing analogues of these fatty acids that were examined (e.g., 12-OH-20:4, 13-OH-18:2, of PGE_2 and $PGF_{2\alpha}$), as well as the fatty acids 18:0, 18:1, 18:2, and 20:4, were ineffective in a comparable concentration range (i.e., 1 to 10 μM). The relatively small stimulatory effect (ca. 15%) produced by 12-OH-20:4 and the fatty acids 20:4 and 18:2 at much higher concentrations (i.e., 50 to 100 μM) suggests that soluble splenic cell guanylate cyclase may be activable by a mechanism involving lipid interaction with hydrophobic enzyme sites. Fatty acid activation of guanylate cyclase has been demonstrated with the soluble enzyme from platelets (6,28), and the particulate enzyme from fibroblasts (7) and adipocytes (29). Some specificity with regard to the fatty acid activator is suggested even with the minimally effective fatty acids (i.e., 20:4, 18:2 and 12-OH-20:4) since monoenic (18:1) and saturated (18:0) fatty acids were totally ineffective even in the high concentration range tested. A high degree of specificity for lipid structural determinants was exhibited by the reduced form of the soluble platelet guanylate cyclase (i.e., assayed with dithiothreitol) which was stimulated by micromolar levels of polyunsaturated fatty acids with n-6 structure (6). These observations suggest the presence of hydrophobic sites on the enzyme from splenic cells and from other cell and tissue sources.

From what is presently known about the metabolism of hydroperoxy fatty acids, it is unlikely that metabolites other than the hydroxy fatty acids are generated in tissue extracts. Since the hydroxy fatty acid analogues of the hydroperoxy fatty acids were found to be relatively ineffective in the 1 to 10 μM range, it is reasonable to conclude that hydroperoxy fatty acids represent effective activators in the low micromolar range while the non-hydroperoxy-containing component may affect the enzyme activity less specifically at higher concentrations. It has been suggested (30,31) that fatty acid hydroperoxides may activate guanylate cyclase from studies in which a reaction mixture containing 20:4 or 18:2 and lipoxygenase led to activation of a partially purified soluble guanylate cyclase from platelets. The identity of the fatty acid products was, however, not established in these studies, but a correlation was made between peroxide equivalents present and the extent of cyclase activation (30).

Although both PGH_2 and PGG_2, when introduced into the reaction, appeared to be

half maximally effective as activators of the cyclase in the range of 2 to 3 μM, and both brought about as much as a 250 to 500% stimulation of the enzyme during the first 6 min of the incubation, it is not certain to what extent the parent compound and/or metabolites of these relatively rapidly metabolized components may have contributed to the activation seen. The apparent activation by PGG_2 or PGH_2 did not require molecular oxygen and the effects induced by both prostaglandin endoperoxides were reversible and preventable by thiol reductants. This indicates that the stimulatory species of prostaglandin, whether represented by the parent compound or metabolites generated in the reaction mixture, are effective by virtue of their oxidizing potential. Some evidence in favor of PGG_2 representing a species of prostaglandin that activates the cyclase is that: a) although 15-OOH-PGE_2 is a major chemical rearrangement product of PGG_2 formed in aqueous solution, 15-OOH-PGE_2 is not generated in the cell extract-containing reaction; b) the major product of PGG_2 metabolism in the extract is PGE_2 which is nonstimulatory; and c) 64% and 35% of the parent endoperoxide still remain at 1 and 6 min, respectively. It remains to be determined whether one or more of the presently unidentified components which together represent no more than 35% of the total constituents present in the PGG_2-containing reaction at 6 min may also be stimulatory to the enzyme.

Since PGG_2 contains both hydroperoxy and endoperoxy functional groups, its effectiveness as an activator would seem predictable from the stimulatory effect all hydroperoxy-containing fatty acids were found to have on the cyclase. What effectiveness to attribute to the endoperoxy function, which also possesses oxidizing potential, is not as clear from these studies. Although the addition of PGH_2 led to activation of the cyclase, it was found that this prostaglandin endoperoxide was virtually all metabolized by 60 sec in the reaction mixture containing cell extract. Although PGH_2 remains a likely candidate as an activator species, evidence was obtained that a metabolite of PGH_2 may represent an alternative or additional stimulator. PGE_2 and a component that comigrated with PGD_2 are two of the major metabolites of PGH_2 that were identified. Neither of these components could be considered effective metabolites because they are among the aqueous rearrangement products of PGH_2 which are all nonstimulatory. The unidentified component to which ca. 50% of the PGH_2 was converted by 30 sec was found to be stimulatory to the cyclase. This PGH_2-derived metabolite promoted enzyme activation that could be prevented or reversed by dithiothreitol which suggests that it contains an oxidizing function. The presence of an oxidizing group on this component was confirmed by the positive reactivity it was found to have with N,N-dimethyl-p-phenylenediamine.

The involvement of protein sulfhydryls and their conversion to disulfides as a result of an oxidation that appears to underlie the activation is also indicated by the relative ineffectiveness of $Na_2S_2O_4$ compared to the effectiveness of dithiothreitol to reverse activation. The inhibition of PGG_2-induced activation by pretreatment of the soluble enzyme preparation with the sulfhydryl reactive N-ethylmaleimide at concentrations of this reagent that did not affect basal enzyme activity also argues in favor of sulfhydryl-disulfide interconversion in the activation. The fact that PGE_2 and $PGF_{2\alpha}$, which were ineffective as activators, also did not interfere with PGG_2 or 15-OOH-PGE_2 activation indicates that the effectiveness of thiol reductants to reverse activation does not derive from the generation of reduction products (i.e., PGE_2 and $PGF_{2\alpha}$) which may serve as inhibitors of the enzyme. The apparent lack of competition between PGE_2 or $PGF_{2\alpha}$ and PGG_2 or 14-OOH PGE_2 also underscores the importance of an oxidizing function for interaction with enzyme (or enzyme complex), but the ineffectiveness of H_2O_2 to activate also emphasizes the hydrophobic requirement for the ligand. The lack of effect of H_2O_2 to activate soluble guanylate cyclase from liver (32) has already been reported, although it has been suggested that H_2O_2 may underlie the spontaneous activation in air of the soluble cyclase from lung (13).

It has recently been suggested (9) that oxidation of protein thiol functions is implicated in the activation of soluble splenic cell guanylate cyclase induced by another oxidant, dehydroascorbic acid. The evidence in support of that conclusion was similar to that obtained with the lipid oxidants, including the reversal of dehydroascorbic acid-promoted activation by thiol reductants and prevention of activation by N-ethylmaleimide. DeRubertis and Craven (16) have also recently demonstrated that the activation of soluble hepatic guanylate cyclase by the chemical carcinogen, N-methyl-N'-nitro-N-nitroguanidine (MNNG), is reversed by dithiothreitol and that the stimulatory effects of MNNG and nitroprusside on the soluble hepatic guanylate cyclase are prevented by N-ethylmaleimide. From these studies, DeRubertis and Craven proposed that stimulation of hepatic guanylate cyclase by these

activators may involve oxidation of tissue sulfhydryls (16). The process of oxidation as a mechanism of guanylate cyclase activation is also implied by studies showing that oxidants such as periodate (33) and a group of chemical agents represented by azide, nitroprusside, hydroxylamine, nitrite, hydrazine (2,14,34-39), and numerous N-nitroso-containing compounds (16,40,41) either elevate cellular cGMP levels and/or stimulate guanylate cyclase activity in a variety of cell-free systems. This latter group of agents are believed to form highly reactive nitroxides as a consequence of their cellular metabolism (40,14). Activation of guanylate cyclase by nitric oxide has indeed been demonstrated (14). The possibility that hydroxyl free radicals may represent a species of oxidizing equivalents within the cell that stimulate the cyclase under certain conditions has also been suggested (32). The fact that thiol reductants have been shown to produce an effect opposite to that of oxidants on cGMP metabolism in intact cells (i.e., lower cGMP steady state levels) and to bring about a corresponding, reversible suppression of the activity of guanylate cyclase from the same cells (9), lends credence to the possibility of an oxidative-reductive mechanism of modulating guanylate cyclase activity.

One characteristic of the lipid hydroperoxy- and/or endoperoxy-induced stimulation of soluble guanylate cyclase from splenic cell is that the activation is a time-dependent process. Although it is possible that one component of the time-dependence may derive from the rate at which effective metabolites are generated from the parent compounds, this is probably not the only or even the major contributing factor since the hydroperoxy fatty acids which probably do not generate secondary oxidizing metabolites and dehydroascorbate which was not found to undergo any detectable metabolic conversion (10), promote activation in a similar time-dependent manner. The rate at which activation occurs appears to increase with increasing concentrations of effector, but essentially the same maximal increases in specific activity can be achieved with 3 or 10 μM concentrations of either PGG_2 or PGH_2 if the incubation is extended for a time (60 to 90 min) to permit completion of the activation process. The demonstration that the rate of activation in the presence of near maximally effective concentrations of PGG_2 is accelerated when another class of oxidant such as dehydroascorbic acid is also included underscores the time-dependent nature of the activation process and also raises the possibility that sulfhydryls associated with separate hydrophobic and hydrophilic sites may undergo oxidation. The time-dependence of the activation process could be explained merely on the basis of time-dependent chemical oxidation of protein sulfhydryls at specific enzyme sites. It is equally likely that oxidation of the free protein thiols occurs relatively rapidly. If so, an initial alteration in the state of the enzyme or of a component in the enzyme complex could occur along with a corresponding increase in enzyme activity represented by the early stimulation seen. The progressively greater increase in activity could result from a secondary, time-dependent, conformational change that ultimately leads to a maximally activated enzyme state. The initial interaction with effector and degree of oxidation it may induce could determine the rate at which the secondary conformational change takes place.

Although the fatty acid and prostaglandin metabolites shown in this study to be effectors of the splenic cell guanylate cyclase are naturally occurring components (in contrast to chemical substances such as those cited above which may provide oxidizing equivalents through nitroxide radicals), the physiological significance of the activation that they produce remains obscure. PGG_2 and PGH_2 formation have been implicated with the enhanced accumulation of cyclic GMP levels that occurs when aggregation of human platelets is induced by collagen or thrombin (42,43, 44). Enhanced cellular accumulation of cGMP in epidermis has also been associated with markedly increased levels of 12-OH-20:4 (45) and it would be expected that correspondingly greater amounts of its precursor, 12-OOH-20:4, would be generated under such conditions. Since hormones and other cell membrane active substances which promote cellular accumulation of cyclic GMP are ineffective as activators of guanylate cyclase (1), other components which may be generated upon cell stimulation could be envisaged to serve as modulators of the enzyme. Although the compounds tested here or their metabolites are attractive candidates for coupling cell membrane-generated signals with the modulation of guanylate cyclase activity, it is not possible at this time to determine whether the stimulatory effects of the lipid components described here play such a role *in situ*.

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Enzymic Regulation of Arachidonate Metabolism in Brain Membrane Phosphoglycerides

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ABSTRACT

The metabolism of arachidonate in brain membrane phosphoglycerides was investigated in vivo by intracerebral injection of labeled arachidonate and by in vitro assay of enzymic systems associated with the metabolism. After intracerebral injection, labeled arachidonate was incorporated rapidly into brain phosphoglycerides with radioactivity distributed mainly in diacyl-*sn*-glycero-3-phosphoinositols (GPI) and diacyl-*sn*-glycero-3-phosphocholines (GPC). Some evidence of a metabolic relationship between diacyl-*sn*-glycerophosphoinositols (diacyl-GPI) and diacylglycerols was observed. Among the phosphoglycerides labeled with [^{14}C] arachidonoyl groups, diacyl-GPI were most rapidly metabolized in brain microsomal and synaptosomal fractions. The decay of diacyl-GPI in brain synaptosomes may be represented by two pools with half-lives of 5 hr and 5 days. Three types of enzymic systems related to metabolism of the polyunsaturated fatty acids in brain were investigated. The first system involves the cyclic events relating the ATP-dependent activation of polyunsaturated fatty acids (PUFA) to their acylCoA by the acylCoA ligase and subsequent hydrolysis of acylCoA to free fatty acids by the acylCoA hydrolase. It is apparent that fatty acid activation and hydrolysis is under stringent control in order to maintain suitable levels of free fatty acids and acylCoA in the brain tissue for various metabolic use. Factors involved in the regulation may include the level of ATP, divalent cations and the nature of substrates. The second enzymic system pertains to deacylation via phospholipase A_2 and reacylation via the acyltransferase of membrane phosphoglycerides. In brain tissue, activity of the acyl transferase is generally higher than that of the phospholipase A_2. Factors known to affect specificity of the acyltransferase include substrate concentration and the nature of the acyl groups and lysophosphoglycerides. The acyltransferase(s) in brain preferentially transfers arachidonate to 1-acyl-GPI. Activity of the acyltransferase can be inhibited by a number of lypophilic compounds including local anesthetics and cell surface agents. Activity of the phospholipase A_2 in brain may depend on the physical form of the substrates, i.e., whether the substrates are in monomeric or micellar form. The third process is associated with the degradation of diacyl-GPI by enzymes present in brain subcellular membranes. Incubation of brain subcellular membranes with 1-acyl-2-[^{14}C] arachidonoyl-GPI yielded labeled diacylglycerols and arachidonate. The phospholipase C action is specific for hydrolysis of diacyl-GPI. The arachidonate released from incubation of labeled diacyl-GPI may be the result of phospholipase A_2 action which is not specific for diacyl-GPI or the hydrolysis by lipase acting on the diacylglycerols formed from the phospholipase C activity. Enzymic hydrolysis of diacyl-GPI is most active in the microsomal fraction, but upon disruption of synaptosomes, enzyme in synaptic plasma membranes is also active in degradating this glycerophospholipid. In general, the results of in vitro studies are in good agreement with those observed in vivo and the information yielded has contributed towards understanding the metabolism of polyunsaturated fatty acids in brain subcellular membranes.

INTRODUCTION

The brain is highly enriched in polyunsaturated fatty acids (PUFA) such as 20:4(n-6), 22:4(n-6) and 22:6(n-3) (1,2). Since these fatty acids are largely esterified with the membrane phosphoglycerides, only trace amounts of the PUFA are present in the free acid form. A rapid increase in the free fatty acid (FFA) level has been shown in brain during ischemia and during stimulation by electro-convulsive shock (3,4). Under these conditions, the PUFA, such as arachidonate, were preferentially released. Apparently, fatty acid metabolism in brain is under strict regulation, and the FFA pool is maintained in a dynamic equilibrium with the phosphoglycerides. Consequently, a disturbance of the equilibrium due to factors affecting the metabolism may result in an accumulation of the FFA.

Among the major PUFA in brain, metabolism of arachidonate is of special interest because it is actively metabolized and also serves as a precursor for the prostaglandins synthesis. Although arachidonoyl groups are present in most brain membrane phosphoglycerides, they are highly enriched in diacyl-*sn*-glycerophosphoinositols in (diacyl-GPI) (5,6). Thus, the stearoyl-arachidonoyl-GPI species account for over 80% of the diacyl-GPI in brain (5). The functional implication of this highly specific acyl group profile of diacyl-GPI in the brain is not well understood. In thyroid tissue, diacyl-GPI is important in releasing the arachidonoyl group for prostaglandin biosynthesis (7,8). The release of arachidonoyl groups from diacyl-GPI was shown to be under regulation by

hormones from the gland. The mode of metabolism of arachidonate in brain phosphoglycerides has not been investigated. In this presentation, the metabolism of arachidonate in brain from results of in vivo and in vitro studies are described.

In Vivo Metabolism of Arachidonate in Brain: Uptake by Phosphoglycerides

Results from earlier studies have demonstrated the feasibility of investigating brain fatty acid metabolism by intracerebral injection of labeled fatty acids into brain tissue (9). When mice were injected intracerebrally with [1-^{14}C]arachidonate, radioactivity of the precursor was incorporated rapidly into the glycerolipids with a half-life around 5 min (10). Between one to 40 min after injection, different modes of arachidonate incorporation by individual glycerolipids were observed. A large portion of the label was distributed in the diacyl-glycerophosphocholines (diacyl-GPC) and diacyl-GPI. However, very little radioactivity was found incorporated into the diacyl glycerophosphoethanolamines (diacyl-GPE) and alkenylacyl-GPE during the initial hour after injection, although these phosphoglycerides also contain a large proportion of arachidonoyl groups. Labeled arachidonate was also incorporated into the diacylglycerols and triacylglycerols (10). Since the less polar glycerolipids are only present in trace levels in the brain (6), the active incorporation suggests that the diacylglycerols and triacylglycerols may also play a role in metabolizing the arachidonate in brain.

The uptake kinetics of [^{14}C]arachidonate and [^{3}H]stearate were compared by simultaneously injecting these two precursors into the brain tissue (11). Results indicated a more rapid uptake and incorporation of the labeled arachidonate into brain phosphoglycerides as compared to the labeled stearate. It is possible that arachidonate which is more unsatuarated than the stearate may be more rapidly activated to arachidonoylCoA by the acylCoA ligase. However, the rapid incorporation of the arachidonoyl groups into diacyl-GPI and diacyl-GPC can also be attributed to the presence of a direct acylation system preferential in transferring the arachidonate to these phosphoglycerides (12). After intracerebral injection of labeled fatty acids into rat brain, a selective synthesis of some molecular species of the glycerolipids was observed (13,14). The results indicated that both *de novo* synthesis and direct acylation are present in brain.

An in vivo study was conducted to examine the effect of carbamylacholine on brain glycerolipids metabolism (14). The stimulatory effect of cholinergic neurotransmitter substances on ^{32}Pi incorporation into phospholipids in nervous tissues is well recognized (16-20). However, a study of the carbamylcholine action in brain in vivo with labeled arachidonate should yield new findings relating the metabolism of phosphoglycerides with other lipid components. In this experiment, injection of 4 μg of carbamylcholine together with the labeled arachidonate resulted in a decrease in the incorporation of labeled fatty acid into the phospholipids (15). The decrease in phospholipid labeling, which was manifested mainly in diacyl-GPI and diacyl-GPC, was accompanied by an increase in the labeling of diacylglycerols and triacylglycerols. These events occurred initially in the synaptosomal fraction at 1 to 3 min after injection of labeled arachidonate and carbamylcholine, but the microsomal fraction also showed some changes during the later time periods. The decrease in arachidonate incorporation into brain phosphoglycerides during carbamylcholine stimulation was attributed to a dilution of the labeled precursor by the increased endogenous free fatty acid pool. Alternately, the decreased acylation may be due to an inhibition of the energy dependent activation of arachidonate to its acylCoA, since regulation of this activation process is likely to occur under different physiological conditions.

Turnover of Arachidonoyl Groups in Brain Membrane Phosphoglycerides

Since over 80% of the labeled arachidonate is esterified and incorporated into membrane phosphoglycerides by 1 hr after intracerebral injection, the pulse-like labeling of brain glycerolipids has proven to be a good model for studying membrane lipid turnover. In this study, mouse brain subcellular fractions were prepared at 1, 12, 24 hr and 3 and 8 days after intracerebral injections of [1-^{14}C]arachidonate (21). Analysis of the glycerolipids gave differences in the decay rate of individual phosphoglycerides depending on the subcellular fraction with which they were associated. Initially, radioactivity was mainly distributed in the microsomal and synaptosomal fractions, but the proportion of radioactivity in the myelin increased from 5 to 16% of the total within 8 days. In the microsomal fraction, the radioactivity of diacyl-GPC and diacyl-GPI declined rapidly between 1 and 24 hr while all other phosphoglycerides were still acquiring the label. The half-lives for diacyl-GPI in the microsomes were 14 hr and 7 days as compared to 12 hr

TABLE I

Effects of Ca^{++} and Mg^{++} on Incorporation of Labeled OleoylCoA into Diacyl-GPC, OleoylCoA Hydrolase Activity and Formation of a Complex with the Brain Microsomal Lipids[a]

Additions	Complex (unidentified)	Diacyl-GPC	OleoylCoA hydrolase	
			FFA	%
		cpm		
None	243	573	12391	100.0
Ca^{++}, 1 mM	3020	4830	7458	60.2
Ca^{++}, 2 mM	5270	3161	7078	57.1
Ca^{++}, 3 mM	7934	5101	6084	49.1
Mg^{++}, 1 mM	381	1234	10897	87.9
Mg^{++}, 2 mM	670	961	7600	61.3
Mg^{++}, 3 mM	640	1258	5605	45.2

[a]Mouse brain microsomes were incubated with [^{14}C]oleoylCoA (0.1 μCi, 10 μM), 0.31 M sucrose with 50 mM Tris-HCl (pH 7.4), and the cations specified. After incubation at 37 C for 10 min, reaction was terminated by adding 4 vol of chloroform/methanol (2:1, v/v). The lipid extract was separated by TLC using solvent system containing chloroform/methanol/15N NH_4OH (130:55:10, v/v). Three lipid areas were obtained for counting: 1. material remaining at the origin (this is considered as a complex since this material is formed only in the presence of calcium), 2. radioactivity of diacyl-GPC (representing the amount of endogenous 1-acyl-GPC in the microsomes); and 3. oleic acid formed from oleoylCoA hydrolase. The inhibition of oleoylCoA hydrolase activity by Ca^{++} and Mg^{++} is expressed as percent of control. (Data from Tang and Sun, unpublished).

and 14 days for diacyl-GPC. In the synaptosomes, only diacyl-GPI showed an initial rapid decline in radioactivity and the mode of diacyl-GPI decay was biphsaic with half-lives of 5 hr and 5 days.

Although similar studies have been conducted with various types of precursors to probe the turnover of brain membrane glycerolipids (22-24), the mode of metabolism of arachidonate among the different phosphoglycerides has not been investigated previously. The results obtained in this study indicated a more rapid metabolism of the arachidonoyl groups in diacyl-GPI as compared to those in other glycerophospholipids. The rapid diacyl-GPI metabolism is probably due to the presence of enzymes specific for metabolizing these glycerophospholipids.

In Vitro Investigation on the Metabolism of Polyunsaturated Fatty Acids in Brain Subcellular Membranes

AcylCoA hydrolases. (EC 3.1.2.2). The acylCoA hydrolases are responsible for hydrolysis of the acylCoA to free fatty acids and CoA. The free fatty acids formed may, in turn, be activated to their acylCoA by the ATP-dependent fatty acid-CoA ligase (EC 6.2.1.3). This cyclic event is probably highly regulated so that a critical level of free fatty acids and acylCoA may be maintained in the brain tissue for cellular metabolism. An active acylCoA ligase activity in brain has been implicated in in vivo studies since labeled fatty acids were rapidly activated and incorporated into the membrane phosphoglycerides (9,10). Activation of the fatty acids requires ATP, Mg^{++} and CoA, and these cofactors may be regulatory for the cyclic metabolism (25). Furthermore, specificity of the enzyme towards different fatty acid substrates is also a possible factor in the regulation. Brophy and Vance (26) reported that specific activity of the ligase in brain is higher with the 18-carbon fatty acids than with the 20-carbon ones. In this regard, it is reasonable to expect that the ligase may prefer the PUFA more than saturated acyl groups.

Brain tissue is especially high in acylCoA hydrolase(s) activity (27,28), implicating possible involvement in neuronal processes. The active acylCoA hydrolase(s) may account for the high susceptibility of brain tissue to free fatty acid accumulation during stimulation, ischemia and postmortem autolysis (3,4,15). AcylCoA hydrolases in brain are present in the soluble and particulate fractions (28). They seem to vary widely in physical properties and substrate specificities. The membrane-associated oleoylCoA hydrolase activity in brain is high in the mitochondrial and microsomal fractions (Sun et al., unpublished data). Nevertheless, all other subcellular membranes, such as myelin, synaptic vesicles and synaptic plasma membranes also exhibited a considerable amount of the hydrolase activity.

We have evidence that divalent cations such as Mg^{++} and Ca^{++} are inhibitors for the acyl-

CoA hydrolase(s) (Table I). Since the forward reaction for activation of fatty acids to their acylCoA requires ATP and Mg^{++} for activity, an inhibition of the hydrolase reaction by Mg^{++} would allow the cyclic event to favor synthesis of acylCoA. Calcium is known to play an important role in regulating neuronal functions. As shown in Table I, calcium also shows a tendency to form a complex with the acylCoA and membrane lipids (Tang and Sun, unpublished observation). This complex may further limit the availability of acylCoA for the acyl transfer reactions. Thus, Ca^{++} intervention in the fatty acid activation and hydrolysis process may be an important factor in regulating the overall membrane lipid metabolism in excitable tissues and may account for the rapid free fatty acid accumulation under the stimulated conditions.

AcylCoA:1-acyl-phosphoglycerides acyltransferase(s) (EC 2.3.1). The enzymic transfer of fatty acids from acylCoA to 1-acylphosphoglycerides is an important mechanism through which PUFA may be directed to specific membrane phosphoglycerides (29). The mechanism of the acyltransferase reaction is intriguing since this type of enzyme utilizes and must interact with two lipophilic molecules as substrates. It has been shown that the enzyme exhibits a high specificity towards the substrate molecules. Besides the chain length and degree of unsaturation of the acyl groups (30,31), the nature of the polar head groups on 1-acyl-phosphoglycerides is also important in determining the activity of the enzyme (32-34). Thus, arachidonoylCoA is preferably acylated to 1-acyl-GPI and 1-acyl-GPC by the acyltrans--ferase of brain microsomes (32). When labeled arachidonate was incubated with synaptosomes in the presence of ATP, CoA and Mg^{++}, the relative order for arachidonoyl transfer was 1-acyl-GPI>1-acyl-GPC>1-acyl-GPE (35). The ester linkage at the C-1 position of 1-acyl-phosphoglycerides is apparently needed for activity because little transfer activity was obtained when 1-alkenyl-GPE was used as the acceptor molecule for arachidonate transfer. The acyltransferase activity depends on the amount of lyso-substrate present in the tissue. In the liver, Holub (36) has shown that a high specificity for arachidonoyl transfer to 1-acyl-GPI occurred when the acceptor concentration was low. Supporting evidence for this type of specificity is the recent finding that the acyltransferase in brain microsomes is mediated by a "ping pong bi-bi" mechanism (37).

Although the acyltransferase is bound to cellular membranes, it is readily accessible to the substrates and does not require detergent for activity. In fact, most non-ionic detergents are potent inhibitors of the acyltransferase (34). The acyltransferase is also easily affected by lipophilic compounds and drugs which may interact with the cell membranes (38-41). We have recently shown that the activity of oleoylCoA:1-acyl-GPC acyltransferase in brain synaptosomes was inhibited by aliphatic alcohols (Sun et al., unpublished data). The inhibitory potency (I_{50}) correlates well with carbon chain length of the alcohols and their lipid solubility. The enzyme is also susceptible to inhibition by various types of lysocompounds which may be generated in the tissues as a result of phospholipase A action (34).

Different types of phospholipases are known to be present in brain subcellular membranes and different cell types (42-47); however, the activity is generally quite low compared to the acyltransferase activity. An increase in phospholipase activity has been shown during nerve degeneration (48) and in pathologic conditions such as during the acute stage of experimental allergic encephalomyelitis (EAE) (49,50). When lyso-compounds begin to accumulate in the brain due to an increase in phospholipase activity, it is possible that the acyltransferase activity may be inhibited, and their specificity in directing PUFA groups to the membrane phosphoglycerides may be altered. Furthermore, the PUFA released may give rise to an increase in the PUFA which, in turn, may affect prostaglandin synthesis. Evidently, a disturbance of the cyclic event mediating the deacylation and reacylation process involving membrane phosphoglycerides may lead to important consequences in altering cellular lipid metabolism in general.

Enzymic degradation of diacyl-GPI by brain subcellular membranes. Labeled diacyl-GPI (1-acyl-2-[^{14}C]arachidonoyl-GPI) were synthesized and incubated with different brain membrane preparations (51). When incubations were conducted in the presence of deoxycholate, labeled diacylglycerols were formed as the major metabolic products. The susceptibility of diacyl-GPI to hydrolysis by a membrane-bound enzyme (PI-C, EC 3.1.4.10) giving rise to diacylglycerols, inositol and cyclic inositol phosphates as products has been described (52). The phosphodiesterase acting on diacyl-GPI in ox brain was found in membrane-bound as well as in soluble forms (53). This type of enzyme is especially active in excitable membranes (54-56), and its activity is stimulated by Ca^{++} as well as by various muscarinic cholinergic compounds. Although the exact function of

TABLE II

Enzymic Degradation of Specifically Labeled Phosphoglycerides By Brain Synaptosomes[a]

Phosphoglycerides	Product	Time (min)			
		0	10	30	60
		%			
1-Acyl-2-[^{14}C]-arachidonoyl-GPI	DG	1.0	5.0	13.6	15.5
	FFA	2.8	3.4	5.7	6.8
1-Acyl-2-[^{14}C]-arachidonoyl-GPC	DG	0.4	0.4	0.5	0.5
	FFA	3.4	4.4	7.8	8.9
1-Acyl-2-[^{14}C]-oleoyl-GPC	DG	0.6	---	---	0.7
	FFA	0.2	---	---	4.6
1-Palmitoyl-2-[^{14}C]palmitoyl-GPC	DG	0.4	---	---	0.8
	FFA	0.6	---	---	2.3

[a]Labeled phosphoglycerides were incubated at 37 C with brain synaptosomes (suspended in 0.32 M sucrose with 50 mM Tris-HCl buffer, pH 7.4) and sodium deoxycholate (0.2 mg/ml) for the time period indicated. Reaction was terminated by adding 4 vol of chloroform/methanol (2:1, v/v) to the incubation mixture. The diacylglycerols (DG) and the free fatty acids (FFA) formed were determined by separating the lipid extract on TLC plates with a solvent system containing chloroform/methanol/15N NH_4OH (130:55:10, v/v). Results are expressed as percent of the labeled products formed with respect to different incubation time. Data are reproduced from reference 58.

this process in cellular metabolism is not yet defined, its role in cell surface receptor function has been suggested (57).

Besides the hydrolysis of diacyl-GPI leading to diacylglycerols and inositolphosphate, a small amount of labeled arachidonate was also released during incubation of diacyl-GPI with the brain membranes (51,58). The release of labeled arachidonate from diacyl-GPI may be the result of an active lipase which has been described in brain (59-62). However, under the condition for incubation, the release of labeled arachidonate is more likely to be due to the action of phospholipase A_2. Unlike the diacylglycerol formation, which is specific for diacyl-GPI, activity of phospholipase A_2 is observed with other glycerophospholipids (Table II). The data in Table II also indicate that the phospholipase A_2 prefers phosphoglycerides with unsaturated and polyunsaturated acyl groups. Among the subcellular membrane fractions, activities for diacyl-GPI degradation (for both diacylglycerols and FFA) were highest in the microsomal fraction (58). However, upon disruption of the synaptosomes, activities were found enriched in the synaptic plasma membranes. The presence of enzymes actively degrading diacyl-GPI in the synaptic plasma membranes suggests an apparent involvement of the processes in neuronal functions.

The presence of an enzymic system for degradation of diacyl-GPI in brain has been implicated in studies in vivo (12,21). Subsequent investigation with carbamylcholine stimulation further indicated a metabolic relationship between arachidonate incorporation into diacyl-GPI and diacylglycerols (15). Analysis of the acyl group composition of diacylglycerols in brain indicated a close resemblance in acyl group profile of diacylglycerols and diacyl-GPI (5,6,63). O'Brien and Geison (64) showed that over 70% of the molecular species of brain diacylglycerols corresponded to the steroyl-arachidonoyl species. The increase in diacylglycerols level during early brain ischemia (61) together with the increase in diacylglycerol labeling during carbamyl-choline stimulation (15) imply that the phospholipase C specific for diacyl-GPI may have an important effect in neuronal processes.

CONCLUSION

It is concluded that the polyunsaturated fatty acids in brain are metabolically active. In vivo experiments have shown that labeled arachidonate is rapidly activated to its acylCoA and subsequently incorporated into the glycerolipids, especially the diacyl-GPI and diacyl-GPC. In the first few minutes after injection, labeled arachidonate is also incorporated into diacylglycerols and triacylglycerols which, in turn, are available for biosynthesis of the phosphoglycerides via the de novo route. Upon stimulation by carbamylcholine, an increase in arachidonate incorporation into diacylglycerols and triacylglycerols with a decrease in labeling of diacyl-GPI was observed. Results are in agreement with the suggested metabolic relationship between diacyl-GPI and diacylglycerols based

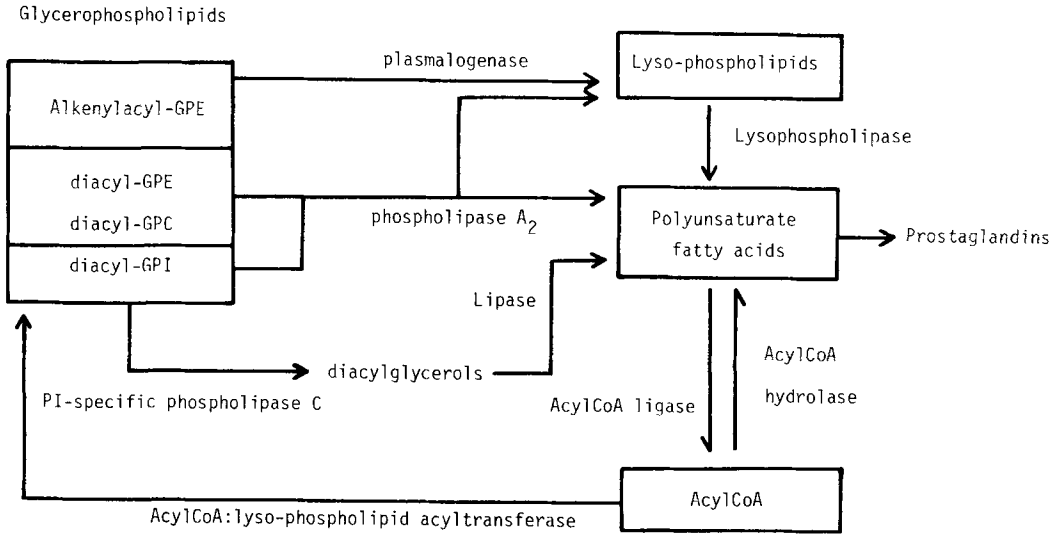

FIG. 1. A scheme showing the metabolic relationship between polyunsaturated fatty acids and membrane phospholipids in brain.

upon similarity of their acyl group profiles. Among the phosphoglycerides prelabeled with arachidonoyl groups, the diacyl-GPI in synaptosomes were metabolized most rapidly with half-lives of 5 hr and 5 days.

In vitro experiments have shown that two types of cyclic events are related to metabolism of brain fatty acids: 1. the ATP-dependent activation of fatty acids to their acylCoA via the acylCoA ligase and subsequent hydrolysis of acylCoA by acylCoA hydrolase(s), 2. the release of polyunsaturated fatty acids from membrane phosphoglycerides via phospholipase A_2 and subsequent reacylation of the lysophosphoglycerides by the acyltransferases. The polyunsaturated fatty acid level in brain may be regulated by enzymes responsible for both cyclic events (Fig. 1). Since activities of the acylCoA ligase and hydrolase in brain are higher than the phospholipase and acyltransferase, regulation by the first cyclic event may be more pertinent in maintaining a constant level of free fatty acids in brain. In this regard, the availability of ATP in the cells and divalent cation concentrations may be important in regulating fatty acid activation and hydrolysis.

Phospholipases A_2 are active in hydrolyzing arachidonate and other unsaturated and polyunsaturated acyl groups from membrane phosphoglycerides in brain tissue. Phospholipase A_2 activity could account for the rapid turnover of phosphoglycerides with polyunsaturated acyl groups. However, the rapid turnover of arachidonoyl groups in diacyl-GPI is attributed largely to the presence of an active phospholipase C specific for hydrolysis of this phospholipid giving rise to diacylglycerols. In the synaptosomes, activity of this enzyme is enriched in the synaptic plasma membrane fraction. The diacylglycerols formed may then be utilized for de novo synthesis of other phosphoglycerides or be further hydrolyzed to give arachidonate for prostaglandin biosynthesis (Fig. 1).

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Cardiac and Renal Lipases and Prostaglandin Biosynthesis

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ABSTRACT

The tissue phospholipids of isolated Krebs perfused rabbit hearts and kidneys can be efficiently labeled with [^{14}C]arachidonic acid. Subsequent stimulation of the prelabeled organ with hormones or ischemia results in release of [^{14}C] prostaglandins (PG). There is a highly efficient acylation mechanism existing in these perfused organs. Thus, tissue lipase activity can only be quantitatively assessed by measuring the [^{14}C]arachidonic acid in the venous effluent in the presence of albumin infusion which "traps" the released fatty acid. Two types of tissue lipases appear to exist. One is a highly specific phospholipase which is hormone sensitive, thus selectively liberates [^{14}C]arachidonate (but not [^{14}C]oleate, linoleate, and palmitate) from tissue lipids. The other nonspecifically responds to noxious stimuli such as ischemia and releases all fatty acids (e.g., including arachidonic and oleic acid) from tissue lipids. The hormone sensitive lipase appears to be predominantly localized in the vascular tissue (in hearts and kidney cortex), while nonspecific lipase activity is widely distributed in myocardial cells and renal tubules. Therefore, hormone stimulation elicits tightly coupled PG synthesis in the vasculature which possess both phospholipase and the cyclooxygenase while ischemia induced deacylation in the entire tissue, but the majority of the arachidonic acid released is not converted to PG due to a relative lack of cyclooxygenase activity in myocytes and renal tubules.

PHOSPHOLIPID LABELING TECHNIQUES AND HORMONE-INDUCED DEACYLATION

The synthesis and release of prostaglandins (PG) requires the activation of tissue lipases to release arachidonic acid (1). The released arachidonic acid is enzymatically converted by cyclooxygenase to the unstable endoperoxide intermediates PGG_2 and PGH_2 which, in turn, are metabolized to several PG end-products having varying biological activities.

To study the relationship between tissue phospholipase activation and prostaglandin synthesis in isolated perfused heart and kidney preparation, we have employed a technique which labels the precursor phospholipid pool (2-4). We found that these perfused organs efficiently (85-90%) incorporated infused [^{14}C]-arachdionate into tisue lipids, predominantly into phosphatidylcholine and phosphatidylethanolamine (4). Much less radioactivity was found in the neutral lipid and the free fatty acid fractions from the chloroform/methanol extract of the tissue homogenate (4). Hormone (bradykinin, angiotensin) stimulation of these prelabeled tissues resulted in the release of only [^{14}C]PGs without [^{14}C]arachidonic acid (3). The lack of detectable radioactive fatty acids in the venous effluent was presumably the result of a highly efficient reacylation mechanism in the tissue, which immediately reincorporated arachidonic acid that had not been converted to PGs into tissue lipids. We resorted to the use of fatty acid-free bovine serum albumin in the perfusate as a "trap" of free fatty acid (3). In the presence of albumin infusion, a substantial release of radioactive arachidonic acid was observed in response to hormone stimulation. Depending on the organ used, the arachidonate release varied from 3 to 10 times greater than the PG release for a given stimulus (3). Indomethacin infusion completely blocked the PG releasing response to bradykinin, but had no effect on the deacylation mechanism (3).

To investigate the specificity of the deacylation reaction, we prelabeled the heart with several radioactive long chain fatty acids. The infused radioactive arachidonate, linoleate, and oleate were all incorporated into tissue lipid with equal efficiency (Table I), predominantly in the 2 position of phosphatidylcholine (4). while [^{14}C]palmitate was esterified into both the 1 and 2 position (4). Bradykinin released comparable amounts of bioassayable PGs (300 ng) from hearts prelabeled with various radioactive fatty acids; however, only [^{14}C]arachidonate (928 CPM) (but not [^{14}C]linoleate, [^{14}C]oleate, or [^{14}C]palmitate) was liberated by bradykinin accompanying the PG release (Table I) (4). This selective bradykinin effect on fatty acid release suggested that hormone stimulation either activates a specific lipase that distinguishes different fatty acids in the 2 position or activates lipase which is selectively compartmented with phospholipids containing arachidonic acid. Ischemia, on the other hand, resulted in a nonselective activation of tissue lipases and released both [^{14}C]oleate as well as [^{14}C]arachidonate (Table I) (4). Furthermore, ischemia was less efficient than bradykinin in PG production in the heart. This is indicated by

TABLE I

Release of PGs and Free Fatty Acids by the Isolated, Perfused Rabbit Heart Prelabeled with Various ^{14}C-Fatty Acids[a]

[^{14}C] FA infused	Incorporation of [^{14}C] FA *into cardiac lipids* (% of total tissue lipids (n=3)			Release of PGs and FA after BK stimulation			Release of PGs and FA after ischemia		
	PL	NL	FA	Total [^{14}C] PG (cpm)	Bioassayable PG (ng)	[^{14}C] FA (cpm)	Total [^{14}C] PG (cpm)	Bioassayable PG (ng)	[^{14}C] FA (cpm)
[^{14}C] arachidonate	82 ± 8	14 ± 1	3 ± 1	928 ± 160 (n=9)	310	1755 ± 282	310 ± 55 (n=4)	60	1582 ± 128
[^{14}C] linoleate	73 ± 12	20 ± 8	5 ± 2	120 ± 95	300	-157[b] ± 136	---	---	---
[^{14}C] oleate	68 ± 6	21 ± 1	8 ± 2	-8 ± 31 (n=3)	350	-154[b] ± 161	32 ± 23 (n=5)	60	1575 ± 280
[^{14}C] palmitate	76 ± 9	17 ± 3	5 ± 2	-45 ± 53 (n=3)	330	-4[b] ± 105	---	---	---

[a]Release of radioactive PG and fatty acid was determined by collecting 80 ml of cardiac effluent (4 min) immediately before (baseline control) and after injection of 2 μg of bradykinin or 5 min of ischemia in the presence of bovine serum albumin infusion (75 mg/ml, 0.2 ml/min). The effluent was extracted, chromatographed in chloroform/methanol/acetic acid/water, 90:8:1:0.8, eluted and counted. Values of each PG and fatty acid were corrected for baseline control. Ischemia was produced by stopping the perfusion to the heart and diverting the perfusate to the bioassay organs. The effluent was collected for 4 min after reestablishing the cardiac perfusion. The incorporation of fatty acid into cardiac lipids was determined by extracting the tissue homogenates of hearts prelabeled with [^{14}C-arachidonate, [^{14}C] linoleate, [^{14}C] oleate and [^{14}C] palmitate with chloroform/methanol (2:1). The lipid extracted was chromatographed in the solvent system of chloroform/methanol/ammonia (65:35:5), and different lipid classes were scraped, counted and expressed as percentage of total counts on the plate. The following abbreviations are employed: PL, phospholipids; NL neutral lipids; FA, fatty acid; BK, bradykinin.

[b]The negative values of fatty acids are due to the declining basal level of the radioactive fatty acid release.

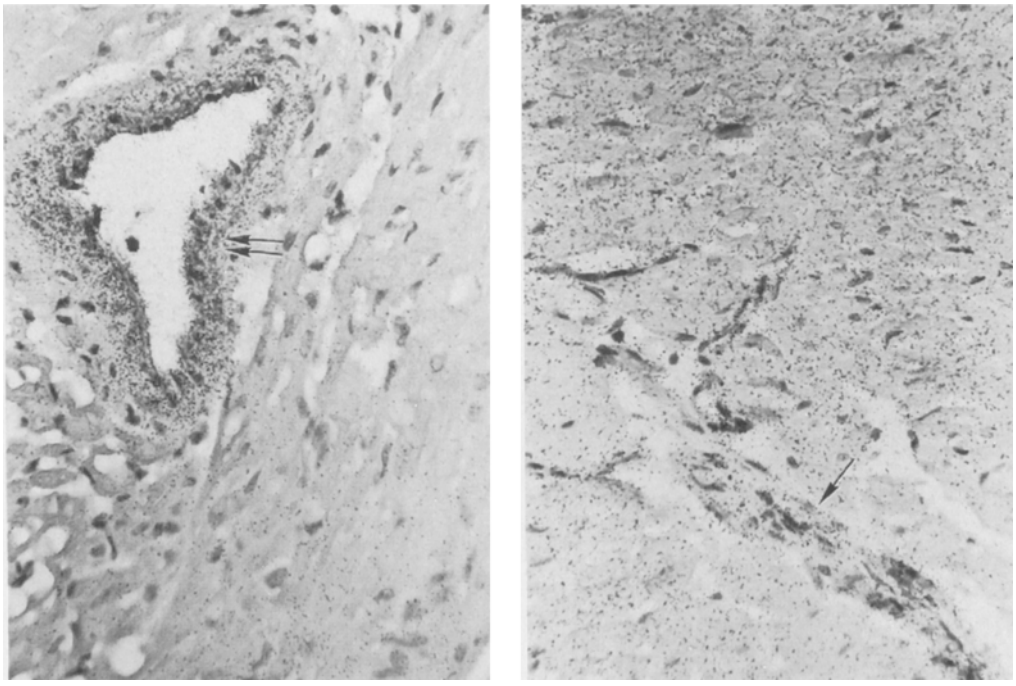

FIG. 1. Autoradiograms of hearts labeled with method A (infusion of [^{14}C]arachidonic acid in the absence of albumin) and method B (recirculation of [^{14}C]arachidonic acid with albumin). Method A resulted in selective labeling of arteries (double arrows) and arterioles (arrow) (the veins and venules are only weakly labeled), while method B resulted in a diffuse and uniform labeling of the entire tissue.

a large amount of arachidonic acid release with a concomitantly smaller amount of PGs than obtained with bradykinin stimulation (Table I). One explanation for this poor coupling between lipase ([^{14}C]arachidonic acid release) and cyclooxygenase ([^{14}C]PGs production) is that ischemia activates deacylation at sites remote from the PG synthesis complex, while bradykinin stimulates deacylation at sites close to or continguous with cyclooxygenase and is more tightly coupled with PG synthesis than is ischemia. Another possibility is that ischemia inhibits cyclooxygenase activity.

SITES OF LIPASE ACTIVATION AND PG SYNTHESIS

In an attempt to correlate the enzyme activity with its anatomical localization, we used two methods to label the tissue lipid with [^{14}C]arachidonic acid: slow influsion of [^{14}C]arachidonic acid into the perfused organ without albumin (method A), and recirculation of [^{14}C]arachidonic acid in Krebs solution containing albumin (method B) (5). Autoradiography of the labeled organs demonstrated that method A selectively labeled the vascular tissue, predominantly arteries and arterioles (method A), while method B nonselectively and uniformly labeled the entire tissue, including vasculature, myocardial cells and interstitium (Fig. 1) (5). Determination of [^{14}C]arachidonic acid and [^{14}C]PG as well as the bioassayable PG (total PG) in the venous effluent from prelabeled organs enables quantitative assessment of the PG synthesis and its specific activity as well as a comparison of the lipase activity of vascular tissue (method A) and nonvascular tissue in heart and kidney.

The pattern of incorporation of [^{14}C]arachidonic acid into tissue lipids was very similar between method A and method B; i.e., the majority of the radioactivity was found in the phospholipids (80-85% in heart, and 70-75% in kidneys) (5), predominantly phosphatidyl choline and phosphatidyl ethanolamine (5). Peptide hormone stimulation (bradykinin) and nonspecific noxious stimulation (with transient ischemia) were employed to elicit lipase activation (i.e., release of [^{14}C]arachidonate) and PG synthesis. In the isolated, perfused heart, the radioactive PG release in response to bradykinin and ischemia was higher with method A than with method B (865 cpm vs. 572 with bradykinin, and 289 cpm vs. 147 with ischemia) (5)

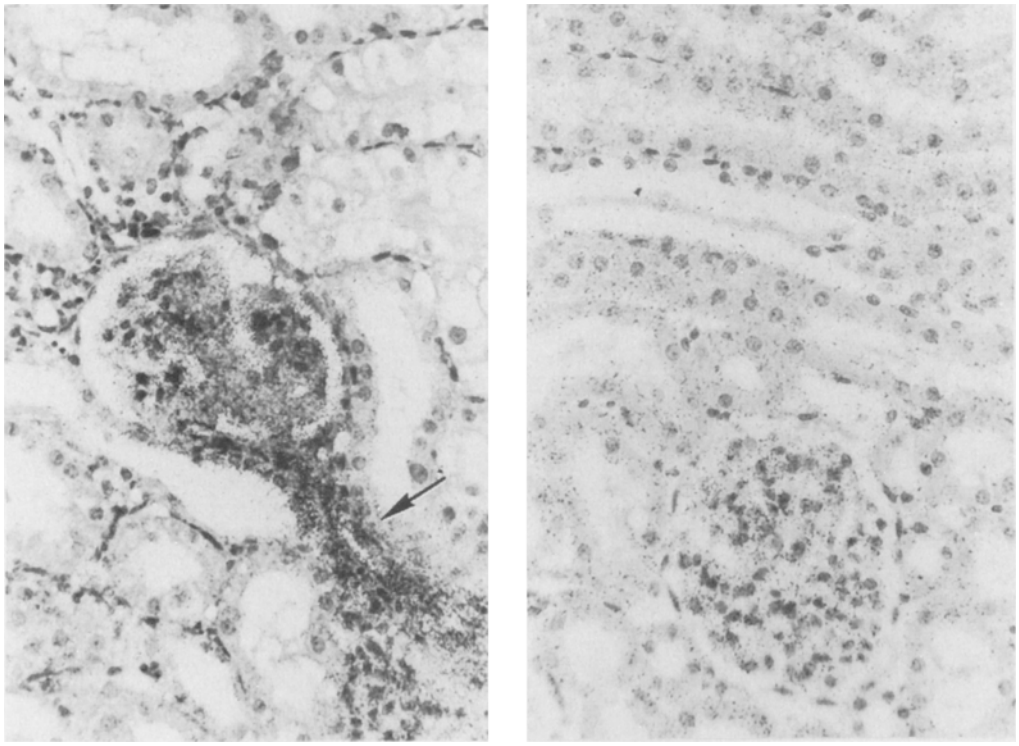

FIG. 2. Autoradiograms of hydronephrotic kidneys labeled with method A (infusion of [^{14}C] arachidonic acid in the absence of albumin) and method B (reciruclation of [^{14}C] arachidonic acid with albumin). Method A resulted in selective labeling of arteries, arterioles (arrow) and glomeruli while method B resulted in a diffuse labeling of the entire tissue.

despite the appearance of comparable amounts of bioassayable PG release (270-300 ng with bradykinin, 40-60 ng with ischemia) (5). As mentioned above, the labeling was confined to the vascular tissue in heart labeled with method A, while the labeling was also in the myocardial cells with method B; therefore, the greater release of radioactive PGs with method A would indicate the sites of PG synthesis in response to both hormone stimulation and ischemia to be primarily in the vasculature. However, the [^{14}C] arachidonic acid ([^{14}C]-AA) release with method B during ischemia was much greater than with method A (2481 cpm vs. 1248) (5), suggesting that the site of lipase activation during ischemia is also in the myocardial cells outside the vascular tissue. However, despite the high lipase activity in myocardial cells, the conversion of released [^{14}C] arachidonate to PG was rather inefficient (shown by the low [^{14}C]PG release with method B), indicating the the relative lack of cyclooxygeanse in these cells. Comparing the [^{14}C]AA/[^{14}C]PG ratio during ischemia between methods A and B (4:4 vs. 19:2) (5) showed that with method A ca. 1 out of every 4 moles of [^{14}C] arachidonate was converted to PG while with method B only one out of 19 moles of [^{14}C] arachidonate was converted to PG. This high [^{14}C]AA/[^{14}C]PG ratio of method B during ischemia suggests that most of the [^{14}C] arachidonate released during ischemia (with method B) was from myocardial cells, while the cyclooxygenase was predominantely in the vasculature, thus only the [^{14}C] arachidonic acid released in the vascular tissue was converted to PGs. These observations have physiological and pathological implications. Thus, the regulation of coronary arterial resistance by hormonal and metabolic factors, nerve stimulation and anoxia could be mediated by PGs (6-9). Furthermore, the lipases in myocardial cells are activated to provide fatty acid for metabolic needs. Thus, stimulation of cardiac lipases and PG synthetase might play an important role in the recovery from coronary ischemia and myocardial injury.

The same labeling methods were applied to perfused hydroephrotic kidneys (5). Autoradiography showed that method A resulted in

selective labeling of the arteries, arterioles and glomeruli in renal cortex, while method B also labeled the tubules and interstitium (Fig. 2) (5). Analysis of tissue lipids showed a similar pattern of incorporation of radioactive arachidonic acid between methods A and B (5). Following bradykinin (250 ng) injection and ischemia, the bioassayable PG (total PG) release was comparable with both methods (2.1-2.3 μg with bradykinin, and 430-432 ng with ischemia) (5). However, the radioactive PG release in response to bradykinin and ischemia with method A was much greater than with method B (3222 cpm vs. 1575 with bradykinin, and 1044 cpm vs. 452 with ischemia) (5), indicating the sites of PG synthesis in the hydronephrotic kidney (cortical region) to be predominantly in the vascular tissue. Furthermore, the [^{14}C]arachidonic acid release in response to bradykinin stimulation in hydronephrotic kidneys was also 3 times higher with method A than with method B (15365 cpm vs. 5542) (5), suggesting that the primary sites of hormone-specific lipase activity are also in the vasculature. However, the [^{14}C]arachidonic acid release in response to ischemia was much higher with method B than with method A (1440 cpm vs. 492) (5), suggesting that the site of nonspecific lipase activation in the hydroephrotic kidney is also in the renal tubules.

This observation is of particular interest since it not only further confirmed the presence of cortical synthesis of PGs (because the majority of the labeling was in the cortex), but also showed synthesis of these potent vasoactive materials to be in the cortical vessels themselves. The local synthesis of the potent vasoactive PGs in the afferent and efferent arterioles themselves may provide an efficient means of regulating renal resistance. Furthermore, PGs have been reported to play a role in the regulation of renin secretion (10,11) which is synthesized and released by specialized cells in afferent arterioles in the cortex.

ACKNOWLEDGMENTS

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Study of the Two Pathways for Arachidonate Oxygenation in Blood Platelets

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ABSTRACT

During collagen-induced blood platelet aggregation, arachidonic acid is set free from membrane phospholipids and subsequently converted into 12-hydroxyeicosatetraenoic acid by arachidonate lipoxygenase and into thromboxane A_2, 12-hydroxyheptadecatrienoic acid (HETE) and malondialdehyde by cyclooxygenase and thromboxane synthase. Lipoxygenase and cyclooxygenase have optimal activity at neutral to basic pH, while the thromboxane synthase is pH-independent between 5 and 9. These enzymes are membrane-bound. The cyclooxygenase is rapidly inactivated upon membrane disruption by nonionic detergents or phospholipid degradation with phospholipase A_2. It was found that platelet phospholipase A_2 preferentially splits off fatty acid with four double bonds. Eicosatetraynoic acid was used to investigate the physiological function of the arachidonate lipoxygenase during collagen-induced aggregation of rat blood platelets. This fatty acid is a more efficient inhibitor of lipoxygenase than of cyclooxygenase. At an inhibitor concentration of 0.6 μg/ml, platelet aggreation, 12-hydroxyeicosatetraenoic acid production as well as 15-hydroxytryptamine release are completely inhibited, while there is an apparent stimulation of the cyclooxygenase. These results indicate that arachidonate lipoxygenase is essential for irreversible blood platelet aggregation.

INTRODUCTION

Arachidonic acid can be converted in a blood platelet suspension by two enzyme pathways (Fig. 1). On the one hand, this fatty acid is converted by arachidonate lipoxygenase into 12-hydroperoxyeicosatetraenoic acid, which is subsequently reduced to give 12-hydroxyeicosatetraenoic acid (HETE) (1,2). The lipoxygenase action can be inhibited by low concentrations of eicosatetraynoic acid (1). On the other hand, prostaglandin endoperoxide synthase or cyclooxygenase produces endoperoxides (1,3). These labile compounds are converted by thromboxane synthase into 12-hydroxyheptadecatrienoic acid plus malondialdehyde and thromboxane A_2, which is a strong aggregatory substance for human blood platelets (3). Thromboxane A_2 is a very unstable

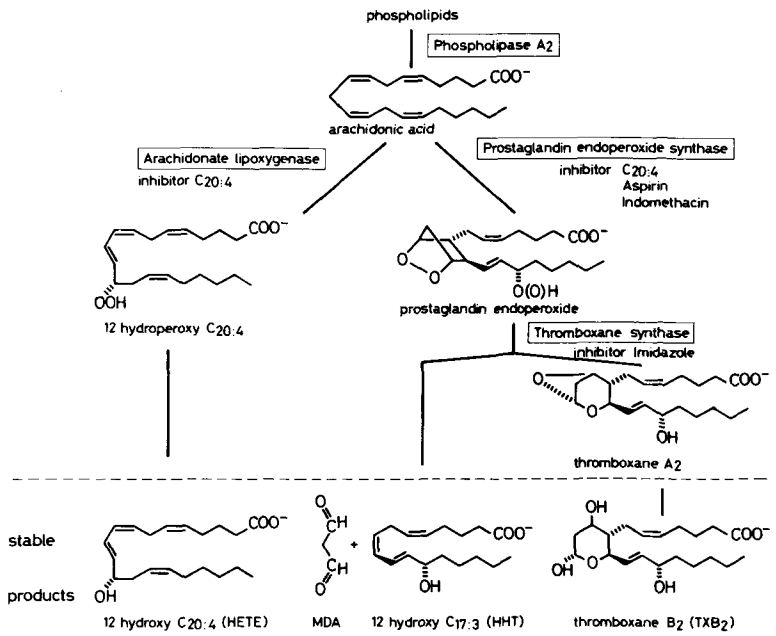

FIG. 1. Pathways for arachidonic acid conversion in blood platelets.

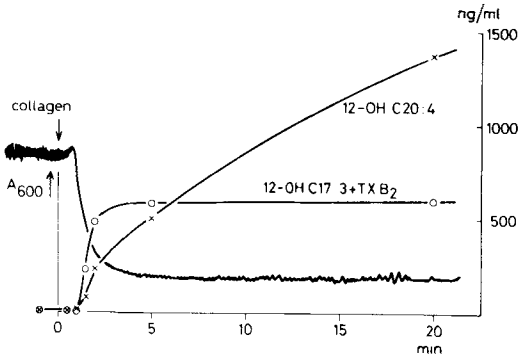

FIG. 2. Production of arachidonate metabolites during collagen-induced aggregation of rat blood platelets (1.6×10^9). The products are quantitated by GLC/MS, using deuterated standards.

product in water and hydrolyses into thromboxane B_2 (3). It is well known that the formation of endoperoxides by cyclooxygenase can be inhibited by non-steroidal antiinflamatory drugs such as aspirin and indomethacin (1), but eicosatetraynoic acid can also be used (1).

Inhibition of the cyclooxygenase with tetraynoic acid is obtained at a higher concentration than inhibition of the lipoxygenase (4). The activity of thromboxane synthase can be measured separately by incubation of blood platelets with prostaglandin endoperoxide. Needleman and coworkers reported that this enzyme system can be inhibited with imidazole (5).

This article deals with some biochemical aspects of the enzyme systems involved in arachidonic acid conversion, and evidence is presented that the activity of the arachidonate lipoxygenase is essential for blood platelet aggregation.

PROCEDURES

Materials. [1-^{14}C]arachidonic acid and ^{14}C-hydroxytryptamine were obtained from Amersham, Radiochemical Centre.

Tween-20 was purchased from Brocades, Triton X-100 from Serva and Dobanol 91-8 from Shell.

Eicosatetraynoic acid and all reference products were from own stocks. Prostaglandin endoperoxide was prepared as described earlier (6).

Platelets. Fresh blood was collected into 0.1 vol of trisodium citrate (38 g/l). Platelet-rich plasma (PRP) was prepared by centrifugation at room temperature for 10 min at 120 g. Washed platelets were prepared by addition of 0.1 vol EDTA solution (28.6 g/l EDTA, 7 g/l NaCl) to PRP in siliconized glass tubes and centrifugation for another 10 min at 600 g. The supernatant was removed completely and the platelet pellet was resuspended in a phosphate-buffered salt solution (pH 7.4) containing glucose (2 g/l). Large amounts of bovine platelets were collected as described earlier (2).

Aggregation reaction. Platelet aggregation was monitored in a Born aggregometer; 20 µl $CaCl_2$ solution (100 mM) was added to 0.8 ml platelet suspension (about 2×10^9 platelets). After 3 min stabilization, 100 µl of a solution of eicosatetraynoic acid in a phosphate-buffered salt solution was added. After 2 min platelet aggregation was induced by addition of collagen together with 20 µl ^{14}C-arachidonic acid solution (50 µg/ml). Four min after induction of the reaction, the enzymic conversions were stopped by addition of 0.2 ml citric acid (2.3 M).

Product analysis. Radioactive products were extracted from the incubates with ether, dried and separated by silica thin layer chromatography (TLC) using chloroform methanol acetic acid water (90:6:1:0.75) as a solvent. Identification was performed by cochromatography of inactive standards. The radioactive zones were scraped off and quantitated by liquid scin-

TABLE I

Fatty Acid Composition of the Lipids and Hydroxy Acids Released during Aggregation with Rat Platelets[a]

	Fatty acid composition (% of total lipids)		Hydroxy acid after aggregation (ng/10^9 platelets)	
Diet	20:4	20:3	12-OH-20:4	12-OH-20:3
With 5 en% linoleic acid	26.4	0.1	810	0
In EFA deficiency	4.5	19.2	12	<5

[a]Platelets were obtained from rats on linoleic acid (5% of total dietary energy, 5 en%) and essential fatty acid deficient diets. The hydroxy acids were determined 20 min after collagen-induced aggregation (20:4 = 5,8,11,14-eicosatetraenoic acid; 20:3 = 5,8,11-eicosatrienoic acid).

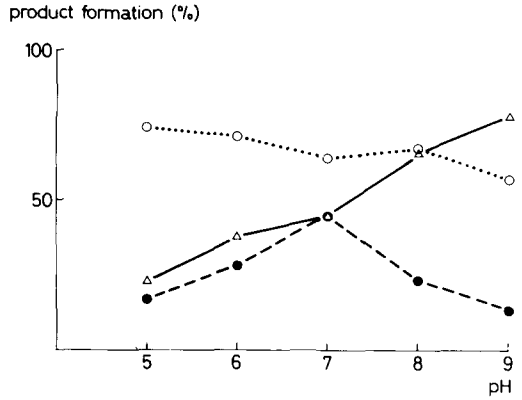

FIG. 3. Conversion of different substrates by washed bovine blood platelets (10^9) at different pH.
△—△ formation of HHT + TXB_2 } from arachi-
●--● formation of HETE } donic acid
○··○ formation of HHT + TXB_2 from PGH_2

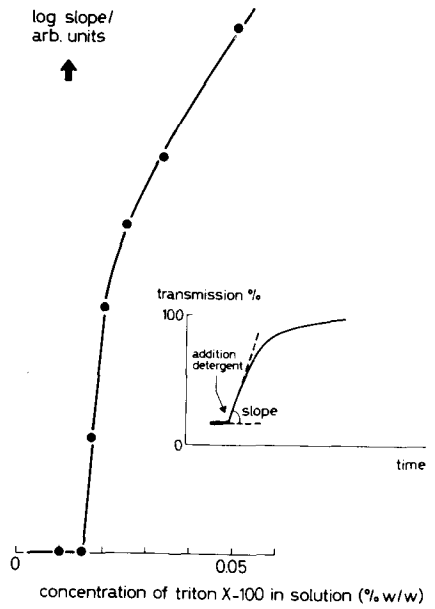

FIG. 4. Solubilization of washed rat blood platelets as a function of Triton X-100 concentration. Inset: Recording of the light transmission of a blood platelet suspension after addition of Triton X-100.

tillation counting. In separate experiments unlabelled reaction products were quantitated after TLC by gas liquid chromatography-mass spectrometry (GLC-MS), using deuterated internal standards.

^{14}C-hydroxytryptamine release was measured according to Smith et al. (7).

RESULTS AND DISCUSSION

Release and Conversion of Endogenous Arachidonic Acid

The production of arachidonate metabolites during collagen-induced aggregation of rat blood platelets is shown in Figure 2. The aggregation curve almost coincides with that of thromboxane formation, whereas HETE formation continues for a much longer period. Very similar results have been obtained with bovine and human platelets.

We also studied platelets of rats which had been on an essential fatty acid-deficient diet. Table I shows that the platelets of these animals contain only small amounts of arachidonic acid, which turns out to be replaced mainly by 5,8,11-eicosatrienoic acid, which is not a substrate for thromboxane formation. Upon addition of 5,8,11-eicosatrienoic acid to washed platelets suspended in buffer, this fatty acid is readily converted by lipoxygenase into 12-hydroxy C20:3 (cf. Ref. 2).

Blood platelets from essential fatty acid-deficient rats do aggregate upon addition of collagen (8), but during this process hardly any metabolites are formed, neither from arachidonic acid nor from 5,8,11-eicosatrienoic acid (Table I). The formation of the oxygenation products is preceded by release of the fatty acids by phospholipase A_2 action, and a possible explanation for the above results with EFA-deficient platelets might be a very pronounced preference of the thrombocyte phospholipase A_2 for the release of fatty acids with four double bonds. Using different techniques, both Derksen and Cohen (9) and Bills et al. (10) reported similar observations.

Influence of pH on Substrate Conversion

To study the lipoxygenase and cyclooxygenase independently of blood platelet aggregation and endogenous substrate release, added ^{14}C-substrate was used to measure the enzymic activity. Both normal and EFA-deficient platelets were found to convert added arachidonic acid or prostaglandin endoperoxide to the same extent, so there is no defect in the arachidonic acid metabolizing enzymes.

Blood platelets can transform large amounts of arachidonic acid into thromboxane A_2, without any aggregation, provided there are no calcium ions present. Bovine platelets were incubated in the absence of calcium with arachidonic acid and with prostaglandin endoperoxide at different pH values.

For washed intact bovine blood platelets, a basic pH optimum was found for the cyclooxygenase as is shown in Figure 3. The same has been reported for cyclooxygenase from other sources (11).

TABLE II

Conversion of 1 μg of ^{14}C-arachidonic Acid by Bovine Blood Platelets (10^9) under Different Conditions (pH 8.0, at 36 C for 15 min)[a]

Platelet pretreatment	Fresh platelets	After aggregation with collagen	After solubilization Triton X-100 (0.5% w/w)	After solubilization Tween-20 (0.5% w/w)
Product				
Thromboxane B_2	20	7	4	17
12-OH-$C_{17:3}$ (HHT)	22	8	7	22
12-OH-$C_{20:4}$ (HETE)	48	70	57	52
Arachidonic acid	10	15	33	9

[a]The percentage of the total radioactivity in the different fractions is indicated.

TABLE III

Conversion of 1 μg ^{14}C-arachidonic Acid by Washed Bovine Blood Platelets (10^9) after Preincubation for 15 min with Different Amounts of Arachidonic Acid[a]

Product	TXB_2	HHT	HETE
Amount of arachidonic acid during preincubation μg			
–	16	16	48
4	12	13	41
10	10	13	31
20	5	7	14

[a]The percentage of the total radioactivity in the different fractions is indicated.

We found that the conversion of prostaglandin endoperoxide by thromboxane synthase is pH-independent (Fig. 3).

We could also demonstrate that endoperoxide methyl ester is converted by blood platelets into the corresponding thromboxane B_2 methyl ester. This means that the carboxyl group is not essential for the thromboxane synthase as it is for lipoxygenase (2) and cyclooxygenase (12,13).

Solubilization of the Enzymes

Solubilization and purification was attempted to be able to study lipoxygenase and cyclooxygenase in more detail. These enzymes are both membrane-bound (14,15) and, therefore, solubilization with nonionic detergents was necessary before purification could be started. Prior to fractionation, it was checked whether the enzyme systems studied are influenced by detergents. The solubilization of washed platelets was monitored by a Born aggregometer, and the platelet proteins were incubated with ^{14}C-arachidonic acid before and after solubilization.

The results of the experiments where Triton X-100 was used as detergent are shown in Figure 4. Above a certain detergent concentration, a sudden and complete solubilization of the thrombocytes was observed. Similar results were obtained with human and bovine platelets, and also when Dobanol, a polyoxyethylene alcohol, was used as a detergent. However, Tween-20 (0.5% w/w), which is a more hydrophilic detergent, was not able to solubilize the blood platelets.

Table II summarizes the results obtained by incubation with arachidonic acid for washed bovine platelets, before and after solubilization or collagen-induced aggregation. It can be seen that especially the cyclooxygenase is a very labile enzyme, which is readily inactivated upon disturbance of its natural membrane environment.

Solubilization of platelet suspensions that had been kept several days at 4 C in EDTA-containing buffer yielded some cyclooxygenase activity in the solubilized fraction. However, Sepharose 6B column chromatography indicated that this fraction had a molecular weight of several hundred thousand dalton and still contained phospholipids. The enzymic activity obtained in this way turned out to be very unstable, especially upon addition of Ca-ions.

In our opinion, the Ca-ions activate the phospholipase A_2 still present in this fraction and, thus, the phospholipids can be degraded. This results in an inactivation of the cyclo-

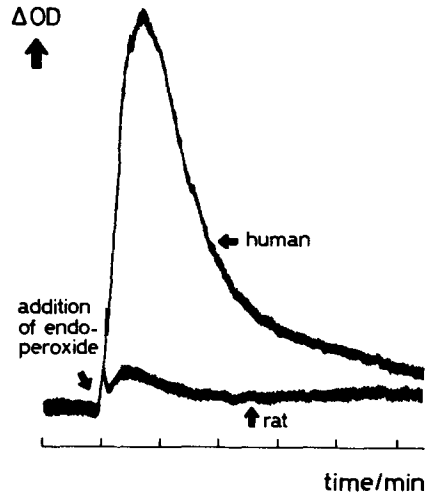

FIG. 5. Effect of prostaglandin endoperoxide on human and rat platelets in suspension.

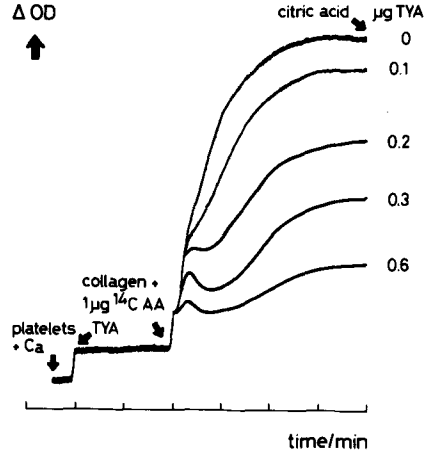

FIG. 6. Effect of eicosatetraynoic acid (TYA) on collagen-induced rat blood platelet aggregation.

oxygenase as was also observed during collagen-induced blood platelet aggregation. Replacement of blood platelet phospholipids by non-ionic detergents also leads to inactivation of the cyclooxygenase and, therefore, we suppose that this enzyme requires a certain amount of phospholipids for its enzymic activity. However, our attempts to reactivate the enzyme by addition of several phospholipids were not successful.

The lipoxygenase appears to be more stable, which can also be concluded from our study of the generation of reaction products during platelet aggregation (Fig. 2).

The inactivation of the cyclooxygenase is

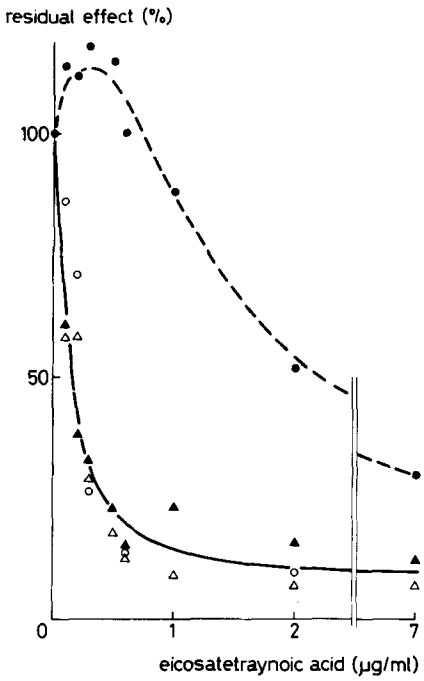

FIG. 7. Effect of eicosatetraynoic acid (TYA) on different parameters during collagen-induced rat blood platelet aggregation.(zero TYA concentration = 100%). ▲ slope aggregation curve (cf. Fig. 6), ○ 15 hydroxytryptamine release, △ HETE production, ● HHT + TXB_2 production.

not caused by product formation as is shown in Table III: preincubation of a platelet suspension with different amounts of arachidonic acid did not essentially change the ratio between thromboxane B_2 + HHT and HETE production from subsequently added ^{14}C-arachidonic acid. This again indicates that not product formation, but rather membrane destruction, can lead to inactivation of the cyclooxygenase.

The solubilized thromboxane synthase, which converts prostaglandin endoperoxide into thromboxane, was found to be fairly stable and could be kept for weeks at 4 C.

Yoshimoto et al. (16) and Hammarström and Fallardeau (17) also tried to solubilize and resolve the thromboxane synthetizing system from blood platelets. They claim a separation of the cyclooxygenase but, unfortunately, they do not give details about the nature and purity of their enzyme preparations. We assume that both groups isolated an unstable enzyme fraction, which still contains membrane fragments.

Physiological Function of Arachidonate Lipoxygenase

Thromboxane A_2 is known to be an active

pro-aggregatory agent for human blood platelets (1). However, as indicated in Figure 5, the effect of prostaglandin endoperoxide, which is rapidly converted into thromboxane A_2, is very different for human and rat blood platelets; in rat platelets there is no aggregation at all. This observation does not imply that thromboxane A_2 formation is not important during rat platelet aggregation. Aspirin inhibits the collagen-induced platelet aggregation and thromboxane A_2 formation both in human and rat platelets. The physiological role of the arachidonate lipoxygenase is, however, not yet known.

Unfortunately no inhibitor specific for this enzyme is known yet and, therefore, we studied the influence of increasing amounts of eicosatetraynoic acid on collagen-induced blood platelet aggregation because this substance inhibits lipoxygenase more effectively than cyclooxygenase (4). To study especially the activity of the enzymes which convert arachidonic acid, we added ^{14}C-arachidonic acid together with the collagen. After 4 min, the reactions were terminated by acidification with citric acid.

The aggregation patterns at different inhibitor concentrations are shown in Figure 6. It is clear that 0.6 μg eicosatetraynoic acid causes complete inhibition of the aggregation. The slope of these aggregation curves was used as a measure of inhibition efficiency. In Figure 7 these slopes have been plotted as a function of inhibitor concentration and it also indicates product formation. It can be seen that a decreased platelet aggregation corresponds to a decreased lipoxygenase action, and this is accompanied by an apparent increase in cyclooxygenase activity. In similar experiments it was demonstrated that 15-hydroxytryptamine release, which is another parameter for blood platelet aggregation, decreases in the same way as the aggregation itself. These results indicate that rat blood platelets do not aggregate unless both lipoxygenase and cyclooxygenase are active. As the 15-hydroxytryptamine release coincides with the second phase aggregation (18), we suggest that HETE production induces this second phase aggregation, while thromboxane A_2 formation only induces the first-phase aggregation (19). We, therefore, suggest that for irreversible blood platelet aggregation, the activity of arachidonate lipoxygenase is possibly as essential as that of the cyclooxygenase. If one of these enzyme pathways is blocked, aggregation of rat blood platelets is always inhibited.

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The Relationship of Dietary Fats to Prostaglandin Biosynthesis

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ABSTRACT

The direct and indirect evidence that the fatty acid composition of dietary fat is involved in the regulation of prostaglandin biosynthesis was reviewed. Direct evidence included effects of essential fatty acid deficiencies and excesses on endogenous tissue levels and production rates of prostaglandins by several tissues. Indirect evidence included lipolytic, platelet aggregatory, hypertensive, inflammatory and immune responses. In general, composition of dietary fat did not affect prostaglandin biosynthesis unless a biochemical essential fatty acid deficiency was induced or the linoleate to saturated fatty acids ratio of the dietary fat was greater than 5. Most results were interpreted in light of changing fatty acid composition; however, very few direct measurements have been made.

In 1964, Bergström et al. (1) and Van Dorp et al. (2) discovered that arachidonate was incorporated into prostaglandin (PG) E_2. In most species arachidonate is derived from dietary linoleate. The control of the conversion of linoleate to arachidonate by diet and physiological parameters is complex and will not be covered in this review. The reader is referred to several excellent reviews by Klenk (3), Holman (4), Mead (5), Brenner (6), and Sprecher (7). It is well accepted that in most tissues the precursor pool of arachidonate that is utilized for PG synthesis is the membrane phospholipids and the availability of arachidonate is regulated by phospholipase A_2 activity (8-11). The objective of the following report is to review the direct and indirect evidence for the relationship between the fatty acid composition of dietary fat and regulation of the PG biosynthesis, through the former's involvment in altering tissue stores of polyunsaturated fatty acids. Very little distinction will be made between the different PG, such as A,D,E,F,I and thromboxane or the 1 and 2 series, due to lack of suitable methodologies and, therefore, information.

DIRECT EVIDENCE

Essential Fatty Acid Deficiency

What is the direct evidence that dietary essential fatty acid (EFA) deficiency lesions are associated with a lack of PG? The scaly lesions of EFA deficiency in the rat are improved by the topical application of PGE (12), and the infertility is corrected by PGE_2 administration (13). The abnormal fatty acid 5,8,11-eicosatrienoate that accumulates in essential fatty acid deficiency (4) is not a substrate for PG synthesis (14,15). It also has the characteristic of allosterically inhibiting the conversion of PGH→E (16).

What is the direct evidence that a dietary EFA deficiency alters PG biosynthesis? The most frequently used research design has been to harvest tissue under a standardized method and determine PG concentration. The measurement has usually been described as indicating endogenous PG content, but one must recognize that the in vivo level is probably much lower because the mildest trauma will markedly stimulate and/or initiate PG production (11,17). The more proper interpretation of this type of measurement is the short term potential for PG production from endogenous precursors.

Van Dorp (18) determined from limited results that EFA deficiency caused a decreased PGE_2 content in a small number of observations using rat epidermis and inner medulla from rabbit kidney. Tan and Privett (19) made rats EFA-deficient by feeding a fat-free diet for 1 year and found a 50% decrease in PGE content of rapidly frozen vesicular glands that had been powdered and extracted with organic solvents.

Another approach to the study of the effect of composition of dietary fat is to allow PG synthesis and release from endogenous precursor pools to occur by incubation an intact tissue or a homogenized tissue for a specified period of time. Time courses have been published for clotting dog blood (20), thrombin-induced aggregating human platelets (21), and slices of rat renal papilla (22). Platelets, fat pads, brain slices, aortic tissue and exudates due to an inflammatory response have been studied.

Vincent and Zijlstra (23) showed that EFA deficiency markedly reduced the thromboxane A_2-like activity, and PG formed when rat platelets were incubated with phospholipase A_2. Epididymal fat pads from EFA-deficient rats when compared to controls showed a reduction of 70% in the release of PGE_2, and

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TABLE I

Effect of Dietary Fat Composition on Prostaglandin Levels in Rat Liver, Lung and Serum

Dietary fat[a]		Endogenous		Incubated for 10 min at 37 C		
Calories (%)	P/S	Lung (ng/g)	Liver (ng/g)	Lung (ng/g)	Liver (ng/g)	Serum (ng/10^9 platelets)
			$PGF_{2\alpha}$			
20	0.4	12.8 ± 1.4[b]	2.8 ± 0.5[c,d]	1320 ± 246[d]	902 ± 143	5.2 ± 0.7
	0.8	8.3 ± 0.5	8.1 ± 2.3	372 ± 93	650 ± 134	4.5 ± 0.8
	5.5	10.2 ± 1.2	9.1 ± 1.4	496 ± 79	1030 ± 491	3.8 ± 0.6
40	0.4	14.8 ± 4.2	7.1 ± 0.8	646 ± 111	993 ± 134	3.2 ± 1.1
	0.8	12.7 ± 1.3	11.0 ± 1.8	500 ± 68	807 ± 155	4.6 ± 1.1
	5.5	11.7 ± 1.3	13.7 ± 0.6	430 ± 47	1170 ± 218	3.8 ± 0.4
			PGE_2			
20	0.4	7.5 ± 1.3	1.8 ± 0.6[c,d]	371 ± 38[d]	637 ± 76	7.3 ± 0.8
	0.8	4.0 ± 0.4	3.8 ± 0.6	182 ± 31	567 ± 91	6.2 ± 1.4
	5.5	5.0 ± 0.6	3.9 ± 0.8	223 ± 44	910 ± 137	5.4 ± 1.5
40	0.4	8.2 ± 2.8	3.8 ± 0.7	316 ± 41	708 ± 54	4.0 ± 1.4
	0.8	6.9 ± 1.1	5.4 ± 0.8	241 ± 29	637 ± 137	5.4 ± 1.4
	5.5	5.6 ± 0.7	6.8 ± 1.1	201 ± 38	729 ± 96	5.9 ± 1.1

[a]Details of diet composition can be found in reference 35.
[b]Mean ± SEM; N=7 or 8.
[c]In factorial analysis of variance, effect of calories was significant ($P<0.01$).
[d]In factorial analysis of variance, effect of P/S was significant ($P<0.01$).

PGE_1 release was abolished (24). Weston and Johnston (25) reported that EFA deficiency decreased PGE synthesis by rat cerebral slices only 30% (P=0.05). Hornstra et al. (26) used a bioassay system to demonstrate an 83% decrease due to EFA deficiency in production of PGI_2 by intact aortic endothelium. No depression was observed when hydrogenated coconut oil was fed for only 2-3 weeks, possibly because a frank deficiency had not been produced. One report (27) has appeared on exudation, and EFA deficiency virtually abolished PGE_2 content.

When the above research approach is used, one must be aware of the fact that EFA-deficient rat tissues, such as lung and small intestine (28) or kidney medulla (29) but not vesicular gland (19), respond by increasing their capacity to convert exogenous precursors to PG. Thus, a lack of substrate could be partially overcome by synthesizing more PG synthetase complex.

Linoleate Excess

Hulan and Kramer (30) utilized a procedure similar to the one published by Tan and Privett (19) to measure endogenous PGE_2 content in rat skin. When corn oil replaced lard as the dietary fat, the PGE_2 level doubled ($P<0.01$.). From their fatty acid analysis of the dietary fats, this would be an increase in linoleate/saturated fatty acids (P/S) ratio from 0.2 to 4.9. Meydani et al. (31) chose to homogenize rat lung in a solution containing an excessive amount (42 mM) of aspirin. They reported an 8-fold increase in $PGF_{2\alpha}$, 13-fold increase in PGE_2 and 11-fold increase in PGE_1, when safflower oil (P/S ratio of 9) replaced beef tallow (P/S ratio of 0.1) in the diet. Using the same techniques (31) but utilizing various levels and mixtures of soybean oil and beef tallow to produce P/S ratios of 0.4, 0.8 and 5.5, no changes in endogenous $PGF_{2\alpha}$ and E_2 levels in lung were shown (Table I). Possibly, extreme differences in P/S ratio are required to markedly alter lung PG content. The endogenous $PGF_{2\alpha}$ and E_2 levels in the liver ca. doubled when the P/S ratio was raised from 0.4 to 5.5 (Table I). There was a strong positive linear correlation (r=.88 for $PGF_{2\alpha}$ and .89 for PGE_2, $P<0.03$) between PG levels and percentage linoleate calories, i.e., 2.3, 3.8, and 8.4% for the 20% fat calories and 4.6, 7.6 and 16.7% for the 40% fat calories. This suggests that the absolute amount, and not proportion, of linoleate was responsible for the increase in PG levels. An increase in consumption of linoleate can easily increase the arachidonate stores in the liver (4).

Measurement of serum levels of PG is analogous to performing an aggregating platelet incubation. In the initial experiment from this laboratory (32), the incubation conditions were not controlled, but we showed a 50% and 37% increase in PGE_1 and a 56% and 27% increase in $PGF_{2\alpha}$ in serum from fed and fasted rats, respectively, when corn oil replaced beef tallow ($P<0.05$). In follow-up studies, rat blood was

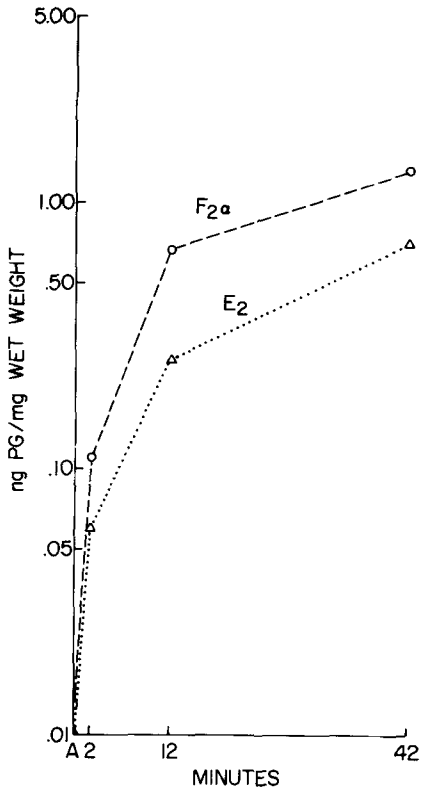

FIG. 1. Ex vivo prostaglandin (PG) synthesis by rat lung homogenates (N=48) in 0.1 M potassium phosphate buffer, pH 7.4, at 37 C. Point A signifies homogenization in presence of 42 mM aspirin.

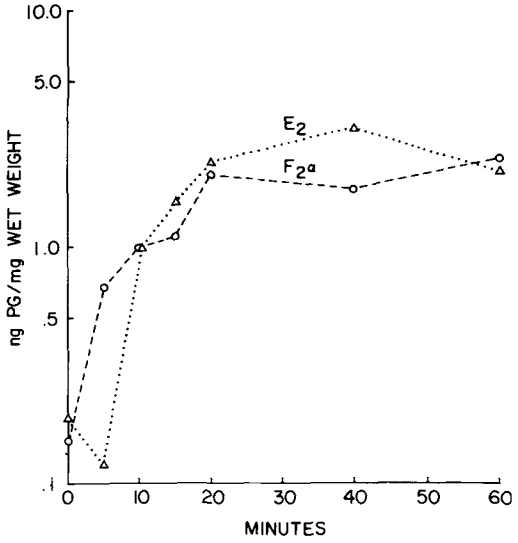

FIG. 2. Ex vivo prostaglandin (PG) synthesis by a rat liver homogenate in 0.1 M potassium phosphate buffer, pH 7.4, at 37 C.

allowed to clot at 37 C for 10 min, then aspirin was added at the level of 0.17 mM and PG determined by radioimmunoassay (20). Varying dietary P/S ratios between 0.4 and 5.5 did not significantly affect serum $PGF_{2\alpha}$ or E_2 levels (see Table I and reference 33). When safflower oil which has a P/S ratio of ca. 9 was fed, however, the serum PG levels increased markedly (34).

Adipocytes in which lipolysis was stimulated by adding norepinephrine showed a 3- and 4-fold increase in $PGF_{2\alpha}$ and E_2 production, respectively, when the dietary P/S ratio was increased from 0.2 to 5.5 (35). PG synthesis by liver and lung homogenates from rats fed diets containing P/S ratios analogous to those discussed above were also determined and are reported herein. Since the hypothesis to be tested was that dietary fat affects PG synthesis by altering the composition of endogenous precursos fatty acid pools, no exogenous substrate was added. This is called an ex vivo incubation. Addition of aspirin at a final concentration of 14 mM was used to stop PG synthesis. The effectiveness of this concentration of aspirin is substantiated in the time course of ex vivo PG synthesis shown in Figures 1 and 2. The effect of dietary fat upon ex vivo PG synthesis by lung and liver was tested at the 10 min time point which was just before the plateau. The results are shown in Table I and differ markedly from the responses observed in endogenous levels, which were discussed above. Dietary fat P/S ratio did not affect liver production ($P > 0.05$) and lung PG production decreased as the P/S ratio increased ($P < 0.01$). Possibly, the endogenous PG precursor pool, arachidonate, was not altered nor released in a positive linear relationship to dietary linoleate. Another plausible explanation is that the tissue stores of linoleate were increased and, if released during PG synthesis, could have acted as a competitive inhibitor to the substrate, arachidonate (36,37). Changes in fatty acid composition of the different lipid classes, unfortunately, were not determined. This type of information could help establish which lipid class is the primary source of the precursor fatty acids for PG synthesis in these tissues.

Other Dietary Fatty Acids

Partial hydrogenation of fats is a common practice in the food industry. The 12-*trans* isomers of dihomo-gamma-linolenate and arachidonate have been shown to be inhibitors of PG synthesis in vitro (38,39). The dietary

precursor of these fatty acids is *trans* linoleate. Hwang and Kinsella (40) fed *trans* linoleate at the level of 5% of the diet and found a 90% reduction in $PGF_{2\alpha}$ production by rat blood allowed to clot at 37 C for 60 min. They concluded that the reduction was due to a marked depression in arachidonate and not due to a build up of *trans* isomers. Feeding a diet containing rapeseed oil, in place of corn oil, depressed ACTH-induced PGE_2 and $F_{2\alpha}$ production by rat adrenal homogenates ca. 40% (41). This was attributed to the accumulation of erucic acid in the cholesterol fraction, which cannot serve as a precursor of PG. Hulon and Kramer (30) fed several varieties of rapeseed oil to rats and measured PGE_2 synthesis in skin. The rapeseed oils did not alter endogenous PGE_2 levels but did depress PGE_2 synthesis from exogenous arachidonate when compared to corn oil.

Arachidonate and dihomo-gamma-linolenate make up a very small portion of human food fats so increasing their consumption could alter PG synthesis. Seyberth et al. (42) fed 6 g of ethyl arachidonate/day for two weeks to three men, and the urinary excretion of the major metabolite of PGE increased 47%. In addition, their platelets were more reactive, which reflects an increase in aggregating PG. When dihomo-gamma-linolenate was fed to laboratory animals, the urinary excretion of the major urinary metabolite of PGE increased 4.6-fold (43), and platelets were less reactive in most cases, which reflects a shift toward PGE_1 production (44-46). What happens in man will be the subject of another paper in this symposium.

INDIRECT EVIDENCE

There is extensive indirect evidence that dietary fat alters PG biosynthesis. Measurement of physiological responses that are well documented to be mediated by PG is the process used. Lipolysis by adipose tissue, platelet aggregation and blood clotting, hypertension, inflammation and immunity are excellent examples.

Lipolysis

Adipose tissue lipolysis has been shown to be feedback regulated by PGE (47-51), and thus, lipolytic rates and/or mobilization of fatty acids should be enhanced when EFA are limited. This enhancement has been demonstrated in several laboratories (24,52-54) but could be an artifact caused by a lowering of lipid content and increasing protein content of adipose tissue due to EFA deficiency (55). A research approach that circumvents this problem is to compare lipolytic rates from rats that have been fed diets that contain adequate and excessive amounts of linoleate. Lipinski and Mathias (35) concluded that published work, which compared various dietary fats, indicated highly variable results. In all cases, fat pads were used to assess lipolytic rats. They optimized the conditions of lipolysis in norepinephrine-stimulated isolated adipocytes and could find no change in adipocyte lipolysis, even though PGE_2 release was altered 4-fold (35).

Platelet Aggregation

It is well established that saturated dietary fat is thrombogenic in rats and rabbits (56-58). In humans, increasing the proportion of dietary linoleate reduces platelet aggregation (59-65). These effects could be mediated by PG, but the exact mechanisms are not understood due to the complexity of PG metabolism in platelets (21,46,66-69). Arachidonate can be converted to the pro-aggregating agents, PGE_2, G_2, H_2 and thromboxane A_2 or to the anti-aggregating agents, PGD_2 and I_2. Dihomo-gamma-linolenate can be converted to PGE_1, an anti-aggregating agent. Thus, dietary linoleate can be converted to either pro- or anti-aggregating agents or, as discussed earlier, act as a competitive inhibitor of total PG synthesis. In addition, there are marked species differences which make interpretation difficult (46). Non-PG mechanisms, such as changes in blood cholesterol or platelet factor 3, are also distinct possibilities. In conclusion, before one can use the fact that dietary fat affects platelet aggregation as indirect evidence that dietary fat alters PG synthesis, much more must be known about the mode of action of different PG in aggregation.

Hypertension, Inflammation and Immunity

These responses are altered in several ways by PG. EFA deficiency induced an increase in blood pressure in rats (70,71), and increased linoleate consumption was associated with a decrease in blood pressure in man (72,73). EFA deficiency altered the inflammatory response in several models (74-76) and linoleate treatment inhibited immune responses (77-82). However, the ambivalent (pro- and anti-) role of PG—a term used by Bonta et al. (27)—in the etiologies of these responses, does not allow further interpretations.

CONCLUSION

In general, it appears that PG synthesis can be depressed by biochemical EFA deficiency

and enhanced when the P/S ratio reaches 5 up to 9. Smaller changes in the P/S ratio only affected PG release by norepinephine-stimulated adipocytes. Under certain conditions the response to dietary fat was due more to inhibition of the PG synthesis by other fatty acids than to change in amount of PG precursor fatty acid.

The obvious connection between composition of dietary fats and PG synthesis is tissue fatty acid composition. As we gain an understanding of the precursor pools (83-85), then meaningful tissue fatty acid determinations can be made. At present the subcellular complexity (86) and the numerous possible pools limit the use of gross tissue fatty acid determinations. The positive aspects is that now we know that we can perturb regulation of PG synthesis by extreme dietary fat manipulation, which presumably does affect tissue fatty acid composition.

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Fatty Acids in Female *Macracanthorhynchus hirudinaceus* (Acanthocephala)

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ABSTRACT

Neutral lipids and phospholipids in the body wall, lemnisci and pseudocoel and neutral lipids of fluid found in the "tube" system of the lemnisci and the lacunar system of the body wall of *Macracanthorhynchus hirudinaceus* (Acanthocephala) were determined by the technique of thin layer chromtography and gas chromatography (GC). Sixteen different fatty acids from nonpolar lipids were identified as follows: 8:0, 10:0, 11:0, 12:0, 13:0, 14:0, 14:1, 16:0, 18:0, 18:1, 18:2 and/or 20:0, 18:3 and/or 20:1, 20:3, 22:1, 24:1 and 22:6. In addition, there were three unidentified GC peaks corresponding to chain lengths greater than 20 carbons. Sixteen different fatty acids from phospholipids were identified in each of the three fractions analyzed. They were as follows: 10:0, 11:0, 12:0, 13:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2 and/or 20:0, 20:2, 20:3, 22:1, 24:1, and 22:6. Four unidentified peaks were also observed. There was a significant difference in the percentage of lipid as well as the concentration of particular fatty acids when each fraction was compared. There was also an abundant supply of sterols and glycerides in each fraction.

INTRODUCTION

Lipids are a major constituent in helminths in which unsaturated fatty acids predominate and occur in equal amounts with triglycerides and phospholipids (1). One might expect the reverse to be the case since in most metazoans oxygen is an essential ingredient for the biosynthesis of unsaturated fatty acids (2). However, the adult acanthocephalan inhabits the vertebrate small intestine where oxygen tension is low or nonexistent (3) and yet possesses a number of unsaturated fatty acids. Unfortunately, it is not known how Acanthocephala metabolize lipids. Kilejian et al. (4) pointed out that in *Hymenolepis diminuta* (cestode) the large degree of unsaturation in its triglyceride fraction was a result of absorption of bile and other intestinal secretions. Apparently, *H. diminuta* has taken advantage of micelle absorption by increasing the uptake of monoglycerides and free fatty acids (5) from the gut contents. Moreover, it has been shown (6) that unsaturated fatty acids are relatively more soluble than their saturated counterparts. This may help explain why these parasites have such a high content of unsaturated fatty acids.

Intestinal helminths apparently use those fatty acids available in the enteric fluid of their host to fill their own needs. These molecules are first absorbed then interconverted into those chain lengths required by the parasite's metabolic machinery. The exact extent to which the various molecules can be changed is not known for Acanthocephala. However, current data (7) indicate that cestodes, trematodes, and Acanthocephala are not capable of de novo "synthesis" of long chain saturated or unsaturated fatty acids. Moreover, some studies (8) indicate that cestodes are incapable of desaturating absorbed fatty acids, whereas specific saturated and unsaturated fatty acids may be elongated. Perhaps the best studied helminth has been *H. diminuta* in which it has been shown (8) that labeled acetate incorporation occurred in phospholipids and neutral lipids at the same rate under aerobic or anaerobic conditions. It has also been shown, although the number of species studied has been minimal, that water soluble components of complex lipids are freely synthesized from various precursors (7).

This study attempts to identify some of the fatty acids present in various body components of female *Macracanthorhynchus hirudinaceus* (Acanthocephala).

MATERIALS AND METHODS

M. hirudinaceus were obtained from pigs at Swift Fresh Meats Company in East St. Louis, IL. Following removal, the worms were transported to the laboratory in Dewar flasks containing gut contents. Female worms were washed and cleaned in 30% artifical sea water (9). A small incision was then made at the caudal end of the body wall and pseudocoel contents "milked" into a test tube and immediately sealed under N_2. The entire length of the body was then split and lemnisci separated from 20 worms prior to extraction. Contents of the lacunar network and large ducts in the lemnisci were collected in micropipettes.

TABLE I

Tissue fraction	Wet weight	Lipid weight	% Lipid
Body wall	0.616 g	39 mg	4.7
Pseudocoel	0.982 g	23 mg	2.3
Lemnisci	0.308 g	32 mg	10.4

Extraction of tissue lipids and separation of major lipid classes followed standard procedures using chloroform and methanol and Silica Gel G plates having a thickness of 250 microns. The first development of the plates consisted of chloroform/methanol/water/ammonium (130:70:8:0.5, by vol) followed by development in the second direction with a mixture of chloroform/acetone/methanol/acetic acid/water (100:40:20:10, by vol). The various spots were detected with iodine vapors.

The technique of Morrison and Smith (10) was followed for esterification of neutral and phospholipids. Dragendorff's reagent (for choline-containing compounds), diphenylamine (for glycolipids), ninhydrin (for free amino compound), 2,7-dichlorofluorescein (for saturated and unsaturated lipids), and Rhodamine 6G were used to identify the various lipid fractions.

The methyl esters were further purified by one-dimensional development on thin layer plates using benzene as the developing solvent.

Methyl esters from neutral and phospholipids were fractionated by gas liquid chromatography (GLC) in a Beckman GC-45 instrument equipped with a flame ionization detector and a 1/8" i.d., 6 ft glass column packed with SP-2340 (76% cyanopropyl) on 100/120 mesh Chromosorb WAW (Supelco, Inc., Bellefonte, PA), programming from 70 C to 210 C, with a helium flow rate of 20 cc/min. Methyl esters were identified by comparing their absolute retention times with those of authentic methyl esters. Absolute identification requires more than a single column since saturated esters may coincide with shorter unsaturated esters on any given column.

RESULTS

The major phospholipids detected were phosphatidylcholine and phosphatidylethanolamine. Small amounts of sphingomyelin and lysophosphatidylcholine were also detected in each fraction by using two-dimensional thin layer chromatography (TLC) and charring with 50% sulfuric acid.

The percentage of lipid found in the body wall, lemnisci, and pseudocoel is shown in Table I. Note that the lemnisci contain much more lipid than either the body wall or pseudocoel.

By comparing the fatty acids of the phospholipids with those of the neutral lipids (Table II and Table III), it is obvious that those fatty

TABLE II

Nonvolatile Fatty Acids of the Neutral Lipids[a]

Fatty acids[b]	Body wall	Lacunar fluid	Pseudocoel	Lemnisci	Lemnisci fluid
8:0		tr			tr
10:0	tr[c]	tr	tr	tr	tr
11:0	0.2		tr	0.1	
W	0.4		1.3	2.3	
12:0	0.3	tr	tr	0.7	tr
13:0	1.5	1.0	0.8	1.0	1.6
14:0	4.8	4.4	1.8	4.0	8.2
14:1	10.1	11.3	3.5	11.3	13.1
16:0	17.2	19.3	5.3	15.0	12.7
18:0	13.4	18.5	8.2	13.9	11.6
18:1	13.8	13.2	9.4	10.5	9.9
18:2, 20:0	10.4	11.4	15.0	8.5	8.3
18:3, 20:1	6.3	8.6	11.7	6.6	7.6
20:3	5.9	5.0	11.1	5.6	7.0
22:1	3.3	2.2	7.0	5.0	6.7
24:1	2.9	1.7	4.3	4.9	6.8
X	3.4	1.3	6.9	5.0	3.9
22:6	2.7	.8	2.9	6.2	1.8
Y	2.6	1.4	9.2	0.2	.9
Z	tr	.3	tr	0.2	.4

[a]Methyl esters as percent of total.

[b]Carbon chain: double bond number; W, X, Y, Z are unknown fatty acids.

[c]Trace amounts present; less than 0.1%.

TABLE III

Nonvolatile Fatty Acids of the Phospholipids[a]

Fatty acids[b]	Body wall	Pseudocoel	Lemnisci
10:0	tr[c]	tr	tr
11:0	tr	tr	tr
12:0	tr	0.1	tr
13:0	tr	tr	0.3
14:0	1.5	1.5	1.9
14:1	3.9	2.8	3.1
16:0	4.7	5.8	5.8
16:1	13.0	10.7	10.3
18:0	14.3	14.7	15.6
18:1	15.9	16.4	16.7
18:2, 20:0	13.3	13.9	15.8
20:2	12.0	10.4	10.9
20-3	6.8	8.1	7.2
22:1	5.1	7.1	4.2
W	3.9	3.1	2.8
24:1	1.9	1.3	1.1
22:6	0.9	0.5	0.4
X	0.2	0.2	tr
Y	4.6	2.7	3.1

[a]Methyl esters as percent of total.

[b]Carbon chain: double bond number; W, X, Y are unknown fatty acids.

[c]Trace amounts present; less than 0.1%.

acids with 18 carbons accounted for the majority of fatty acids in each fraction. The degree of unsaturation of identified neutral lipid fatty acids averaged about 60% while the degree of unsaturation of phospholipids was about 71%. This large degree of unsaturation agrees with that observed in cestodes (1) and other acanthocephalans (11). Previous work by Beames and Fisher (11) did not indicate nonpolar lipids of a chain length greater than 20 carbons, but as shown in Table II, 22:1, 24:1 and 22:6 are present along with unknown fatty acids X, Y, and Z, which also have a chain length greater than 20 carbons. Table II indicates that the phospholipids also contain fatty acids longer than 20 carbons. In making a comparison between fatty acids found in this study with those reported by Beames and Fisher (11), it should be noted that the odd carbon, short chain fatty acid 13:0 was detected in both studies. However, 11:0, 14:1 and 20:3 are reported for the first time in this study. The differences observed between these two studies are probably the result of different extraction procedures or esterification processes. The method of Morrison and Smith (10) which was used in this study was designed to esterify fatty acids of a higher carbon chain length by the addition of benzene and methanol to the reaction mixture.

The fatty acid percentages of phospholipids do not appear to be different between different worm fractions; but if one considers the fatty acids of the neutral lipids, one can see that there is a substantial difference between the body wall, lemnisci, and pseudocoel, particularly with respect to 14:1, 16:0, 18:2, 18:3, 20:1, 20:3 and 22:1. The lemnisci fluid and lacunar fluid from their respective tube systems are also significantly different for most carbon lengths.

DISCUSSION

Only limited information is available on lipids in Acanthocephala. Neutral lipids and phospholipids of male and female *M. hirudinaceus* and *Moniliformis dubius* were studied by Beames and Fisher (11). In this study, the fatty acids of the whole worm were extracted and analyzed by GLC. These authors reported that males of both species had 1.7 times more lipid than females, and both species had C-10 through C-21 fatty acids. They reported 16 nonvolatile acids in *M. hirudinaceus* and 29 fatty acids in *M. dubius*. Only fatty acids from the neutral lipids were analyzed by GLC. Fatty acids from the phospholipids were not studied by Beames and Fisher (11), nor have they been reported previously from Acanthocephala (7,12).

The mechanism of fatty acid absorption, metabolism, and incorporation has not been determined in Acanthocephala, although it is clear from the studies of Byram and Fisher (13) that pinocytosis is a major activity of the tegument in *M. dubius* and may participate in

the uptake of these molecules throughout this phylum. However, the importance of other structures in internal manipulation of lipids is poorly understood even including the lemnisci, which have long been suspected of participating in lipid metabolism (14). Pflugfelder (15) reported that the lemnisci and praesoma were sites of fat absorption. Later Crompton and Lee (16) proposed that lipid entered through the lemnisci and then into the body cavity following absorption through the praesoma area. The lipid was then thought to move through the body wall and into the radial layer of muscle, where it was utilized. However, Hammond's (17) studies ruled out the lemnisci as the site where lipid entered this parasite. He showed, with the use of labeled substrates and radioautographs, that lipid absorption took place over the entire body surface (metasoma area). Hammond indicated that lipid precursors, produced from carbohydrate metabolism, were absorbed by the lemnisci from the pseudocoel. Once in the lemnisci, they aggregated into lipid bodies and were synthesized into neutral fats. He suggested that lipid then traveled through the lacunar system to the praesoma area for excretion to the outside.

Table I is in agreement with the work of Crompton and Lee (16) and Hammond (17,18) in that the lemnisci contain a much higher percent lipid than the body wall. However, this does not necessarily support the idea of Bullock (19) that the lemnisci contain lipase activity, but it does suggest that the metabolic role of the lemnisci is different from that of the pseudocoel or body wall. If the work of Hammond (17) is verified, then the praesoma and lemnisci have no unique role, if any, in lipid absorption, but have a major role in fatty acid chain elongation following absorption by the tegument. Nevertheless, the chloroform-methanol extracts of the lemnisci as well as those of the body wall have a green-brown discoloration not observed in samples from the pseudocoel. The simplest explanation of this color seems to be that it represents bile pigments from the intestinal fluid, and this suggests that the absorption issue may not yet be resolved satisfactorily.

Fatty acids of neutral lipids (Table II) apparently undergo some biochemical modification as they enter the pseudocoel. As indicated in these results, there is a substantial reduction in the percentages of 14:1, 16:0, 18:0 and 18:1 in the pseudocoel as compared to the body wall and lemnisci. At the same time, there is a substantial increase in the amount of 18.2, 20:0, 18:3, 20:1 and 20:3 in the pseudocoel. It is known that in other metazoans (20) palmitic acid (16), which has the largest percentage change of fatty acids in the pseudocoel, is the one most readily modified. It is possible that both elongation and desaturation may be operative in *M. hirudinaceus* as the fatty acids enter the pseudocoel because, in order to increase the amount of 18:2, 20:0 and 20:3, there must be two-carbon additions. This same type of change in fatty acid composition of phospholipids (Table III) does not occur. A similar modification of C-16 and C-18 fatty acids, as suggested above, has been observed by Buteau et al. (21). Finally, it should be noted that *M. hirudinaceus* does not smell like it excretes volatile fatty acids, certainly not to the extent of many of the ascarids.

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Trans Fatty Acids: Positional Specificity in Brain Lecithin

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ABSTRACT

Fifteen-day-old rats were divided into three groups: one group received an intracerebral injection of 5 μCi of 9-*trans*,12-*trans* [1-^{14}C] octadecadienoic acid; the second group was given 5 μCi of the same compound plus an equal wt of nonradioactive all *cis* arachidonic acid; the third group was given 5 μCi of 9-*trans* [1-^{14}C] octadecenoic acid. All animals were sacrificed 8 hr after injection. Glycerophosphocholine (GPC) was isolated and partically deacylated with phospholipase A_2 from *Crotalus Adamanteus* venom. The results of this study were as follows: 1) after *t*[1-^{14}C] 18:1 injection, there was twice as much radioactivity in the 1-position as in the 2-position; 2) when *tt*[1-^{14}C] 18:2 was injected, more than 90% of the total radioactivity was found in the 2-position; 3) following *tt*[1-^{14}C]-18:2 + nonradioactive arachidonate injection, ca. 75% of the total radioactivity still remained in the 2-position; and 4) all of the injected [1-^{14}C]-tracers showed evidence of undergoing β-oxidation to form acetyl-CoA, which was converted to radioactive palmitate. The possibility is discussed that the observed distribution pattern of the injected radioactive tracers may be attributed to tissue metabolic specificity. Ramifications of the deposition of dietary *trans* fatty acids in the brain during the developmental stage of the central nervous system are also discussed.

INTRODUCTION

Selectivity of acyltransferases that control the incorporation of nutrient fatty acids into membrane lipids is a subject of active investigation (1). Akesson et al. (2,3) have reported on the in vivo synthesis of phosphatidate (PA) after intraportal injection of [^{3}H] palmitate and [^{14}C] linoleate. They found that 90% of the palmitate was in the 1-position in PA, whereas, 90% of the linoleate was found in the 2-position. Trewhella and Collins (4) administered [^{32}P] phosphate, [^{3}H] choline, and [^{14}C]-ethanolamine simultaneously to rats. They reported that 1-palmitoyl-2-linoleoyl glycerophosphocholine (GPC) was formed mainly via CDP choline and diglyceride, the diglyceride being formed from phosphatidic acid. This selectivity may not be universal for all tissues. For example, when [^{14}C] linoleate was injected intracerebrally (5), 53-61% of the radioactivity was found in the 2-position of brain GPC, indicating that an appreciable portion of the radioactivity of [^{14}C] linoleate was in the 1-position. This distribution is markedly different from that reported in rat liver GPC (6,7). *Trans* fatty acids behave differently from their *cis* counterparts; for example, 9-*trans* octadecenoic acid was found predominantly in the 1-position of GPC whereas its *cis* counterpart, oleic acid, was found predominantly in the 2-position of liver GPC (8). The absence of reports on the esterification of *trans* fatty acids in the brain led us to undertake this study. Our results show that the all *trans* 9,12-octadecadienoic acid was predominantly esterified in the 2-position of brain GPC whereas, 9-*trans* octadecenoic acid was esterified in the proportion of 65:30 in positions 1 and 2, respectively.

MATERIALS AND METHODS

9-*Trans*,12-*trans* [1-^{14}C] octadecadienoic acid (linoelaidic acid), 52.5 mCi/mM, and 9-*trans* [1-^{14}C] octadecenoic acid (elaidic acid), 55 mCi/mM, were purchased from DHOM Products (Hollywood, CA). A fatty acid-albumin complex was prepared as described earlier (9).

Five 15-day old rat pups, with an approximate mean weight of 23 g, were given 5 μCi of *tt*[1-^{14}C] 18:2 as the albumin complex by intracerebral injection. These animals were sacrificed 8 hr later. Another group of five 15-day-old pups (the same size as above) was given 5 μCi of *tt*[1-^{14}C] 18:2 + equal μM of nonradioactive 20:4 and killed 8 hr later. A third group of ten 15-day-old rat pups was given 5 μCi of *t*[1-^{14}C] 18:1 and sacrificed 8 hr later.

The brain tissue was excised, washed free of blood, and quickly blended in cold chloroform/methanol (2:1, v/v). The extract was purified by the method of Folch et al. (10) to obtain total lipids.

Distribution of radioactivity in various lipid components was determined by thin layer chromatography (TLC) methods using pentane/ether/acetic acid (80:20:1, v/v) for neutral lipids and chloroform/methanol/acetic acid/water (100:50:16:8, v/v) as the solvent systems for phospholipids (11).

Isolation and Purification of Lecithin

The total lipids were fractionated according to the method described by Rouser et al. (12).

TABLE I

Percent Incorporation and Distribution of Radioactivity in Various Nonpolar and Polar Lipid Components of Brain Lipid 8 hr Following Intracranial Injection

	Tracer			
	[1-^{14}C] 9 *t*, 18:1	[1-^{14}C] 9,12 *tt* 18:2	[1-^{14}C] 9,12 *tt* 18:2 + all *cis* 20:4	[1-^{14}C][a] 9,12 *cc* 18:2
% Dose incorporated[b]	10.6	8.8	11.9	15.9
% Distribution in total lipids				
Sterol esters	0.2	0.3	0.3	0.3
Acyl glycerides	6.5	1.8	2.2	2.1
Free fatty acids	1.9	1.4	1.2	1.3
Free sterol	7.8	7.5	6.7	5.7
Polar lipids	83.5	88.5	89.7	90.7
% Distribution in major PL				
Choline phosphoglycerides	50.3	52.4	55.4	52.5
Ethanolamine phosphoglycerides	28.0	36.0	24.6	16.8
Serine phosphoglycerides	21.1	11.2	19.9	14.9

[a]Values as reported earlier in Ref. 15.

[b]Dose incorporated = $\frac{\text{Radioactivity in TL/g wet brain}}{\text{Dose injected } (\mu\text{Ci})} \times 100$.

The isolated lecithin fraction was first tested for purity by TLC and then subjected to hydrolysis by *Crotalus Adamanteus* (Sigma, St. Louis, MO) as suggested by Robertson and Lands (13). The completeness of hydrolysis of fatty acids at the 2-position was checked at 1/2 hr intervals by withdrawing a small aliquot and testing this on a small TLC plate using chloroform/methanol/formic acid/water (70:28:7.5:2.5, v/v) as the developing solvent (14). After hydrolysis, the mixture of free fatty acids, (liberated from the 2-position), and lysolecithin, were separated from each other on a 5 x 20 cm SiO_2-TLC plate using the above solvent mixture. Brief exposure to iodine fumes resulted in visualization to mark the lipid areas. The plate was scanned for radioactivity by a Packard Radiochromatogram TLC scanner (Model 7200). Areas under the peaks were measured by triangulation. Visualized bands from several TLC plates were then collected to obtain adequate amounts of fatty acids and lysolecithin, which were methylated overnight (14) to obtain fatty acid methyl esters. Analysis of the fatty acid methyl esters obtained from the lysolecithin fraction represented the fatty acids at position 1, and the methyl esters obtained from the fatty acids liberated by the venom from the 2-position represented those at the 2-position. Distribution of radioactivity was measured using a Packard Radio-GLC model 824 as described before (14), which measures mass and radioactivity simultaneously.

RESULTS

Table I shows the % of the injected dose that was retained in the brain 8 hr after injection. It seems that more radioactivity was retained in the brain after *cc*-18:2 injection than after *tt*-18:2. The distribution of radioactivity in

TABLE II

Positional Distribution of Fatty Acids in Brain Lecithin (GPC)[a]

Fatty acid	1-Position	2-Position	Reconstituted Pos. 1 + Pos. 2 2	Total GPC
16:0	65.0	42.1	53.5	49.5
16:1	1.7	5.5	3.6	5.9
18:0	17.6	3.2	10.4	7.9
18:1	13.3	26.2	19.7	20.8
18:2	---	3.4	1.7	3.2
20:4	---	11.7	5.8	7.4
22:6	---	3.8	1.9	2.6

[a]Average of triplicate analysis of pooled brain samples.

TABLE III

Positional Distribution of Radioactivity in Brain Lecithin 8 hr Following I.C. Injection of [1-^{14}C] *t*-18:1, [1-^{14}C] *tt*-18:2 and Radioactive *tt*-18:2 + Nonradioactive 20:4

	% Radioactivity by TLC scanner			
	9-*trans* 18:1 injection	9,12-*tt* 18:2 injection	9,12-*tt* 18:2 + all *cis* 20:4	9,12-*cc* 18:2[a] injection
Position 1	65.6	9.4	25.2	42.9
Position 2	29.4	90.3	74.8	57.1

[a]Average of values reported by Baker and Thompson (5).

various components of the total brain lipids indicated that the free fatty acid fraction contained less than 2% of the total activity indicating almost complete incorporation of all the tracers into lipids. In most cases, polar lipids and free sterols accounted for the major portion of the total radioactivity. As far as radioactivity in choline phosphoglycerides is concerned, about 1/2 of the total radioactivity was found in this component irrespective of the tracer injected. The differences in distribution of radioactivity after the all *trans* and the all *cis* injection (15) were noticed in other polar lipid fractions. For example, the glycosphingolipids contained about 16% of the total radioactivity following all *cis*-18:2 administration, whereas, when the other tracers were injected, less than 1% was found in this component.

Table II shows the average values of triplicate analysis of fatty acids obtained from total and partially hydrolyzed GPC from pooled brain tissue lipids. Unsaturated fatty acids containing two or more double bonds were not found among the fatty acids from position 1 of GPC. On the contrary, polyunsaturated fatty acids formed ca. 19% of the fatty acids in the 2-position of GPC. Reconstituted values, obtained by averaging the fatty acid concentrations at positions 1 and 2, were very close to those found when the total unhydrolyzed lecithin was subjected to methanolysis and fatty acid composition was determined. This indicated that hydrolysis was specific to the 2-position and that all the fatty acids were liberated from the 2-position. The products of phospholipase-A_2 hydrolysis were analyzed for radioactivity distribution by the radio-TLC scanner (Table III). After *t*[1-^{14}C] 18:1 injection, there was twice as much radioactivity in the 1-position than in the 2-position. When *tt*[1-^{14}C] 18:2 was injected, more than 90% of the total radioactivity was found in position 2. Finally, when nonradioactive arachidonate in equimolar amount was injected mixed with radioactive *tt*-18:2, ca. 75% of the total radioactivity still remained in the 2-position. The radioactivity in the 1-position, however, increased almost 3 times when 20:4 was simultaneously administered as compared to linoelaidate alone.

The total radioactivity in any position may not come from the injected tracer, since the possibility of metabolic transformation of the injected tracer does exist. Therefore, radio gas liquid chromatography (GLC) analysis was done to examine this possibility. Table IV shows that the injected tracers had indeed been metabolized to give rise to other radioactive fatty acids in the brain. It is thus necessary to compare the values in Table IV with those in Table III. After injection of *t*[1-^{14}C] 18:1, 87% of the total radioactivity in position 1 came from the injected tracer and the remaining 12.5% came from palmitate synthesized from

TABLE IV

Radioactivity in Fatty Acids of 1- and 2-Positions of Brain Lecithin Following I.C. Injection of [1-^{14}C] 9-*trans*-18:1, [1-^{14}C] 9,12-*trans, trans*-18:2 and Radioactive 9,12 *trans, trans*-18:2 + Nonradioactive Arachidonic Acid

Fatty acid (Ratio-GLC)	9 *t*-18:1		9,12 *tt*-18:2		9,12 *tt*-18:2 + 20:4	
	1 Pos	2 Pos	1 Pos	2 Pos	1 Pos	2 Pos
16:0	12.5	24.2	trace	20.9	69.6	13.4
c-18:1	---	---	---	8.8	---	---
t-18:1	87.4	75.7	---	---	---	---
tt-18:2	---	---	trace	70.3	30.4	86.5

acetyl CoA derived from β-oxidation of the tracer fatty acid. In addition, the values in Table IV show that although only about 30% of the total radioactivity was originally present in the 2-position of the lecithin, this radioactivity was contributed largely by radioactive elaidate, the injected tracer (Table III). In the case of *tt*-18:2 injection, the radioactivity in the 1-position of brain GPC was only 10% of the total and, therefore, inadequate for radio GLC analysis. However, the radioactivity (90.3%, Table III) in the 2-position consisted largely of the injected tracer (Table IV). When equimolar amounts of radioactive *tt*-18:2 and nonradioactive all *cis* 20:4 were administered simultaneously, there was a slight decrease in the radioactivity in the 2-position, and again this radioactivity consisted largely of the injected tracer. However, the radioactivity in the 1-position (25.2%, Table III) was largely due to palmitic acid, which was formed from the injected radioactive *tt*-18:2. The values reported after injection of *c,c*-18:2 are from the report of Baker and Thompson (5) and are included for comparison only.

DISCUSSION

Fatty acyl substituents of phospholipids are, for the most part, nonrandom and may indicate tissue specificity. It has been found that lecithins from lung and the brain tissue showed many similarities but differed from the liver lecithins. In addition, lecithins containing one saturated and one polyunsaturated fatty acid constituent were abundant in the liver, whereas the brain lecithins were largely of the 1-palmitoyl-2-oleoyl variety (7). When labeled fatty acids were injected directly into the brain (5) or were incubated with respiring slices of rat cerebrum (16), it was observed that radioactive linoleic acid, for example, was not restricted to the 2-position in lecithin, but was more or less evenly distributed between both acyl positions. This is in contrast to the results found in the liver following an intraportal in vivo injection of linoleate (3,4). Esterification of *trans* fatty acids in lecithin biosynthesis in the liver was studied by Lands et al. (17). They found that the *trans* configuration was slightly favored over the saturated fatty acids at the 1-position. In these experiments, linolaidate was esterified rapidly at the 1-position when the substrate was 2-acyl GPC. Feeding fats supplemented with various *trans* isomers, Privett et al. (18) found that in rat liver lecithin, the *trans, trans* 18:2 isomer was esterified predominantly at the 1-position. The differences between the present study and those in the liver may be attributed to tissue metabolic specificity and possibly due to the method of introduction of the labeled compounds. In the liver, de novo synthesis, deacylation-reacylation reactions, and the stepwise methylation pathways, can lead to the biosynthesis of lecithin, but the brain lacks the capacity to carry out the stepwise methylation of glycerophosphoethanolamine to GPC, although the other reactions have been clearly demonstrated (19,20). It has also been recognized that the distribution of fatty acids between positions 1 and 2 is influenced by competition from other fatty acids (17,18). It was found that the *trans*-9,*cis*-12-18:2 isomer distributed evenly between both positions in the absence of competition from the all *trans* 9,12-18:2; but in the presence of the all *trans* isomer, the *trans,cis* isomer favored esterification at the 2-position. Similarly, Privett, et al. (18) found that in the presence of saturated acids, the all *trans* isomer was incorporated into the 2-position rather than the usual 1-position. Since earlier work (5,7) had indicated that all *cis* 20:4 predominantly sought the 2-position of brain as well as liver GPC, whereas *c,c*-18:2 is distributed about evenly between the 1 and 2 position (5), simultaneous injection of 20:4 and *tt*-18:2 was included in the present work. This resulted in some decrease in the amount of radioactivity from *tt*-18:2 found in the 2-position, in agreement with the observations cited above. Thus, all *trans* 18:2 sought the 2-position, whereas *trans* 18:1 favors position 1 in the brain GPC. The finding of radioactivity in palmitate at the 2-position, in general, could be due to the fact that brain lecithin contains ca. 15% dipalmitoyl lecithin (21). After the injection of *tt*-18:2, there was only a very small amount of activity in the 1-position of brain lecithin, and it could not be further analyzed by radio-GLC due to instrument limitation.

If *trans* fatty acids are deposited in the brain during the developmental stage of the central nervous system as a result of dietary intake, they would be incorporated into the brain lecithin. The *trans* fatty acids could be removed by the action of phospholipases and utilized for the synthesis of cholesteryl esters.

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A Comparative Study of the Lipid Composition of Isolated Rat Sertoli and Germinal Cells

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ABSTRACT

The lipid composition of enriched preparations of sertoli cells and of germinal cells, isolated from the testes of mature rats, has been investigated. Sertoli cells contained a much lower content of phospholipids (in particular, much less phosphatidylcholine and phosphatidylethanolamine) and a higher content of triacylglycerols than did germinal cells. In addition, the Sertoli cells had a higher ratio of esterified to unesterified cholesterol than did germinal cells. Total lipids of Sertoli cells contained considerably lower levels of palmitic and docosa-4,7,10,13,16-pentaenoic acids and higher levels of stearic and oleic acids than did the total lipids of germinal cells. The major phospholipid classes and the triacylglycerols of Sertoli cells similarly contained less palmitic and docosa-4,7,10,13,16-pentaenoic acids, more stearic and oleic acids and also more arachidonic acid than did the corresponding lipid classes of the germinal cells. Minor differences between cell types were also noted for the content of palmitoleic, linoleic, docosa-7,10,13,16-tetraenoic, docosa-4,7,10,13,16,19-hexaenoic and tetracosa-9,12,15,18-tetraenoic acids.

INTRODUCTION

It has become increasingly evident in recent years that lipids are of importance to the development and functioning of testicular tissue. Of particular interest is the fact that 22-carbon polyenes (docosa-4,7,10,13,16-pentaenoic acid in the rat; docosa-7,10,13,16-tetraenoic acid in the rooster; and docosa-4,7,-10,13,16,19-hexaenoic acid in the human) accumulate in testes in large quantities during the time of sexual maturation (1,2). Numerous factors which adversely affect testicular development are associated with decreased levels of these fatty acids (3).

The testis is an organ composed of many different cell types, of which some are hormonal in nature while others act as precursors of spermatozoa. Thus, in order to define more clearly the role that specific lipids may have in testicular tissue, it is necessary to study their biochemistry at a cellular level. Recently, we initated such a study and reported the lipid composition of enriched fractions of the major germinal cell types of rat testes, separated by centrifugal elutriation (4). Of major interest was the finding that spermatids contained large quantities of docosa-4,7,10,13,16-pentaenoic acid ($\cong$20% of the total fatty acids), while spermatocytes contained much smaller amounts of this polyene ($<$10% of the total fatty acids).

In the present communication, we summarize the lipid composition of enriched fractions of Sertoli cells and compare it with that of the germinal cells isolated from the testes of mature rats by means of enzymatic treatments. Sertoli cells, although nongerminal, have been implicated in a diverse number of functions concerned with the regulation of spermatogenesis (for a review see (5)). One such role of the Sertoli cells is to provide nutrition for developing germ cells. In this regard, many authors have suggested that Sertoli cell lipid droplets are absorbed into spermatids during specific stages of spermatogenesis in which the spermatids are embedded in Sertoli cell cytoplasm (6,7). The lipid biochemistry of Sertoli cells may, therefore, have an important role in testicular development and in spermatogenesis.

EXPERIMENTAL PROCEDURES

Sprague-Dawley rats of 60-150 days of age maintained on Purina laboratory chow were used in these studies. Enriched fractions of Sertoli cells and of germinal cells were prepared from testicular tissue essentially according to the method of Steinberger et al. (8) with minor modifications. Each rat testis was sliced with a razor blade into 10-12 pieces and incubated in 30 ml of Ca^{++} free Hank's balanced salt solution containing 0.25% (w/v) trypsin for 15 min at 37 C. The tubules were then washed on top of a 125 μ stainless steel grid with cold Hank's balanced salt solution (containing Ca^{++}), collected off the grid, and incubated in 30 ml of the same buffer containing 0.1% w/v collagenase for 65 min at 37 C with frequent and vigorous agitation. Five minutes before the end of this incubation, 20 μg of DNAse was added to the suspension. Following the collagenase treatment, the cell suspension was filtered through a 75 μ stainless steel grid. Germinal cells and peritubular cells passed through the grid while Sertoli cells remained interconnected in clusters on top of the grid. They were thus easily separated. Both the filtrate (primarily

TABLE I

Composition of Total Lipids in Enriched Fractions of Sertoli and Germinal Cells Isolated from Mature Rats[a]

Lipid class	Cell type		P value
	Sertoli	Germinal	
Phospholipids	5.3 ± 0.58[b] (8)	17.4 ± 0.59 (8)	<0.001
Triacylglycerols	5.0 ± 0.25 (5)	3.2 ± 0.13 (5)	<0.001
Cholesterol	2.5 ± 0.33 (4)	4.0 ± 0.21 (6)	<0.01
Cholesteryl esters	1.1 ± 0.13 (4)	0.4 ± 0.1 (4)	<0.01

[a]Data are expressed as μmol/g wet weight of cells. Analyses were done as described under Experimental Procedure.

[b]Mean ± S.E.; number of samples in parentheses.

germinal cells) and the cell clusters (Sertoli cells) were then pelleted and resuspended in a small volume of Hank's balanced salt solution. Air-dried smears of the fractions were fixed and stained according to Meistrich et al. (9) for differential counting of cells. The identification of cells was done using criteria described by Meistrich et al. (9). The wet weight of the cell fraction was obtained after pelleting in a tared centrifuge tube prior to the extraction of lipids. Procedures for the extraction and hydrolysis of lipids, extraction of fatty acids, and quantitative gas chromatographic analysis have been described previously (10,11). The content of lipid phosphorus, triacylglycerols, cholesterol, and cholesteryl ester was determined by standard procedures (12-14). Classes of lipids were separated by thin layer chromatography using the solvent system light petroleum/diethyl ether/acetic acid (80:20:1, v/v), and major classes of phospholipids were separated using the method of Skipski et al. (15). Quantification of the major phospholipids was done by phosphorus determination performed directly on silicic acid following the chromatographic separation. Separated classes of lipids and of phospholipids were trans-esterified by the methods of Morrison and Smith (16) prior to fatty acid analysis by gas chromatography.

RESULTS

The cell clusters obtained from the collagenase-digested rat testes contained 80-90% Sertoli cells, the remainder being mostly germinal cells. The germinal fraction contained primarily spermatids, but spermatocytes and peritubular cells were also present in smaller numbers.

The content of the major classes of lipids of Sertoli and of germinal cells is given in Table I. Sertoli cells contained less phospholipid and more triacylglycerol than germinal cells. In addition, the Sertoli cells had more cholesteryl esters and less unesterified cholesterol. The content of mono- and diacylglycerols and of unesterified fatty acids was judged by thin layer chromatography not to be significant and was not investigated further.

The fatty acid patterns of the total lipids of the separated cell fractions are given in Table II. Sertoli cells contained less palmitic and docosa-4,7,10,13,16-pentaenoic acids and more stearic and oleic acids than did the germinal cell fractions. Significant differences between the cell types were also found for palmitoleic, linoleic, docosa-7,10,13,16-tetraenoic, docosa-4,7,10,13,16,19-hexaenoic and tetracosa-9,12,15,18-tetraenoic acids.

The amounts of various types of phospholipids in these cell fractions are given in Table III. The Sertoli cells contained considerably smaller amounts of phosphatidylcholine and phosphatidylethanolamine than did the germinal cells. A greater proportion of the phospholipid of Sertoli cells than of germinal cells was composed of phosphatidylserine, phosphatidylinositol, sphingomyelin, lysophosphatides and a fraction migrating with neutral lipids. Subsequently, it was shown that the latter fraction was composed of a number of minor phosphorus-containing compounds .

The fatty acid profiles of the major phospholipid classes and of the triacylglycerols in Sertoli and germinal cells are given in Table IV. The fatty acid profiles of phosphatidylcholine, phosphatidylethanolamine, and phosphatidyl serine all contained less palmitic and docosa-4,7,10,13,16-pentaenoic acids and more stearic, oleic, arachidonic, and docosa-7,10,13,16-tetraenoic acids than did the corresponding phospholipids of the germinal cells. The fatty acid

TABLE II

Fatty Acid Composition of the Total Lipids of Enriched Fractions of Sertoli and Germinal Cells Isolated from Mature Rats[a]

Fatty acid[b]	Cell type		P Value
	Sertoli	Germinal	
16:0	28.4 ± 0.4[c]	39.1 ± 0.6[c]	<0.001
16:1	3.4 ± 0.26	1.2 ± 0.20	<0.001
18:0	12.8 ± 0.4	4.4 ± 0.5	<0.001
18:1	18.4 ± 0.7	10.0 ± 0.4	<0.001
18:2	5.0 ± 0.47	3.2 ± 0.27	<0.01
20:3	1.1 ± 0.11	0.9 ± 0.09	
20:4	17.2 ± 0.5	17.0 ± 0.6	
22:4	2.5 ± 0.46	1.3 ± 0.07	<0.001
22:5	8.3 ± 0.39	19.0 ± 0.6	<0.001
22:6	0.91 ± 0.13	2.4 ± 0.45	<0.01
24:4	1.1 ± 0.2	0.6 ± 0.10	<0.05

[a]Data are expressed as weight percent of total fatty acids.

[b]Number of carbons: number of double bonds.

[c]Mean ± S.E. of 7 samples.

TABLE III

Phospholipid Composition of Enriched Fractions of Sertoli and Germinal Cells Isolated from Mature Rats[a]

Phospholipid class	Cell type		P value
	Sertoli	Germinal	
Phosphatidylcholine	20.8 ± 1.8[b]	44.4 ± 0.5	<0.001
Phosphatidylethanolamine	17.8 ± 1.5	25.5 ± 1.2	<0.01
Phosphatidylserine	15.4 ± 1.4	11.7 ± 0.5	<0.05
Phosphatidylinositol	5.6 ± 1.0	2.0 ± 0.75	<0.05
Sphingomyelin	14.2 ± 0.5	6.3 ± 0.35	<0.001
Lysophosphatidylcholine	9.4 ± 0.86	4.3 ± 0.07	<0.001
Lysophosphatidylethanolamine	4.0 ± 0.67	1.9 ± 0.52	<0.05
"Other Phospholipids"[c]	12.3 ± 2.1	3.7 ± 0.47	<0.01

[a]Data are expressed as percent of total lipid phosphorus.

[b]Mean ± S.E. of 4 samples.

[c]Consisting of minor quantities of various phospholipids including cardiolipin, phosphatidic acid and phosphatidylglycerol.

patterns of the total phospholipids of Sertoli and germinal cells were also determined and were essentially in agreement with the data for the individual phospholipid classes. The triacylglycerols of the Sertoli cells contained considerably more stearic and arachidonic acids and less docosa-4,7,10,13,16-pentaenoic acid than did the triacylglycerols of germinal cells. Minor differences between triacylglycerols of Sertoli and germinal cells were also found in the content of palmitic, palmitoleic, oleic, docosa-7,10,13,16-tetraenoic, docosa-4,7,10,13,16,19-hexaenoic, and tetracosa-9,12,15,18-tetraenoic acids.

DISCUSSION

The lipid composition of the total germinal cells of rat testes is reflective of the data obtained from whole tissue (17) or from isolated spermatids (4). This is not surprising as germinal cells, and, in particular, spermatids, make up the majority of the testicular volume. The lipid composition of Sertoli cells, on the other hand, is quite distinct. Significant differences between Sertoli and germinal cells were found in the content of every major lipid and phospholipid class and in the content of most of the fatty acids within those classes.

Unlike germinal cell lipid, which is predominately phospholipid, Sertoli cell lipid is quite rich in triacylglycerols. It is worth noting that the testes of immature rats contain about twice as much triacylglycerol as do those of mature rats, despite the fact that spermatocytes contain less triacylglycerol than do spermatids (17). Thus, the greater concentration of triacyl-

TABLE IV

Fatty Acid Composition of the Major Phospholipid Classes and of the Triacylglycerols of Sertoli and Germinal Cells Isolated from Mature Rats[a]

Fatty acid	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine		Triacylglycerols	
	Sertoli[b]	Germinal[b]	Sertoli	Germinal	Sertoli	Germinal	Sertoli	Germinal
16:0	37.3 ± 1.2	46.7 ± 1.4[c]	23.9 ± 0.8	33.9 ± 0.9[c]	13.6 ± 0.8	25.7 ± 0.9[c]	32.0 ± 1.0	35.0 ± 0.9[e]
16:1	1.6 ± 0.42	1.0 ± 0.06	0.7 ± 0.05	0.7 ± 0.12	0.6 ± 0.15	0.5 ± 0.07	4.6 ± 0.78	2.4 ± 0.41[e]
18:0	9.5 ± 0.04	2.6 ± 0.13[c]	12.9 ± 0.8	5.8 ± 0.18[c]	35.7 ± 0.8	18.2 ± 0.8	8.7 ± 0.86	3.8 ± 0.44[c]
18:1	17.6 ± 1.0	13.2 ± 0.7[d]	11.7 ± 1.1	6.0 ± 0.38[c]	11.9 ± 0.9	7.6 ± 0.64[e]	16.0 ± 0.35	13.1 ± 1.1[e]
18:2	3.1 ± 0.28	3.8 ± 0.25	2.4 ± 0.3	1.4 ± 0.21[e]	1.4 ± 0.21	1.0 ± 0.40	5.3 ± 1.00	3.3 ± 0.38
20:3	1.0 ± 0.40	1.3 ± 0.16	2.2 ± 0.83	1.3 ± 0.16	1.0 ± 0.4	1.5 ± 0.86	0.85± 0.15	1.0 ± 0.05
20:4	24.0 ± 1.1	16.4 ± 0.3[c]	30.6 ± 1.0	16.4 ± 0.3[c]	26.6 ± 2.1	21.7 ± 1.4[e]	15.7 ± 0.4	9.0 ± 0.29[c]
22:4	1.3 ± 0.40	0.5 ± 0.12[e]	4.4 ± 0.30	0.5 ± 0.12[c]	3.5 ± 0.18	1.4 ± 0.45[d]	2.2 ± 0.20	3.6 ± 0.21[d]
22:5	3.3 ± 0.42	13.3 ± 0.4[c]	9.7 ± 0.25	26.0 ± 1.4[c]	4.7 ± 0.58	17.8 ± 1.9[c]	10.2 ± 0.5	22.0 ± 0.47[c]
22:6	1.0 ± 0.11	1.0 ± 0.14	1.6 ± 0.26	1.0 ± 0.24	0.98 ± 0.28	2.3 ± 0.70	1.7 ± 0.08	2.4 ± 0.21[e]
24:4	---	---	---	---	---	---	2.1 ± 0.08	4.1 ± 0.27[c]

[a]Data are expressed as weight percent of total fatty acids.

[b]Mean ± S.E. of 4 samples.

[c]$P \leq 0.001$.

[d]$P \leq 0.01$.

[e]$P \leq 0.05$.

glycerols in immature rats may likely be due to the larger proportion of Sertoli cells in this tissue. Sertoli cells also have a higher ratio of esterified to unesterified cholesterol than do germinal cells. Evidence has been accumulating suggesting that cholesteryl esters may serve as a source of cholesterol for steroid hormone synthesis by adrenals (18), ovaries (19), and testes (20). Perhaps cholesteryl esters play a similar role in Sertoli cells, since this cell type is known to metabolize (21) steroid hormones.

Differences between germinal and Sertoli cells in fatty acid patterns of the various lipid classes and of various types of phospholipids reflected differences seen in the fatty acid profiles of total lipids. The major differences in the total lipid fatty acid patterns of the two cell types (a lower concentration of palmitic and docosapentaenoic acids and a higher concentration of stearic and oleic acids in Sertoli cells) were present in both the triacylglycerols and the phospholipids. The higher levels of palmitoleic, linoleic and docosatetraenoic acids in the total lipids of Sertoli cells compared to germinal cells may be explained by the higher levels of palmitoleic and linoleic acids in Sertoli cell triacylglycerols and the higher level of docosatetraenoic acid in Sertoli cell phospholipids than in the corresponding lipid classes of the germinal cells. The lower concentration of docosahexaenoic acid in Sertoli cell total lipid was reflected in its lower concentration in Sertoli cell triacylglycerols. Arachdionic acid was present in higher concentration in Sertoli cell than in germinal cell triacylglycerols and phospholipids but not in total lipids. This apparent discrepancy is explained by the fact that the phospholipids of both cell fractions were richer in arachidonic acid than were the triacylglycerols, and the Sertoli cells had a higher triacylglycerol/phospholipid ratio than did the germinal cells. Similarly, tetracosatetraenoic acid was present in higher concentration in total lipids of Sertoli cells than in total lipid of germinal cell despite its lower level in Sertoli cell triacylglycerols because of the relative greater abundance of triacylglycerols in Sertoli cells compared to germinal cells.

The amounts of the various phospholipid classes in Sertoli cells were quite different from those of germinal cells. Germinal cells contained mostly phosphatidylcholine and phosphatidylethanolamine, while the Sertoli cells had a more evenly distributed phospholipid pattern. In terms of absolute quantities of phospholipid per gram of cells, the amounts of most of the minor phospholipid classes were quite similar in the two fractions. However, phosphatidylcholine and phosphatidylethanolamine were present in much lower concentrations in the Sertoli cell fraction.

This study demonstrates that the testis accumulates large quantities of docosa-4,7,10,-16,19-pentaenoic acid in the germinal cells but not in the Sertoli cells. Our previous report (4) further localized the docosapentaenoic acid of the germinal cells to the spermatids. It still remains to be determined, however, which cell type is responsible for the biosynthesis of this polyene and what the specific function of the polyene in the spermatid might be.

It has been suggested (6,7) that lipid droplets in Sertoli cells are transported into maturing spermatids (6,7). The chemical nature and biological function of this lipid are of interest. The abundance of triacylglycerols in Sertoli cells suggests tha the lipid droplets are triacylglycerol-enriched. Both spermatids (4) and Sertoli cells contain significant quantities of docosa-4,7,10,13,16-pentaenoic acid esterified as triacylglycerols (2.27 and 1.53 μmoles/g cells in spermatids and Sertoli cells, respectively). Possibly the Sertoli cells synthesize and rapidly transport docosapentaenoic acid via the lipid droplets into spermatids where the polyene serves some vital role. Further studies are required to elucidate these problems.

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In Vitro Conversion of Erucic Acid by Microsomes and Mitochondria from Liver, Kidneys and Heart of Rats

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ABSTRACT

Microsomes and mitochondria of liver, kidneys, and heart were incubated with [14-^{14}C]erucic acid in three assay media: one favorable for chain elongation (NADPH + KCN), another favorable for β-oxidation and the last one for shortening (NADP + KCN). Elongating reactions occurred mainly in microsomes, those of kidneys being very active; the mitochondria also showed some activity, heart mitochondria being, however, more active than the microsomes, when considering the amount of erucic acid activated. In the medium for β-oxidation, practically no shortened fatty acids were found. On the contrary, when β-oxidation was inhibited, and in the presence of NADP, the formation of shorter monoenes, probably in the outer membrane of the mitochondria, was observed, namely eicosenoic acid in high amount, oleic acid and hexadecenoic acid. Mitochondria from liver were very active as were those of heart, when compared with the quantity of activated erucic acid. In heart, the mitochondria shortened erucic acid into oleic acid and hexadecenoic acid, which were then probably used as energy substrates. With carnitine and without NADP, shortened fatty acids were formed in the mitochondria of liver, probably by the first reactions of β-oxidation. In this case, the proportions of oleic acid and hexadecenoic acid were higher than with NADP alone. In the presence of carnitine and NADP, the level of the chain-shortening reaction did not differ from that observed with NADP alone. It appears, therefore, that the activated erucic acid is mainly directed towards shortening reactions and not towards transfer reactions across the mitochondrial membranes.

INTRODUCTION

In several animal species, the presence in the diet of rapeseed oil rich in erucic acid (*cis*-13-docosenoic) causes various pathological disorders, especially in the heart. Many reports have dealt with the morphological aspects of the affected organs (1-5), others with the metabolic modifications brought about by the diet (6-9). As erucic acid has often been considered responsible for such disorders, many studies have been done on the metabolism of this fatty acid. The results have shown that, although erucic acid was a source of expired carbon dioxide (10-11), it was also shortened into oleic acid (12-14), particularly in the liver, and lengthened into nervonic acid (15), especially in the kidneys (16) and the lungs (17). Until now, studies on metabolic conversions have been carried out in the entire animal (12–17), in isolated organs (18) or in tissue culture (19). The purpose of this work was to determine in which cellular organelles, mitochondria or microsomes, these shortening and lengthening reactions in liver, kidneys and heart occur.

EXPERIMENTAL PROCEDURES

Isolation of the Subcellular Fractions

Experiments were performed with male Wistar rats, weighing ca. 250 g, which had been fed a balanced diet prepared by Usine Alimentation Rationnelle, Villemoisson-sur-Orge, France, and containing, as percentage by wt, 25% casein, 35% cornstarch, 25% sucrose, 5% cellulose, 5% corn oil, 4% salt mixture (20) and 1% vitamin mixture providing in mg/kg of diet:thiamine HCl, 7; riboflavin, 6.5; panthotenate, 16.5; pyridoxine HCl, 2.2; vitamin B_{12}, 0.02; DL α-tocopherol acetate, 33; menadione, 2.5; nicotinic acid, 75; folic acid, 0.5; biotin, 0.04; choline chloride 1,580; vitamin A, 8,500 IU and vitamin D, 2,020 IU. The animals were starved 20 hr before experiments. They were killed by decapitation, the organs were removed in a cold room at a temperature of 5 C and kept at 0 C. Rinsing and homogenization of tissues were done with a mixture of 0.25 M sucrose and 10 mM triethanolamine at pH 7.4. The liver was cut with scissors into very small pieces and rinsed several times. The kidneys were first taken out of their connective tissue capsule and the medullary part cut away. For the heart, only the ventricular tissue was used, and the homogenizing medium contained 10 mM EDTA at pH 7.4, to complex the calcium ions which cause mitochondrial swelling.

The fragments of tissues were homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The membranes, nuclei, and unbroken cells were sedimented at 800 x g for 10 min at 0 C. Then the mitochondria were separated by centrifuging the supernatant at 8,000 x g for 10 min at 0 C. They were resuspended and recentrifuged under the same conditions to remove contaminants. The supernatant of the first mitochondrial sediment was first centrifuged at

14,000 x g, and microsomes were fractionated from the supernatant at 105,000 x g for 45 min at 0 C. The pellet obtained was resuspended and resedimented for 30 min to rinse away the traces of cytoplasmic proteins. Mitochondrial or microsomal protein was measured according to Layne (21).

Criteria of Purity

The procedures described above were designed to minimize problems associated with contamination from other cellular components. It was not possible to check the purity of the subcellular preparations for each experiment. However, when techniques were being perfected, the integrity of the mitochondrial membranes and the essential absence of contaminating elements were checked by electron microscopy (22-23). Moreover, the mitochondrial oxygen consumption, as measured in a Gilson respirometer, indicated normal P/O ratio and normal respiratory control (24). For the microsomes, the absence of mitochondrial contamination was demonstrated by the absence of oxidation of the erucate substrate (see Results section).

Incubation Conditions

The medium used for the elongation studies was modified from Mohrhauer et al. (25) and Sprecher (26), and contained the following: Tris-HCl buffer, 120 mM (pH 7.4); inorganic phosphate, 100 mM; $MgCl_2$, 4 mM; ATP, 5 mM; CoA, 0.1 mM; NADPH, 1.25 mM; KCN, 1 mM; acetyl-CoA, 0.15 mM (for mitochondria) or malonyl-CoA, 0.15 mM (for microsomes).

The medium used for the oxidation studies contained the normal requirements for activation, plus carnitine for the transport of acyl-CoA across the inner mitochondrial membrane (27): Tris-HCl buffer, 10 mM (pH 7.4); inorganic phosphate, 10 mM; KCl, 35 mM; $MgCl_2$, 4 mM; ATP, 1 mM; CoA, 0.02 mM; DL carnitine, 2 mM; sodium malate, 1 mM.

The medium used for the shortening studies contained: inorganic phosphate, 50 mM (pH 7.6); KCl, 33 mM; $MgCl_2$, 4 mM; ATP, 1 mM; CoA, 0.05 mM; $NaHCO_3$, 2 mM; KCN, 1 mM. In addition NADP, 1 mM, which is required for this type of reaction (28), was present.

Purified [14-^{14}C]potassium erucate (35 nmol) (Centre d'Etudes Nucléaires de Saclay, Gif-sur-Yvette, France.) bound to bovine serum albumin (4 nmol) (Fraction V, fatty acid free, Sigma, St. Louis, MO) was added to 2 ml of each of the three media. The media were preincubated for 15 min at 37 C, before the addition of 2 mg of mitochondrial or microsomal protein and prior to incubation for 10 min at 37 C. Incubations were stopped by the addition of 10 ml of 10% perchloric acid for study of the acid-soluble products or by the addition of 2 ml of 10% methanol-KOH for analysis of total fatty acids.

ANALYTICAL TECHNIQUES

Products Recovered after Incubation

The perchloric acid-treated sample was filtered on Millipore filters (diameter of pores 0.45 μ). The radioactivity of the filtrate corresponds to that of the small molecules formed during β-oxidation and soluble in aqueous perchloric acid. They are referred to as acid-soluble products (ASP). The deposit on the filter was washed clean of the remaining free fatty acids by three five-hour contacts with petroleum ether. The radioactivity which remained on the filter corresponds to that of fatty acid molecules activated by CoA, present as acylcarnitines if the medium contained carnitine, or incorporated into phospholipids. The erucic acid activated was designated as the total radioactivity remaining on the filter and in the filtrate (acid-soluble fraction).

Negligible amounts of radioactive CO_2 were formed during these short times of incubation, especially in the presence of cyanide, as was anticipated. Thus, the formation of radioactive CO_2 was not measured in these present studies.

Fatty Acid Analysis

The methanol-KOH-treated sample was saponified for 1 hr at 70 C as prescribed by Sprecher (26). After acidification with HCl, the fatty acids were extracted by 10 ml of chloroform, then methylated in a sealed tube in methanol-H_2SO_4 (50:1, v/v) at 100 C for 3 hr. After concentration of the medium with nitrogen and neutralization with 5% K_2CO_3, the methyl esters were extracted by pentane, separated by gas liquid chromatography, collected individually on glass wool, as prescribed by Bézard et al. (29), and their radioactivity was measured.

Measurement of Radioactivity

Prior to radioactivity determination, aqueous samples were solubilized in an appropriate scintillating liquid (Instagel, Packard) and a solution of Permafluor III (Packard)-Xylene (1:9, v/v) was added to lipid samples. All measurements for radioactivity were carried out with a Packard Tricarb 544 Spectrometer.

RESULTS

For the first series of experiments, a medium

TABLE I

Amounts of Erucic Acid Activated by CoA[a] and Percentage[b] Transformed into Nervonic Acid in a Medium Favorable for Elongation

	Liver		Kidneys		Heart	
	Mitochondria	Microsomes	Mitochondria	Microsomes	Mitochondria	Microsomes
nmol of activated erucic acid[c]	14.4; 14.7[d]	6.7; 7.3	8.6; 8.8	2.1; 2.5	10.3; 11.2	3.7; 4.4
Percent found in nervonic acid	0.2; 0.4	2.4; 2.8	0.4; 0.7	18.2; 21.4	0.3; 0.5	0.1; 0.6

[a]Comprises as end products erucoyl-CoA recovered unreacted or transformed after incubation (See experimental section).

[b]Percentage of the activated erucic acid.

[c]Produced in the presence of 35 nmol of erucic acid and 2 mg of mitochondrial or microsomal protein.

[d]The two values were obtained from two measurements on a preparation of mitochondria or microsomes from ten rats.

TABLE II

Amounts of Erucic Acid Activated by CoA[a] and Percentage[b] Transformed into Oleic Acid (18:1) or Recovered as Acid-soluble Products (ASP)[e], in a Medium Favorable for β-Oxidation

		Liver		Kidneys		Heart	
		Mitochondria	Microsomes	Mitochondria	Microsomes	Mitochondria	Microsomes
nmol of activated erucic acid[c]		12.0; 12.3[d]	2.7; 2.8	3.4; 3.7	0.6; 0.9	5.1; 5.9	0.3; 0.4
Percent found in	18:1	0.3; 0.5	0.5; 1.0	1.3; 1.9	0.1; 0.5	0.5; 0.9	0
	ASP	38.8; 40.6	0	19.6; 21.5	0	51.0; 70.0	0

[a,b,c,d]See footnotes in Table I.

[e]Small molecules formed during β-oxidation and soluble in aqueous perchloric acid.

favorable for elongation of fatty acid was used. The results (Table I) show that the greatest lengthening activity was possessed by the microsomal fraction. Kidney microsomes were the most active, whereas those of heart were not very active at all. The activity of liver microsomes was intermediate, but the effects should not be underestimated, considering the mass of microsomes in the entire organ. Liver mitochondria were nine times less active than the corresponding microsomes.

For the second series of experiments, a medium favorable for β-oxidation was used. The results (Table II) show that the mitochondria of the three organs studied degraded erucate, but the extent varied according to the organ. The liver mitochondria activated more erucic acid than did those from kidneys or heart. The activated erucic acid β-oxidized (ASP formed) was found in high percentage in the three series of mitochondria, but in each case the amount of radioactive oleate appearing in the medium was almost negligible. The presence of oleate in only trace amount, and the absence of any radioactivity in the acid-soluble products with microsomes from the three organs proved their inability to produce smaller molecules from erucate.

Moreover, the absence of radioactivity demonstrated the absence of contamination of the microsomal fraction with mitochondria.

In the third series of experiments, attempts were made to determine whether the chain-shortening reaction might be the reverse of elongation. Thus, oxidation was stopped by the addition of cyanide, and the absence of carnitine prevented the penetration of fatty acids into the mitochondria and also a possible accumulation of fatty acids bound to carnitine. NADP was used in place of NADPH, the latter being necessary for the reducing reactions of elongation.

Under these conditions (Table III), it can be seen that the radioactivity of the acid-soluble products was very low in each case, or even nonexistent. But, contrary to previous observations, monounsaturated fatty acids shorter than erucic acid appeared, mainly after incubation

TABLE III

Amounts of Erucic Acid Activated by CoA[a] and Percentage[b] Recovered in Eicosenoic Acid (20:1), Oleic Acid (18:1), Hexadecenoic Acid (16:1) and in the Acid-soluble Products (ASP)[e], in a Medium Favorable for Shortening

		Liver		Kidneys		Heart	
		Mitochondria	Microsomes	Mitochondria	Microsomes	Mitochondria	Microsomes
nmol of activated erucic acid[c]		13.4; 13.9[d]	3.9; 4.3	2.2; 2.4	1.9; 2.1	1.9; 2.1	0.3; 0.4
Percent found in	20:1	5.7; 6.2	2.0; 2.7	5.0; 6.1	1.4; 2.1	6.7; 7.6	
	18:1	1.2; 1.5	0.1; 0.4	1.9; 2.9	0.7; 0.9	5.1; 5.8	[f]
	16:1	0.3; 0.5	0.1; 0.4	0.7; 1.1	0.4; 0.7	2.5; 3.2	
	ASP	4.6; 4.8	0.2; 0.6	0.3; 0.8	0	0.1; 0.7	0

[a,b,c,d,e]See footnotes in Table II.

[f]Activation and shortening did not occur to a sufficient extent to give any significant figures.

TABLE IV

Amounts of Erucic Acid Activated by CoA[a] and Percentage[b] Recovered in Eicosenoic Acid (20:1), Oleic Acid (18:1), Hexadecenoic Acid (16:1) and in the Acid-soluble Products (ASP)[e], after Shortening by Liver Mitochondria[f]

		Shortening medium			
NADP 1 mM		–	+	–	–
DL Carnitine 2 mM		–	–	+	+
nmol of activated erucic acid[c]		13.3; 13.7[d]	12.7; 13.0	17.4; 17.7	17.5; 17.7
Percent found in	20:1	2.0; 2.2	12.2; 12.5	7.5; 7.7	11.6; 12.0
	18;1	0.6; 0.8	5.6; 5.8	4.7; 4.9	5.3; 5.5
	16:1	0.2; 0.3	3.8; 4.0	5.7; 6.0	3.6; 3.8
	ASP	7.9; 8.1	6.7; 7.0	5.3; 5.7	3.3; 3.6

[a,b,c,e]See footnotes in Table II.

[d]The two values were obtained from two measurements on a preparation of liver mitochondria from 6 normally fed rats.

[f]The mitochondria were incubated for 15 min at 35 C.

with mitochondria. Those were eicosenoic acid (20:1 n-9), oleic acid (18:1 n-9) and hexadecenoic acid (16:1 n-9), as previously shown in vivo (12,16). The microsomes were less active but nevertheless capable of shortening reactions. For the mitochondria, the high percentage of 20:1 was noteworthy, for only small quantities are found in vivo (14). The conversion of 20:1 to 18:1 and then to 16:1 seems to be achieved more easily with heart mitochondria than with those of liver.

To determine whether the presence of a large quantity of 20:1 in the fatty acids formed by shortening erucic acid, in vitro, results from the accumulation of this fatty acid in the first steps of the intramitochondrial β-oxidation when using cyanide, a fourth series of experiments was undertaken. Liver mitochondria were used, as they are active in the shortening of erucic acid and because, in vivo, essentially only 18:1 was found in liver (14). They were incubated in the same medium as for the previous experiments but in the presence or absence of NADP in order to confirm the role of this coenzyme in shortening reactions, and also in the presence or absence of carnitine, to see whether the facilitated transport of acylcarnitines into the mitochondria to the sites of β-oxidation modifies the quantities and the proportions of the fatty acids formed by shortening. In each case, β-oxidation was stopped by addition of cyanide.

The results (Table IV) showed that in absence of carnitine the shortening reaction was significant only when NADP was added. However, with carnitine, but without NADP, the shortening reaction occurred and the percentage of acids formed was relatively high. If both NADP and carnitine were added, the percentage of the three fatty acids formed was not higher but nearly the same as with NADP alone.

In each case, the percentage of radioactivity present in the acid-soluble products was very low, which demonstrates the efficiency of cyanide in the inhibition of β-oxidation.

DISCUSSION

When a medium favorable for elongation was used, it was observed that microsomes showed the greatest activity, especially those from kidneys. The localization of erucic acid elongation in the microsomes is thus the same as had already been determined for other fatty acids, whose length did not exceed 18 carbon atoms (28,30). In mitochondria, the extent of elongation appears to be very small, since, in liver, they were nine less active than microsomes. The possibility that chain elongation in mitochondria might originate from microsomal contamination cannot be discarded.

From the results obtained in the media favorable for oxidation and shortening, it appeared that the shortening of erucic acid did not follow the same pathway as intramitochondrial β-oxidation. Nor does it follow the pathway of the reverse reaction of elongation, since microsomes, which can elongate, should also shorten erucic acid. On the other hand, mitochondria, which can actively shorten erucic acid, are able to elongate only poorly. This suggests the existence of two separate enzymatic systems, one for chain-shortening, essentially localized in mitochondria, the other one for elongation, essentially localized in microsomes.

The capacity for chain-shortening was observed to vary with the origin of mitochondira. Those from liver were seen to be particularly active, and this can be explained by their very great activating possibilities (31).

In absence of carnitine, erucic acid cannot pass through the inner mitochondrial membrane, which is, in addition, impermeable to NADP. It can, therefore, be deduced that the shortening system is not localized in the inner mitochondrial membrane, nor inside the mitochondria.

The addition of carnitine to the incubation medium without NADP increased the quantity of shortened fatty acids. Those were probably formed at the beginning of the β-oxidation reactions, as concluded by Stanley and Tubbs (32) from their results obtained with rat liver mitochondria in similar conditions. Since, moreover, the quantity of the short acid-soluble molecules was low, it seems that the shortened fatty acids then left the mitochondria as acylcarnitines, the presence of a carnitine-acyltransferase in the mitochondrial matrix facilitating this passage (33-35). Carnitine thus greatly facilitates also the passage outwards of shortened fatty acids formed in the matrix, just as it facilitates the entry of erucic acid into the mitochondria.

When both NADP and carnitine were added to the incubation medium, it might be expected that the two shortening processes (true chain-shortening and β-oxidation) would take place simultaneously. However, the very low radioactivity of the acid-soluble products suggests that carnitine played no major part, in this case, to facilitate the entry of erucic acid inside mitochondria towards the sites of β-oxidation. On the other hand, the relative proportions of the three shortened fatty acids in the activated fraction were the same, in the presence of NADP alone or associated with carnitine. It thus seems probable that erucic acid activated by CoA was mainly channeled towards shortening reactions, before it was transesterified with carnitine. This suggests that the shortening system is more likely localized in the outer mitochondrial membrane. If the relative amounts of the different fatty acids formed by shortening are now compared, it appeared that the proportions of oleic and hexadecenoic acids were the highest with heart mitochondria. These two common fatty acids are better oxidized than erucic acid by the cardiac mitochondria, as has already been demonstrated (24,36). The easier entry of these two fatty acids into the mitochondria would explain their low percentage found in the whole heart in vivo (14).

The hypothesis that erucic acid is used by the cardiac cells after shortening has already been suggested by Pinson and Padieu (19), from results obtained with myocytes in culture. Evidence for the shortening of erucic acid by heart mitochondria is now presented in this paper. The results also strongly suggest that heart mitochondria then utilize the fatty acids thus formed by shortening for β-oxidation reactions.

The hypothesis that there is a shortening step for the use of erucic acid by the heart can explain a certain number of facts observed in that organ when animals are kept on a diet rich in rapeseed oil. Thus, the early accumulation of triglycerides rich in erucic and eicosenoic acid (37,38) probably reflects a deficiency of the mitochondrial shortening systems, which would normally work very little with the usual fatty acids. The accumulation of these fatty acids would then lead to an increase in the shortening capacity by a large increase of the number of mitochondria in the cardiac cells, which can in fact be very clearly observed

after forty days on diet (23,39). The presence of megamitochondria could constitute an abnormal exaggeration of this adaptation.

Finally, one last point merits discussion: the necessity of NADP in shortening reactions. In the cell, the de novo synthesis of fatty acids is considered to supply most of the oxidized NADP. With a diet rich in lipids, this synthesis is lowered, which can lead to a reduction of the shortening processes and favor an accumulation of erucic acid in the myocardium, especially in young animals where the enzyme systems have not yet reached their full potential. Moreover, the shortening reactions are in competition with the reactions of the pentose cycle which also require NADP. This could lead to a reduction in the synthesis of ribose and deoxyribose, and eventually of nucleic acids and thus to a deterioration in metabolic regulations. On the other hand, the increase in NADPH concentration in the cell, following an active chain-shortening of erucic acid, may direct the glucose towards lipogenesis, eliminating it from the oxidation pathway and thus modifying the oxidative metabolism of the cell.

From these few examples, it appears that utilization of NADP in shortening processes can considerably modify the cellular metabolic equilibria. These modifications are able to cause pathological changes, especially in very specialized cells, such as cardiac cells, where there is not much metabolic diversity. The reverse may be true in the liver where the metabolic diversity allows an abnormal situation to be confronted, which can prevent any pathological effect.

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Sterols and Fatty Acids of *Epifagus virginiana*, a Nonphotosynthetic Angiosperm[1]

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ABSTRACT

The sterol and fatty acid compositions of *Epifagus virginiana*, which is a nonphotosynthetic flowering plant, have been examined by combined gas liquid chromatography-mass spectrometry (GLC-MS) and nuclear magnetic resonance (NMR). The organism exhibited a typical higher plant Δ^5-sterol profile with the C-24 alkyl group oriented "α" for the major sterol. Trace amounts of 24-methylenecycloartanol and cycloeucalenol established connection with the first cyclic intermediate (cycloartenol) in photosynthetic plant sterol biosynthesis. In a separate in vitro experiment, excised root and stem tissue were incubated with two labeled triterpenoid precursors, [2-^{3}H]lanosterol and [2-^{3}H]cycloartenol. Both the radioactive compounds were converted to labeled [^{3}H]sitosterol indicating the presence of an active biosynthetic pathway in *Epifagus*. Characteristic photosynthetic higher plant fatty acids were also present, and the polyenic acid was identified as α-linolenic acid.

INTRODUCTION

During the last decade, the use of sterols, sterol precursors, and unsaturated fatty acids have been shown to be a means of determining phylogenetic relationships (1-4). There are three structural features of lipids which have been used to discriminate between higher evolved and lower evolved plants as well as to distinguish between photosynthetic and nonphotosynthetic organisms. The configuration of the sterol side chain at C-24 (α or β) and the position of the double bonds in an unsaturated fatty acid ($\Delta^{6,9,12}$ or $\Delta^{9,12,15}$) are used as biochemical markers to differentiate between higher and lower evolved plants. Differentiation between photosynthetic and nonphotosynthetic organisms has been made through the study of the first tetracyclic intermediate after the cyclization of squalene. There are two frequent products of squalene cyclization, cycloartenol and lanosterol. Cycloartenol is found in all photosynethetic organisms but is never biosynthesized by mammalian tissues (3,5). Conversely, lanosterol, which is ubiquitous in mammals and other nonphotosynthetic organisms, has only been isolated and identified in a few photosynthetic plants (3,5). Since cycloartenol and lanosterol are equally effective in photosynthetic plants as the primary cyclic intermediate following the cyclization of squalene, it has been suggested that a cycloartenol-lanosterol bifurcation exists, which for phylogenetic purposes can be used to distinguish between photosynthetic and nonphotosynthetic organisms (1,6). Recently, the use of biological and cell-free systems has confirmed the view that either cycloartenol or lanosterol can be an acceptable precursor in the biosynthesis of the major sterols in photosynthetic plants (6). Thus, the sterol biosynthetic pathways through cycloartenol or lanosterol must have a phylogenetic rather than a functional basis (5).

Many higher evolved photosynthetic plants have been examined to obtain sterol and fatty acid compositional data. However, only a few nonphotosynthetic flowering angiosperms have been examined for their lipid composition (7,8). All six of the previous species examined exhibited sterols typical of photosynthetic higher plants. These sterols could be assimilated from the host through haustorial contact and transported to stems and roots of the parasite. In three species, however, it was demonstrated that the sterol biosynthetic pathway was functioning in the organisms independently of the host, and that de novo sterol biosynthesis followed the cycloartenol route used by photosynthetic plants (8). Fatty acid data from the three species was less conclusive. From a phylogenetic point of view, parasitic nonphotosynthetic plants are of interest because they are representative of a range of plant families and are, therefore, not monophyletic. In order to extend the compositional and biosynthetic data on sterols and fatty acids in this area, the broomrape, also known as Beech Drops, *E. virginiana* (Orobanchaceae), was selected for comparison with the other three genera of nonphotosynthetic plants which have been investigated previously (7,8).

EXPERIMENTAL PROCEDURE

Materials and Methods

E. virginiana (Beech Drops) was collected

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from beneath a large stand of beech trees on the Patuxent Wildlife Refuge in Maryland. Fresh material was washed and divided into stems and roots, freeze-dried and extracted with chloroform methanol (2:1) in a Soxhlet apparatus. Processing of the lipid fraction and separation of fatty acid and sterol fractions were by the method of Orcutt and Patterson (9). Sterols were isolated by digitionin precipitation and by chromatography on a lipophilic Sephadex LH-20 column (10). Some fractions were also purified by acetylation and separation of the acetates on argentation this layer chromatography (TLC). Identifications of sterols were made by GLC on SE-30, QF-1, and OV-17 columns and by GLC-mass spectroscopy and NMR in the case of sitosterol. Fatty acid separations and identifications were made by argentation TLC and by GLC. Tentative qualitative and quantitative determinations on root and stem tissues were made on a 1% SE-30 GLC column (sterols) and a 15% HI-EFF 8BP column (methylated fatty acids).

In separate labeling experiments, [2-^{3}H-]-lanosterol (8 μC) and [2-^{3}H]cyloartenol (4.5 μC) synthesized from the unlabeled compounds, as described by Thompson et al. (11), were incubared with 25 g portions of fresh sliced tissue (0.5 cm slices) in 0.01 M citrate buffer at pH 6.0 for 12 hr at room temperature. Tissue was washed twice with buffer and then freeze-dried before extraction and analysis as in nonradioactive experiments. Radioassays were made on a Tricarb 6 liquid scintillation spectrometer Model 500 P.

RESULTS AND DISCUSSION

The fatty acid composition of *E. virginiana* (Table I) is similar to that of a normal photosynthetic higher plant (12,13) and in particular is comparable to the two *Monotropa* species which have been analyzed (7). The 18:3 acid was identified as α-linolenic acid by comparison with α- and γ-linolenic acid standards. The α-linolenic acid content of *E. virginiana* occurred in greater concentrations in the root than the stem. Radioactively labeled [^{14}C-]-acetate was incorporated into the C-18 unsaturated fatty acids indicating that the fatty acid biosynthetic pathway was present.

E. virginiana has also been examined for its total sterol composition (Table II). Confirmation of structure of the sterols and triterpenoids was by GLC and mass spectroscopy. GLC on three columns and GC-MS showing peaks due to a 9,19 cyclopropane ring and a nine-carbon side chain confirmed that the 4-methyl and the 4,4-dimethyl compounds were of the cycloartane and not the lanostane type. Only sitosterol was examined by NMR. The NMR spectrum of sitosterol when compared to an authentic standard (14) was unequivocably that of a 24 α-ethyl sterol with no detectable clionasterol (24β-ethylcholesterol) present. Thus, based on the configuration at C-24 as "α," the classification of *E. virginiana* by plant taxonomists as a more highly evolved plant has been confirmed and the position of the Orobanchaceae, placed above the Leguminosae, on a linear scale, is in harmony with the classification of Tracheophyte families by using the configuration at C-24 of the sterol side chain proposed by Nes et al. (15).

The roots and stems of *E. virginiana* have been incubated in vitro with various radioactively labeled precursors to check whether the sterol biosynthetic pathway was operative. Both tritiated lanosterol (4×10^7 dpm/mg) and cycloartenol (5×10^7 dpm/mg) were converted to tritiated sitosterol and diluted 100-fold by unlabeled sitosterol. Labeled sitosterol was purified by Sephadex chromatography and subsequent GC analysis showed that it approached 100% purity. The sample was rechromatographed on TLC until a constant specific activity was obtained. The specific activity of iso-

TABLE I

Percentage Fatty Acid Composition of *E. virginiana* Root and Stem Tissue[a]

Fatty acids	Roots	Stems
12:0	1.1	---
14:0	2.1	tr
16:0	32.9	8.2
18:0	0.8	0.6
18:1	1.9	14.0
18:2	42.8	69.3
18:3 (α)	18.6	7.9
Total fatty acids (% dry weight)	0.11	0.97

[a]As % of total fatty acid.

TABLE II

Percentage Sterol Composition of *E. virginiana* Root and Stem Tissue[a]

	Root (%)	Stem (%)
24-methylene cycloartenol	tr	tr
cycleucalenol	tr	tr
cholesterol	tr	tr
24-methyl cholesterol	1.5	2.0
stigmasterol	6.8	8.5
sitosterol	91.7	89.5
28-isofucosterol	tr	tr
Total sterol (% dry weight)	0.12	0.22

[a]As % of total sterol.

lated sitosterol (dilution accounted for) was 4.7 x 10^4 dpm/mg for the cycloartenol experiment and 4.2x10^4 dpm/mg for the lanosterol experiment. The percentage conversion of cycloartenol and lanosterol to sitosterol was 0.3% and 0.2%, respectively. Labeled ^{14}C-acetate and ^{14}C-mevalonate were also converted to sitosterol in stems and roots in vitro.

From identification of the major sterols extracted from *E. virginiana,* it would appear that the organisms possess cycloartenol metabolites, a typical photosynthetic higher plant Δ^5-sterol composition, and α-linolenic acid ($\Delta^{9,12,15}$), which is in agreement with previous work on other nonphotosynthetic plants (7,8). Rohmer et al. (8) have also extended their data, through the use of labeled precursors, to show as this study has done, that heterotrophic plant parasites are capable of synthesizing their own sterols. Thus, from the combined lipid studies on nonphotosynthetic organisms, we conclude that nonphotosynthetic angiosperms are of photosynthetic origin, and that *E. virginiana* sterols and fatty acids are produced primarily, if not exclusively, in the parasite itself.

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The Effects of Phenobarbital on Biliary Lipid Metabolism in Cholesterol Gallstone Subjects[1]

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ABSTRACT

The effect of 1.7-2.2 mg/day oral phenobarbital over short (1 MO) and long term (6-24 MO) treatment on primary bile acid (BA) secretion, composition, synthesis, pool size, and enterohepatic cycling rates as well as phospholipid (PL) and cholesterol (C) secretion rates and biliary composition was determined in 12 asymptomatic cholesterol gallstone subjects while 5 normals had only short term studies. Phenobarbital enhanced BA and C secretion (BA-636 ± 166 to 2110 ± 382 mg/hr, $p<0.001$ and C-42 ± 5 to 224 ± 48 mg/hr, $p<0.001$) and BA cycling rate in all subjects studied during stimulated enterohepatic circulation but, during fasting, it only enhanced BA secretion (451 ± 129 vs. 759 ± 159 mg/hr, $p<0.05$) in gallstone subjects. Cholic acid (CA) production rate (171 ± 28 to 395 ± 9 mg/hr, $p<0.05$) and pool size (727 ± 80 to 1209 ± 132 mg/hr, $p<0.05$) were increased during long term treatment of gallstone subjects, while the proportion of CA in bile and deoxycholic acid (DCA) in feces increased. Treatment decreased biliary cholesterol from supersaturated to saturated levels (9.5 ± 0.6 vs. 6.1 ± 0.9 moles %, $p<0.02$) in all fasting gallstone subjects and decreased cholesterol crystal loads during long term treatment; but, while prohibiting gallstone growth, it did not affect stone dissolution over 24 months' treatment. Phenobarbital also failed to affect biliary lipid composition or bile acid pool size in short term treatment of normals. Thus, phenobarbital affected hepatic metabolism of CA by enhancing production rate, secretion, and pool size; and intestinal metabolism of both CA and chenodeoxycholic (CDC) acids by increasing their cycling rates. Phenobarbital may have failed to produce stone dissolution by enhancing CA production and pool size more than that of CDC.

INTRODUCTION

The sine qua non of cholesterol gallstone disease is the secretion of bile supersaturated with cholesterol (1,2). Defective cholesterol solubilization as the cause for cholesterol gallstone formation may occur in association with a reduced bile acid pool (3,4) or with excessive secretion of biliary cholesterol in obese gallstone subjects (3,5). Pharmaceutical therapy aimed at decreasing cholesterol saturation by altering the bile acid pool is as yet experimental on the North American continent, although chenodeoxycholic acid and its derivative, ursodeoxycholic acid, have been reported to be effective in dissolving cholesterol gallstones in subjects with functioning gallbladders (6-8). While the mode of action of chenodeoxycholic acid-induced gallstone dissolution appears related to decreased biliary cholesterol secretion, other agents capable of enhancing bile acid synthesis or secretion have been sought to gain an effective medical treatment of this common malady (9,10). We found that phenobarbital enhanced bile acid synthesis, secretion, and pool size but not cholesterol secretion in bile of rhesus monkeys (11), so that biliary cholesterol saturation decreased. Reports of the effect of phenobarbital on biliary lipid metabolism in man have been contradictory, since some have reported enhancement of bile acid pool size (10,12) and decreased biliary cholesterol saturation (9,13), while others could demonstrate no effect on biliary lipid composition, secretion, or pool size in subjects with gallstones (14) or in normals during short term treatment (15). Since length of treatment as well as subject selection may have contributed to these diverse findings, a definitive study addressed to characterize the metabolic effects of short term in normals and both short and long term treatment in asymptomatic subjects with cholesterol gallstones was carried out.

EXPERIMENTAL PROCEDURES

Subjects, Length of Study, Drug Administration

Approval was obtained for these studies from the Research Council's Subcommittee on Research Involving Human Research at the University of Western Ontario, and all subjects provided informed consent and entered the study with normal liver function and serum lipids. None of the patients was morbidly obese, although 3 males were in excess of 110% ideal body weight. Twelve asymptomatic subjects with radiolucent cholesterol gallstones

[1]Portions of this work were presented at the National Meeting of the American Federation of Clinical Research, Atlantic City, April 29, 1973 and at the Annual Meeting of the Canadian Society of Clinical Investigation, Winnepeg, Manitoba, January 21, 1975 as well as that of the Royal College of Physicians and Surgeons of Canada, January 27, 1977 at Toronto, Canada.

in a functioning gallbladder (10 men, aged 51 to 63; two women, ages 37 and 72) were studied while on long term phenobarbital treatment 1.7 to 2.2 mg/kg: 6 subjects during 6 to 12 months of therapy, the rest to 24 months. By contrast, 5 normal subjects with no radiologic evidence of gallstones (aged 24 to 59) received identical therapy: 4 subjects for 1 month and the other for 6 months. Six treated gallstone subjects had studies performed 1 to 6 months following cessation of treatment, whereas 3 were also studied during 1 month of short term therapy. Another group of 5 untreated subjects with asymptomatic cholesterol gallstones were also followed for 1 to 3 years. Cholesterol gallstones were verified by examination of biliary sediment by polarizing microscopy for cholesterol crystals, the finding of radiolucent stones by X-ray, and stone analysis in 3 subjects requiring cholecystectomy. Termination of the study occurred at the patient's request, with drug intolerance, the finding of a nonfunctioning gallbladder by cholecystogram, onset of symptoms characteristic of gallstone disease, or after 24 months' treatment.

EXPERIMENTAL STUDIES AND ANALYSES

Biliary Lipid Composition and Secretion Rates

Composition. Biliary drainage studies were carried out on all gallstone subjects before and every 3-6 months during phenobarbital treatment while 6 of these subjects had studies following cessation of treatment (1-6 months) also. Drainage studies were carried out in 5 normals before and after short term treatment (1-6 months) and 5 untreated subjects at irregular intervals over 3 years. Three gallstone subjects also had studies carried out after 1 month of treatment. Bile-rich fluid was obtained by duodenal intubation with a small 0.2 mm ID double-lumen tube in overnight fasted subjects after stimulation of the gallbladder with intraduodenal $MgSO_4$. Six cc of bile in total was collected as 1-2 cc samples over 1 hr and the most concentrated sample used for comparison of biliary lipid composition. In all samples the bile was immediately centrifuged (1500 g x 15 min at 25 C) and the sediment examined immediately for cholesterol crystals by polarizing microscopy. The supernatent was frozen to -20 C, and bile salt (16), phospholipid (17), and cholesterol (11) content determined within 2 weeks. The relative proportions of individual biliary lipids were plotted on triangular coordinates according to the method of Admirand and Small (1) and limits of cholesterol solubility determined as established by Hegardt and Dam (18).

Secretion rates. Biliary lipid secretion rates were assessed on and off treatment with phenobarbital for 1-2 months in 3 of the gallstone subjects and 2 normals by a modification of the intestinal perfusion method developed by Grundy et al. (19). A double-lumen tube was positioned fluoroscopically in fasting subjects at 7 a.m. with proximal perfusing port at the level of the ampulla of Vater and aspirating port 15 cm distal in the duodenum. Steady state conditions in the perfused segment were established by perfusing 0.15 M saline and 10 mg per dl sulfobromophthalein (BSP), adjusted to pH 6, for 6 hr at 2 ml/min with hourly aliquots aspirated for analysis as described below. Cholecystokinin (75 Ivy dog units, Karolinska Institutet, Stockholm, Sweden) was then administered intravenously and immediately thereafter 30 μc 2-4 ^{3}H cholic acid in 20 ml $NaHCO_3$ and 10 μc ^{14}C chenodeoxycholic acid in 10 ml ethanol were flushed through the proximal port followed by 25 cc $NaHCO_3$ to label the primary bile acids for consequent assessment of primary bile acid pool sizes. Thereafter, an amino acid mixture (230 mM amino acid solution adjusted to pH 6-8 containing L-leucine, L-phenylalanine, L-methionine, L-lysine, L-isoleucine, L-valine, L-histidine, L-threonine, L-tryptophan, L-alanine, L-arginine, L-proline, L-tyrosine to provide only 26.3 cal/l [Eastman Kodak Co., Rochester, NY]) to provide a maximal stimulus to bile flow (3) with BSP 10 mg/dl was infused at 2 ml/min over the next 6 hr, but hourly bile samples were collected by continuous aspiration only over the last 4 hr of this period as described below. Lipid in the form of 10% intralipid (Intralipid 10%, [Pharmacia Ltd., Dorval, Quebec]). The contribution of phospholipid in the aspirate provided by egg lecithin in intralipid was calculated knowing the proportion of lipid to exogenous marker BSP in the infusate) was then added to the mixture to provide 1.1 cal/cc and perfused for another 5-6 hr at the same rate. Biliary lipid secretion rates and bile acid specific activities were assessed from 4 to 6 cc accumulated hourly samples with continuous aspiration from the distal port into a syringe prefilled with 2 ml methanol. Bile acid secretion rate was assessed following the method of Grundy et al. (19) using the formula

$$\text{BA secretion (mg/hr)} = \text{BA Concentration (mg/ml in aspirate)} \times \frac{\text{BSP infused mg/hr}}{\text{BSP conc in distal aspirate (mg/ml)}}$$

Secretion rates of the other biliary lipids (i.e., phospholipid and cholesterol) were similarly calculated from respective analysis of these samples (19). Bile acid specific activities were used in the assessment of bile acid pool size described below.

Bile Acid Composition in Bile, Urine, and Feces

Nonsulfated bile acids in bile. For most bile samples, hydrolysis and purification were carried out as described previously (11) with quantitation by gas liquid chromatography (HP-Model 5710) using 4-ft coiled columns packed with QF-1% on Gas Chrom Q (80:100 mesh) and programmed oven temperatures of 210-230 C. 5α-Cholestane was added to each sample and each run accompanied by bile acid standards to determine individual bile acid masses with an Autolab System 1 computing integrator. Thoroughness of bile acid recovery was checked by addition of ^{14}C-CDC to the samples at the beginning of analysis and 60-95% efficiency was obtained.

Total bile acid in bile, feces, and urine. BILE–In four gallstone subjects treated with phenobarbital, simultaneous collections of feces, urine (3 day and 1 day pooled samples, respectively) and concentrated duodenal bile were analyzed for nonsulfated and sulfated bile acids before and during long term treatment. Internal standards of ^{14}C sulfated LCA prepared according to the method of Stiehl et al. (20) and ^{3}H nonsulfated DCA or CDC (Amersham-Searle) were added prior to analysis to follow recovery of both sulfated and nonsulfated bile acids. All radioisotopes were >95% pure as determined by thin layer and/or gas liquid chromatography. Recovery efficiencies of greater than 60% for nonsulfated and sulfated bile acids were accepted.

FECES–Fecal samples containing ca. 3 g of fecal water homogenate (1:2) were subjected to mild saponification prior to petroleum ether extraction to remove nonsaponifiable neutral lipids according to the method of Grundy et al. (21). All alkaline mixtures were then subjected to 2.5 N KOH for 4 hr in a sandbath at 170 C to complete hydrolysis and the bile acids then extracted with equal volumes of normal butanol. After drying, the residue was taken up in 25 ml 1% propionic acid and transferred to an activated florisil column according to the method of Campbell and McIvor (22). The fatty acids were eluted with 50 ml 1% propionic acid, followed by 100 ml acetic acid/ethyl ether (1:9) to elute the nonsulfated bile acids. After evaporation these bile acids were extracted with ethyl acetate from the aqueous solution and prepared for gas liquid chromatography as described previously (11). Following elution of the nonsulfated bile acids, the sulfated bile acids were eluted from the column with 100 cc 2% HCl in methanol and then solvolized according to the method of Palmer and Bolt (23) prior to preparation for GLC. Similar bile acid standards as above were used and recovery efficiencies of greater than 50% accepted.

URINE–30 ml urine samples were lyophilized, then taken up in 4 ml water and placed on an XAD-2 column. Urinary bile acids were eluted from this column according to the method of Makino et al. (24) and prepared for GLC as above.

Bile Acid Pool Size, Production Rate, and Half-Life

Bile acid turnover studies were performed by the method of Lindstedt (25) before and after short term phenobarbital (1 month) treatment in 3 gallstone subjects and 3 normals, as well as in 3 gallstone subjects after long term phenobarbital treatment (6 month-2 years) after administration of 15 μc ^{14}C-CDC in a small amount of ethanol administered orally in orange juice and 15 μc ^{14}C-CA or 30 μc ^{3}H-CA in $NaHCO_3$. Serial bile samples were obtained over the next 7 days and CA and CDC specific activities assessed by the method of Vlahcevic et al. (4), whereby cholic and CDC pool sizes, production rates, and half-lives were determined from the dilution of respectively administered radioactive bile acids by newly synthesized bile acids.

Bile acid pool sizes were also determined by a single intubation method modified after that described by Duane et al. (26) in 5 subjects (2 gallstone and 3 normals) before and after 1 month treatment with phenobarbital as well as in 3 gallstone subjects treated for 12 to 24 months. Five μc of ^{14}C-CA and ^{14}C-CDC (or 10 μc 2-4 ^{3}H) was administered orally before the evening meal 15-18 hr prior to duodenal aspiration next morning for assessment of CA and CDC specific activity in bile. In addition, during biliary lipid secretion studies after labeling as described above, individual BA specific activities were determined 8 to 24 hr following isotopic administration and BA pool size calculated according to the formula

$$\text{Pool Size mg} = \frac{\text{Dpm Ingested}}{\text{S.A. (Specific Activity)}}$$

In selected instances, these values were compared to values obtained in the same patients by the Lindstedt technique (25).

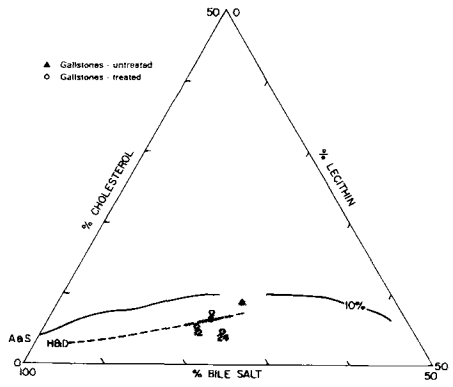

FIG. 1. Bile Acid Composition is depicted by a triangular coordinate graph showing only the pertinent portion of the triangle. Cholesterol saturation lines are shown for 10% solids by the method of Admirand and Small (A&S) (1) as well as by Hegardt and Dam (H&D; dashed line) (18). Open circles represent composition from treated subjects at 6, 12, and 24 month intervals, while the triangle represents composition before treatment. The upper shaded area represents overlaps of ± 1 SE of the mean for all untreated gallstone subjects including pre and posttreatment controls, while the lower area represents that for treated and normals with respect to the relative proportion of each biliary lipid in moles percent.

Cholecystograms and Microscopic Examination

Cholecystograms were performed and evaluated by radiologists independent of the study before and at 6 months to 1 year intervals of treatment by radiological technique employing abdominal markers, constant focal points, and standard views to evaluate changes in stone size in phenobarbital-treated and control subjects. The number of cholesterol crystals per hpf in bile sediment was graded as 0, small 1-2, moderate 2-10, or multiple >10 by polarizing microscopy.

Statistical evaluation. The paired Student t-test was applied to control vs. treatment data for all indices studied including biliary lipid composition, secretion rates, bile acid composition, bile acid pool size, and turnover. The unpaired Student t-test was applied to data for assessment of differences between normals, treated and untreated gallstone subjects (27).

RESULTS

Biliary Lipid Composition and Microscopic Examination

Figure 1 is a summary of changes observed in biliary lipid composition as plotted by the method of triangular coordinates for all treated gallstone subjects. Changes in biliary lipid composition were noted in most subjects within

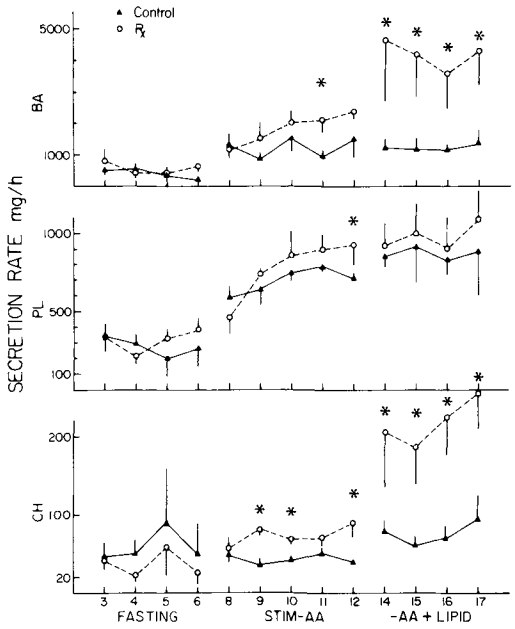

FIG. 2. Biliary Lipid Secretion Rates. Mean ± 1 SE for bile acid (BA), phospholipid (PL), and cholesterol (CH) secretion rates in mg/hr are shown for control (untreated) studies by triangles and paired treatment studies (R_X) by open circles in five subjects studied during fasting and stimulated EHC by intraduodenal amino acid perfusion (AA) or AA + lipid. Asterisk values indicate significant difference from control $p<0.05$ or better.

6 months' treatment, and, while some fluctuation in cholesterol saturation occurred over 24 months of treatment, decreased biliary cholesterol saturation persisted in most subjects throughout the study (9.5 ± 0.6 vs. 6.05 ± 0.9 moles %, $p<0.02$ @ 24 mo). Posttreatment bile analysis in 6 subjects (1-6 months) revealed an increase in biliary cholesterol saturation from treatment in all but one of these subjects (5.92 ± 1.0 vs. 8.5 ± 0.65 moles %, $p<0.05$). Biliary lipid composition was similar in gallstone subjects before (9.5 ± 0.6) and following cessation of treatment (8.5 ± 0.7) compared to untreated controls (9.2 ± 0.8 moles %). Biliary lipid composition in subjects treated for greater than 1 year was not significantly different from composition in normals (e.g., C-6.05 ± 0.9 vs. 5.7 ±0.4 moles %, NS). Biliary cholesterol saturation decreased in short term studies only in gallstone subjects (8.64 ± 2.2 vs. 3.85 ± 1.0 moles %, $p<0.05$) but not in normals (6.27 ± 2.0 vs. 3.30 ± 1.1,NS).

Biliary Lipid Secretion Rates

Figure 2 is a summary of biliary lipid secretion rates determined in 5 subjects (3 gallstone

TABLE I

Bile Acid Composition in Bile and Feces[a]

	Bile		Feces			
	Control	Treatment	Control		Treatment	
NS	%		%	%	Absolute mg	Absolute
CA	38.7 ± 2.1	42.8 ± 2.0[b]	8.9 ± 1.9	8.8 ± 2.4	18.4 ± 4	37.2 ± 10[b]
CDC	37.2 ± 2.6	36.1 ± 2.4	12.9 ± 2.0	9.3 ± 1.5[b]	26.8 ± 4	39.4 ± 6
DCA	23.0 ± 2.5	20.1 ± 3.2	35.7 ± 2.3	39.2 ± 4.7	73.8 ± 5	165.8 ± 19[b]
LCA	1.1 ± 0.2	1.0 ± 0.3	42.4 ± 3.4	42.6 ± 5.8	87.8 ± 7	180.2 ± 25[b]
S						
CA	0	0	1.9 ± 0.5	9.1 ± 4.5[b]	0.6 ± 0.2	3.8 ± 0.2[b]
CDC	0	0	14.5 ± 6.2	9.9 ± 3.1	4.8 ± 2	4.0 ± 1
DCA	0	0	36.5 ± 4.4	32.6 ± 5.5	12.2 ± 1	13.4 ± 2
LCA	less than 5% of total		47.0 ± 6.0	51.3 ± 6.8	15.8 ± 2	21.0 ± 2

[a]Values indicate mean ± 1 SE for percent composition of individual nonsulfated (NS) and sulfated (S) bile acids in bile and feces as well as absolute amounts in feces for simultaneous collections.
[b]Indicates significant difference from control ($p<0.05$ or better).

and 2 normals) by the perfusion technique before and following 1 to 2 months' treatment. Intubation and consequent perfusion of the duodenum with saline and BSP marker for 6 hr resulted in relatively steady state conditions of biliary lipid secretion within 3 to 4 hr. No significant differences in the rates of bile acid, phospholipid, or cholesterol secretion between control and treatment periods were noted during the fasting (i.e., unstimulated) period when all studies were analyzed, but increased bile acid secretion was noted for gallstone subjects (451 ± 129 to 769 ± 159 mg/hr, $p<0.05$). Within 5 hr, the stimulated bile flow produced by amino acid perfusion increased the secretion rates of bile acid and phospholipid three-fold compared to fasting in both control (i.e., BA-360 ± 85 to 950 ± 197 mg/hr, $p<0.001$ and PL-261 ±77 to 794 ± 97 mg/hr, $p<0.001$) and treatment experiments (i.e., BA-636 ± 166 to 2110 ± 382, $p<0.001$ and PL-383 ± 111 to 898 ± 44 mg/hr, $p<0.001$). Increased cholesterol secretion rates not noted during amino acid stimulation in control studies occurred during treatment studies (i.e., 42 ± 5 vs. 90 ±21 mg/hr @ 12 hr AA Stimulation, $p<0.01$). Addition of lipid to the infusion mixture resulted in further enhancement (i.e., up to five-fold over fasting) of all lipid secretion rates during treatment but not control studies which was significant for both BA and cholesterol secretion rates (i.e., @ 12 hr AA BA-114 ± 191 vs. 17 hr AA + lipid 4305 ± 1128 mg/hr, $p<0.01$ and C-70 ± 15 vs. 224 ± 48 mg/hr, $p<0.01$). Similar differences between control and treatment periods in phospholipid secretion were not as obvious, however (i.e., 893 ± 489 vs. 1089 ± 213 mg/hr).

Bile Acid Composition in Bile, Urine, and Feces

Phenobarbital increased the proportion of cholic acid (38.7 ± 2.1 vs. 42.8 ± 2.0 %, $p<0.005$) in bile shown in Table I, but had no other effects on biliary bile acids over all lengths of treatment. Sulfated bile acids amounted to less than 5% of total biliary bile acids in all cases and were not affected by treatment. Analysis of urine for bile acids revealed traces of mainly sulfated bile acids similar in both control and treatment studies.

Phenobarbital increased the excretion of total bile acids in feces two-fold during treatment. Table I also shows the effect of treatment on both the proportion and absolute quantities of individual bile acids in feces. Treatment decreased the proportion of total CDC in feces (13.2 vs. 9.3 %, $p<0.05$) and increased the proportion of sulfated cholic acid (1.9 ± 0.5 to 9.1 ± 4.5%, $p<0.05$). Thus, except for CDC, the absolute amounts of all bile acids in feces increased during treatment. Changes in sulfated bile acids were minor, however, and accounted for less than 15% of the bile acids found in feces.

Bile Acid Pool Size and Turnover

Figure 3 illustrates the effects of phenobarbital treatment upon primary bile acid pool size in subjects treated for 1 month or longer (6 months to 2 years). Phenobarbital had no significant effect on bile acid pool size in normals treated for 1-6 months but did increase cholic acid pool size in gallstone-treated subjects in both short and long term treatment (i.e., 1 mo 1011 ± 88 to 1637 ± 171 and 6 mo 727 ± 80 to 1209 ± 132 mg, $p<0.05$). Chenodeoxycholic acid pool size was increased

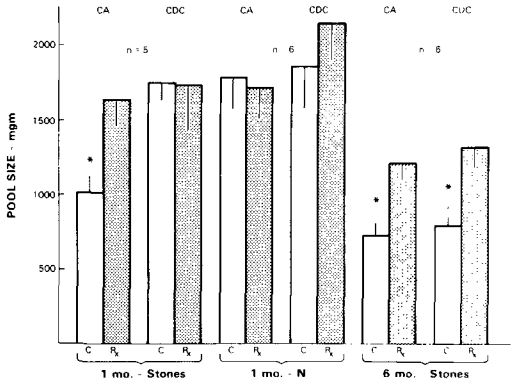

FIG. 3. Pool sizes for cholic acid (CA) and chenodeoxycholic acid (CDC) in mg are shown by height of bars (clear untreated control periods vs. stippled treatment periods) with 1 SE represented by vertical inserts. Treatment for 1 month in normals, and 1 month and 6 or more months in gallstone subjects are compared with asterisks denoting significant difference from control ($p<0.05$).

following long term (789 ± 137 to 1321 ± 134 mg, $p<0.05$) but not short term treatment (1751 ± 115 to 1745 ± 289 mg, NS). Table II shows the effect of phenobarbital on bile acid turnover as determined by the Lindstedt method (25). As noted in Figure 3 where all data was summarized, CA pool size did not increase in normals during phenobarbital treatment, although a trend toward enhanced CDC pool size was evident (i.e., CA-772 ± 88 to 611 ± 58 mg and CDC-946 ± 89 to 1421 ± 215, NS). Following 6 or more months of treatment in gallstone subjects, CA pool was enhanced in all instances (i.e., 365 ± 103 to 741 ± 233 mg, $p<0.05$). Not dissimilar findings were noted for CDC pool size during long term treatment (i.e., 1243 ± 343 to 1734 ± 276 mg) but, because of patient variability, these values did not achieve statistical significance. The production rate was likewise increased for cholic acid only for gallstone subjects treated for more than 6 months (i.e., 1 month-n 254 ± 67 to 244 ± 31 mg/day,NS; 6 month stones 171 ± 28 to 395 ± 9 mg/day, $p<0.05$). The production rate of CDC was, however, increased significantly during short term treatment in normals (i.e., 150 ± 33 to 236 ± 32 mg/day, $p<0.05$) but not for gallstone-treated subjects (i.e., 6 month 304 ± 64 to 484 ± 155 mg/day, NS).

Table III shows the effect of phenobarbital on cycling of individual primary bile acid pools in 4 subjects treated for 1-2 months in which BA pool size and secretion rate were determined. Phenobarbital increased bile acid secretion rate in these subjects in both unstimulated and stimulated periods but only increased cholic acid pool size (i.e., CA-1001 ± 94 to 1510 ± 166, $p<0.05$ and CDC-1270 ± 191 to 1313 ± 236, NS). Bile acid cycling was more noticeably enhanced in the stimulated periods (i.e., AA-CA-8.0 to 14.1 and CDC-7.7 to 18.3, and AA + lipid CA-9.7 to 25.3 and CDC-9.3 to 32.8 cycles per day) than in the unstimulated periods during phenobarbital treatment (i.e., CA-3.1 to 2.9 and CDC-3.7 to 4.8). Phenobarbital treatment, however, appeared to enhance CDC cycling slightly more than that of CA.

Cholecystograms and Bile Sediment. No significant differences in gallstone size were noted during treatment of gallstone subjects in cholecystograms read blindly by two independent observers. While increase in gallstone size was not noted during the study, a decrease in biliary cholesterol crystal load (from >10 to <10/hpf), was noted, however, in the majority of treated subjects. Three subjects, however, noted to have an increase of the number of small stones by X-ray, were also observed to have increased crystals in bile and developed

TABLE II

Bile Acid Pool Turnover, Lindstedt Method, (25)[a]

		Pool Size mg		Prod rate mg	
		C	R_x	C	R_x
Normals (1 month) n=3	CA	772 ± 88	611 ± 58	254 ± 67	244 ± 31
	CDC	946 ± 89	1421 ± 215	150 ± 33	236 ± 32[b]
Gallstones (6 month) n=3	CA	365 ± 103	741 ± 233[b]	171 ± 28	395 ± 99[b]
	CDC	1243 ± 343	1734 ± 276	304 ± 69	484 ± 155

[a]Values represent mean ± 1 SE for pool size and production rate of cholic acid (CA) and chenodeoxycholic acid (CDC) for control vs. R_x studies in three normals and three gallstone subjects.

[b]Indicates significant difference from control ($p<0.05$ or better).

TABLE III

Effect of Phenobarbital on EHC of Bile Acids[a]

		Secretion rate (g/24 hr)		Pool size (mg)		EHC cycles/day	
		CA	CDC	CA	CDC	CA	CDC
Unstimulated							
	off	3.06 ± 0.7	3.71 ± 0.9	1001 ± 94	1270 ± 191	3.1	2.9
	on	5.65[b] ± 1.4	6.37[b] ± 1.6	1510 ± 166	1313 ± 236	3.7	4.8
Stimulated AA							
	off	8.06 ± 1.6	9.79 ± 2.0	1001 ± 94	1270 ± 191	8.0	7.7
	on	21.28[b] ± 5.5	24.01[b] ± 6.2	1510 ± 166	1313 ± 236	14.1	18.3
Stimulated AA + lipid	off	9.69 ± 1.1	11.77 ± 1.3	1001 ± 94	1270 ± 191	9.7	9.3
	on	38.23[b] ± 10.0	43.14[b] ± 11.3	1510 ±166	1313 ± 236	25.3	32.8

[a]EHC in cycles/day of both cholic acid (CA) and chenodeoxycholic acid (CDC) have been determined by dividing respective secretion rates by pool size during unstimulated and stimulated periods with amino acid (AA) or amino acid + lipid for control and treatment periods in three gallstone subjects and one normal. Values indicate mean ± 1 SE.

[b]Indicates significant difference from control ($p<0.05$ or better).

symptoms leading to cholecystectomy. In two of three subjects, bile composition did not change with this development while the third showed increased cholesterol saturation. At surgery, cholesterol gallstones which were soft and easily fragmentable were verified.

DISCUSSION

Our studies revealed a difference in the effect of phenobarbital on normals and gallstone subjects during short term therapy. No significant effects of short term therapy were noted in fasted normal subjects in respect to bile composition, biliary lipid secretion, or bile acid pool size while such treatment decreased biliary cholesterol saturation in gallstone subjects by enhancing bile acid secretion rates even during fasting. An increase in cholic but not chenodeoxycholic acid pool size was, however, apparent after short term treatment in gallstone subjects. An increased production rate of CDC with a trend toward increased CDC pool size, however, was noted in normals while enhanced biliary bile acid and cholesterol secretion rates were noted during stimulated bile flow in both normals and gallstone subjects after short term therapy. Enhancement of biliary lipid secretion by amino acids was heightened by the addition of lipid and therefore calories to the infusate during control studies, but these effects were further enhanced by phenobarbital. In addition, phenobarbital increased cholic and chenodeoxycholic acid cycling frequencies. This intestinal effect of phenobarbital, however, appeared to increase CDC cycling more than that of CA. Since long term treatment with phenobarbital was not feasible in normals, only such treatment in gallstone subjects resulted in persistent lowering of biliary cholesterol saturation throughout their treatment, with reversion to pretreatment levels on discontinuance of phenobarbital within 1 to 6 months. While phenobarbital enhanced both CA and CDC pool size, it increased the production rate of CA more during long term studies.

Phenobarbital enhanced fecal excretion of bile acids almost two-fold as determined by gravimetric fecal analysis, which also paralleled the enhanced production rate of bile acids (i.e., 240.6 vs. 464 mg/day, $p<0.01$) determined by the Lindstedt method (25) during long term treatment. The greater proportion and quantities of cholic acid in bile and stool, along with its bacterial degradation product deoxycholic acid in feces, appeared to result from increased hepatic production and secretion of cholic acid during treatment. Enhanced chenodeoxycholic acid reabsorption from the intestine may have contributed to the decreased proportion of chenodeoxycholic acid in stool since, while its cycling frequency was enhanced even more than that of CA, the proportion of its bacterial degradation product lithocholic acid did not change with treatment. In spite of increased cholic acid cycling during stimulated EHC, the proportion of DCA decreased in bile as well, indicating possible enhanced intestinal reabsorption of cholic acid also. While enhancement of bile acid pool size was found during long term treatment with phenobarbital by both the Lindstedt technique and the single intubation method of assessing bile acid pool size by isotope dilution, the latter method was

unreliable in evaluating pool size earlier than 8 hr after labeling, probably as a consequence of inadequate mixing of ingested isotope, as well as after 18 hr, as a consequence of isotope dilution by newly synthesized bile acid. While the modified Duane method slightly overestimated bile acid pool size compared to the Lindstedt technique in the same subjects (i.e., CA-917 ± 203 vs. 1001 ± 94 mg), the latter technique was extremely useful in allowing greater flexibility in the study of bile acid pool size in ambulatory subjects. Stimulation of the enterohepatic circulation during the secretory studies did not appear to significantly alter pool size determinations when compared to other studies.

In summary, three factors were identified which may have prohibited optimal conditions for stone dissolution in gallstone subjects even though decreased crystal loads and failure of stone growth were noted during phenobarbital treatment. First, phenobarbital appeared to enhance cholic acid production, pool size, and secretion more than that of chenodeoxycholic acid in gallstone subjects. It has been shown that expansion of the bile acid pool with chenodeoxycholic acid but not cholic acid affects stone dissolution (6). Of interest was the apparent enhancement of CDC rather than CA production rate and pool size in normals only. Second, phenobarbital also enhanced cholesterol secretion during stimulated EHC so that normal biliary cholesterol desaturation was less evident during such periods. Third, while cholesterol saturation was decreased during fasting from supersaturated levels after long term treatment, bile did not become less than saturated by the criterion of Hegardt and Dam (18). Finally, a species difference in the effect of phenobarbital on biliary lipid metabolism between human and rhesus monkey was found since man responded to phenobarbital treatment by increasing cholesterol secretion while the monkey did not (11). These studies, however, show that phenobarbital has well defined effects on hepatic metabolism of biliary lipids and suggested enhancement of intestinal absorption and bile acid cycling frequency. Phenobarbital may, therefore, be of use in enhancing intestinal reabsorption of bile acids in bile acid enteropathies and protect against bile acid pool depletion and bile acid-induced diarrhea. These studies show that phenobarbital, although not affecting stone dissolution by not allowing enhanced crystal formation or stone growth, should be safe in the study of pool depletion syndromes where the risk of stone formation may be increased (28). The difference in bile acid metabolism observed in normals vs. gallstone subjects also suggests an underlying metabolic difference in the response to phenobarbital between these groups. It may be that only subjects with reduced bile acid pools respond to phenobarbital treatment by enhancing cholic acid metabolism.

ACKNOWLEDGMENTS

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The Content and Composition of Sterols and Sterol Esters in Sunflower and Poppy Seed Oils

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ABSTRACT

The composition and proportion of free sterols and sterol esters in crude sunflower and poppy seed oils were determined, using preparative thin layer chromatography followed by gas chromatography with cholesterol as an internal standard. Free sterols and sterol esters were also isolated in a liquid fraction obtained by low temperature crystallization (-80 C) of the oils and enriched with minor lipid classes. This enrichment procedure provided a liquid fraction suitable for studies of minor components in the oils. However, selectivity towards sterol esters was observed since sterols esterified to very long chain fatty acids (C_{20}-C_{24}) were preferentially retained in the precipitate. The proportions of free and esterified sterols were found to be 0.34% and 0.28%, respectively, in the sunflower oil, whereas the corresponding figures for poppy seed oil were 0.33% and 0.05%. Sunflower oil was characterized by a relatively high percentage of Δ7-sterols, preferentially obtained in the esterified fraction, and by very long chain saturated fatty acids of sterol esters. The sterols in poppy seed oil were composed almost entirely of campesterol, stigmasterol, sitosterol and Δ5-avenasterol, although their percentage distributions were remarkably different in the free and esterified fraction.

INTRODUCTION

Sterols are the major nonglyceride lipids in many vegetable oils. In the crude oil, they mainly exist as free sterols and as sterol esters, although sterol glucosides and acylated sterol glucosides are also present (1,2). After the degumming of sunflower oils, only free sterols and sterol esters remain (3). The total sterol patterns after saponification are used to characterize the vegetable oils and to detect adulterations (4). However, the compositions of free and esterified sterols are available for very few oils (3,5).

Sterols are partly removed in the industrial processing of the oil, and artifacts are formed during treatment with bleaching earth (6). It is likely that free and esterified sterols behave differently under such conditions and the content of free and esterified sterols in the oil is therefore of interest.

This paper presents data on free and esterified sterols as well as on the fatty acids of the sterol esters for one variety of sunflower and three of poppy seeds. Oils from sunflower seeds are used in the margarine industries, and sunflower and poppy seeds are of potential interest as oil crops in the Swedish agriculture.

MATERIAL AND METHODS

Materials

High quality seeds were supplied by the Swedish Seed Association, Svalöv. One cultivar of sunflower, Sunbred, and three cultivars of poppy seeds, viz., Soma, Indra and Bialy Oleisty, were investigated. Reagents and chromatographic materials used were similar to those described in a previous investigation (5).

Extraction

Poppy seeds were extracted with hexane/ethanol (3:1, v/v) (5). The same extraction procedure was used for sunflower seeds with the exception that the seeds were crushed before the solvent extraction took place. Three g of sunflower seeds and four steel balls were shaken in the tubes for 30 min. The solvent was then added and the procedure continued as previously reported (5).

Isolation of Free Sterols and Sterol Esters

Nontriacylglycerols were enriched in a liquid fraction by low temperature crystallization of the oils (5). Free sterols and sterol esters were isolated from the liquid fraction and from the crude oil by preparative thin layer chromatography (TLC), using hexane/diethyl ether/acetic acid (70:30:1, v/v) as developing solvent. Sterol esters were hydrolyzed in methanolic NaOH, and the sterols and fatty acids were isolated by TLC. Sterols were silylated and fatty acids were methylated as previously described (5).

Sterol patterns were obtained in the liquid fraction and in the crude oil. Quantitative analyses were made on the crude oil using cholesterol as an internal standard.

Gas Liquid Chromatography (GLC) and Gas Chromatography-Mass Spectrometry (GC-MS)

The silylated (TMS) sterols were analyzed by GLC on two different columns with 3% SE-30

TABLE I

The Free and Esterified Sterols in Crude Sunflower and Poppy Seed Oil[a]

Species and cultivar		Composition of sterols[b] (%)										
		Cholesterol	Brassicasterol	I	II	Campesterol	Stigmasterol	III	Sitosterol	Δ5-Avenasterol	Δ7-Stigmastenol	Δ7-Avenasterol
Sunflower												
Sunbred[c]	free sterols	1.2	---	0.8	2.9	7.3	15.3	---	55.0	8.5	5.4	3.5
	esterified sterols	---	0.3	0.4	---	8.6	2.8	4.9	54.3	6.8	14.1	7.9
Sunbred[d]	free sterols	0.4	tr	0.8	3.6	7.3	15.2	---	64.0	4.2	3.1	1.4
	esterified sterols	---	tr	0.6	---	10.1	3.0	4.2	47.4	9.9	14.2	10.6
Poppy seed												
Soma[c]	free sterols		---			18.9	3.3		71.3	6.5		tr
	esterified sterols		tr			40.0	3.3		31.5	25.3		tr
Soma[d]	free sterols		---			18.9	3.8		69.4	7.9		tr
	esterified sterols		---			35.9	1.5		29.9	29.1		3.5
Indra[d]	free sterols		---			19.3	3.3		71.0	6.4		tr
	esterified sterols		tr			32.9	2.8		38.1	25.6		0.6
Bialy Oleisty[d]	free sterols		---			18.7	2.4		72.9	6.0		tr
	esterified sterols		tr			31.8	2.7		37.8	26.7		1.0
RRT[e] of individual components on OV 17		0.63	0.71	0.81	0.81	0.81	0.87	0.94	1.00	1.10	1.15	1.44
RRT[e] of individual components on SE 30		0.59	0.64	0.72	0.77	0.81	0.87	0.91	1.00	1.00	1.11	1.11

[a]The sterols were isolated by direct preparative TLC or by low temperature crystallization of the oil followed by preparative TLC and determined by GLC.

[b]I, II, and III unidentified.

[c]The sterols were isolated by direct preparative TLC of the oil.

[d]The sterols were isolated by low temperature crystallization of the oil followed by preparative TLC of the liquid fraction.

[e]Retention time for sitosterol is taken as 1.00.

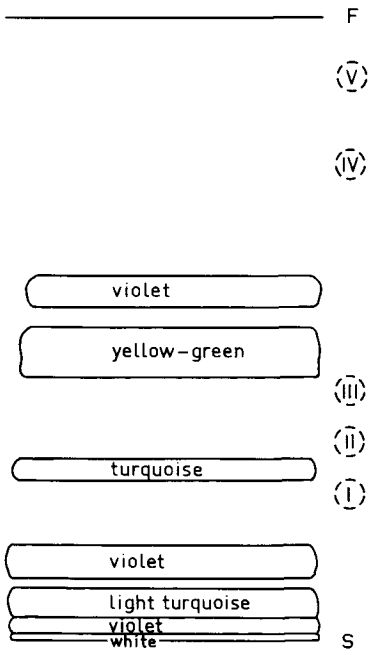

FIG. 1. Thin layer chromatogram of a precipitate from the crystallization of crude, laboratory-extracted sunflower oil (Sunbred). Absorbent: Silica Gel H. Solvent: hexane/diethyl ether/acetic acid (70:30:1, v/v). The colors noted are those observed for non-sterolic components fluorescent under UV-light (365 nm). The location of the known standards, visible under UV-light after spraying with dichlorofluorescein, are denoted by Roman numerals. I = sterols (R_f = 0.17-0.21), II = triterpene alcohols (R_f = 0.27-0.30), III = free fatty acids (R_f = 0.37-0.41), IV = triacylglycerols (R_f = 0.73-0.78), V = sterol esters (R_f = 0.87-0.90).

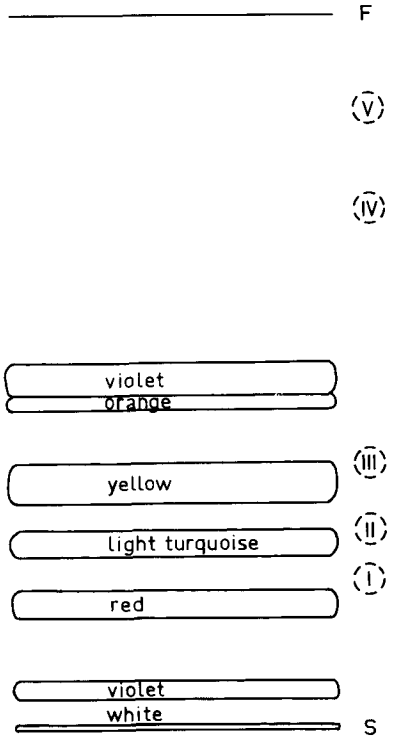

FIG. 2. Thin layer chromatogram of a precipitate from the crystallization of crude, laboratory-extracted poppy seed oil (Soma). Absorbent: Silica Gel H. Solvent: hexane/diethyl ether/acetic acid (70:30:1 v/v). The colors noted are those observed for non-sterolic components fluorescent under UV-light (365 nm). The location of the known standards, visible under UV-light after spraying with dichlorofluorescein, are denoted by Roman numerals. I = sterols (R_f = 0.17-0.21), II = triterpene alcohols (R_f = 0.27-0.30), II = free fatty acids (R_f = 0.37-0.41), IV = triacylglycerols (R_f = 0.73-0.78), V = sterol esters (R_f = 0.87-0.90).

and 1% OV-17 as stationary phases. The sterols marked I and II in Table I were calculated from analyses on the SE-30 column and the rest from analyses on OV-17. Fatty acid methyl esters were analyzed on 6% EGA and corrected for contaminants accumulated in a blank sample during the TLC procedure. The figures presented are the mean of duplicate GLC analyses. The analyses were performed in a Varian Aerograph Model 2100 gas chromatograph, and a Varian 480 digital integrator was used for the calculation of peak areas. The mass spectra were obtained on a LKB-9000 gas chromatograph-mass spectrometer (5).

RESULTS

Enrichment of Nontriacylglycerols

The liquid fractions obtained by the low temperature crystallization of the oils were greatly enriched with "minor" lipid classes, whereas the precipitate consisted almost entirely of triacylglycerols. Besides those lipid classes generally obtained as "standard unsaponifiable," e.g., hydrocarbons and free sterols, the liquid fraction contained fatty acid esters of sterols, triterpenes, and higher alcohols as well as extremely polar substances which are generally lost during the saponification procedure. The liquid fraction also contained large amounts of triacylglycerols, smaller amounts of mono- and diacylglycerols, and free fatty acids. The complex composition of the liquid fractions has previously been demonstrated in studies with olive oil (7).

In the present work, ca. 6% of the sunflower and ca. 3-4% of the poppy seed oil were recovered in the liquid fraction. The standard unsaponifiables usually amounted to 0.8-0.9%

TABLE II

Content of Free and Esterified Sterols in Crude, Laboratory-Extracted, Sunflower and Poppy Seed Oils[a]

Species	% of oil		
	Free sterols	Esterified sterols	Calculated sterol esters
Sunflower (Sunbred)	0.34	0.28	0.46
Poppy Seed (Soma)	0.33	0.05	0.08

[a]Isolation was performed by direct preparative TLC of the oil, and cholesterol was used as an internal standard.

TABLE III

Some Characteristic Fragmentations and Their Relative Intensity of Δ5-Avenasterol, Δ7-Stigmastenol and Δ7-Avenasterol in GC-MS

Fragmentation	Δ5-Avenasterol	Δ7-Stigmastenol	Δ7-Avenasterol
Molecular ion	484 (14)	486 (100)	484 (14)
M-(C_{23}-C_{29} + 1 x H)[a]	386 (100)	388 (---)	386 (56)
M-(C_{23}-C_{29} + 1 x H + TMSOH)	296 (70)	298 (2)	296 (5)
M-(side chain + 2 x H)[b]	343 (4)	343 (3)	343 (100)
M-(side chain + TMSOH)	255 (---)	255 (70)	255 (8)

[a]Arises from part of side chain by a McLafferty rearrangement and is typical for sterols containing a Δ24(28)-bond.

[b]Characteristic of Δ7,24(28)-sterols and also registered for two Δ7,24(28)-mono/methyl-sterols, see ref. 27.

in the sunflower oil (8) and 0.5% of the poppy seed oil (9).

After separation of the lipid classes in the liquid fraction by TLC, some components showed fluorescent colors when the plate was viewed under UV-light (Figs. 1 and 2). Characteristics of poppy seed oil were a bright yellow band located below the free fatty acids and a red band below the sterols. A distinct turquoise band located between the sterols and the triterpene alcohols was typical of the liquid fraction of sunflower oil. These fluorescent areas were helpful markers in the location of lipid classes.

Composition of Free Sterols and Sterol Esters in Sunflower Oil

The free sterols and esterified sterols determined by direct preparative TLC accounted for 0.34% and 0.28% of the oil, respectively (Table II). The sterol esters, calculated as sitosterol linoleate, amounted to 0.46% of the oil.

The free sterols displayed a different composition from that of the esterified sterols. The differences were related to Δ7-sterols, stigmasterol, and some unidentified sterols, whereas the percentages of sitosterol (55%), campesterol (8%) and Δ5-avenasterol (8%) were almost the same in the two fractions (Table I). Δ7-Stigmastenol and Δ7-avenasterol amounted to 22% in the esterified fraction and 9% in the free fraction, while the corresponding figures for stigmasterol were 3% and 15%, respectively. Two unidentified components (ca. 4%), with relative retention times shorter than that for campesterol, appeared preferentially in the free fraction. A third, unidentified component (ca. 5%) was eluted between stigmasterol and sitosterol and appeared only in an esterified form. One sterol from sunflower oil with the same relative retention time has previously been identified as 24-methyl-cholest-7-enol (Δ7-campesterol) (10). Small amounts of cholesterol in the free fraction and brassicasterol in the esterified fraction were also observed.

Fragmentation patterns, obtained by combined GC-MS of TMS sterol derivatives, agreed with those reported by Knights (11). This author has, however, not reported the pattern of Δ7-stigmastenol. The relative intensity of some fragments of Δ5-avenasterol and Δ7-avenasterol compared to those of Δ7-stigmastenol is given in Table III. Other characteristic fragments of Δ7-stigmastenol appeared at m/e 471 (M-15) (24%), m/3 396 (M-90) (90%), m/e 381 (M-90-15) (15%), m/e 229 (M-side chain-TMSO-27) (28%) and m/e 213 (M-side chain-TMSOH-42) (29%).

The composition of sterols isolated in the liquid fraction was slightly different from that obtained in the crude oil (Table I). For sitosterol, the difference was as much as 9% in the free sterol fraction and 7% in the esterified fraction.

The total sterol composition was calculated to be as follows: 7.9% campesterol, 9.7% stigmasterol, 54.7% sitosterol, 7.8% Δ5-avenasterol, 9.3% Δ7-stigmastenol, 5.5% Δ7-avenasterol and 5.1% other sterols.

Linoleic acid amounted to 35% of the fatty acid content of the sterol esters, and this can be compared to 77% in the total fatty acids which were mainly derived from triacylglycerols (Table IV). Fatty acids esterified to sterols also contained some longer chain fatty acids, (12% of 20:0, 22% of 22:0 and 9% of 24:0), whereas the percentages of palmitic acid, stearic acid and oleic acid were almost the same as in the total fatty acids.

Enrichment of sterol esters by the low temperature crystallization technique reduced sterols esterified to 20:0 and 22:0, compared to the direct preparative TLC technique (Table IV).

Composition of Free Sterols and Sterol Esters in Poppy Seed Oil

Free sterols from seeds of the cultivar, Soma, amounted to 0.33% of the oil and esterified sterols to 0.05% (Table II). The sterol esters, calculated as sitosterol linoleate, amounted to 0.08% of the oil.

Although qualitatively the same sterols were obtained in the two fractions, with the exception of traces of brassicasterol, which only appeared in esterified form, the composition of free sterols was remarkably different from that of the esterified sterols (Table I). Sitosterol predominated (ca. 70%) in the free sterol fraction and campesterol and Δ5-avenasterol accounted for 19 and 7%, respectively. In the esterified fraction, only 40% appeared as sitosterol while the percentages of campesterol (32%) and Δ5-avenasterol (25%) were much higher than in the free fraction. These figures were obtained directly from the crude oil and differed by less than 4% from those obtained from the liquid fraction after crystallization of the oil.

Only small differences were observed in the sterol patterns of the cultivars investigated (Table I). The morphine-"free" cultivar, Soma, displayed, however, slightly lower percentages of sitosterol and higher Δ5-avenasterol in the esterified fraction than Indra and Bialy Oleisty. The total sterol pattern of the Soma cultivar was as follows: 21.6% campesterol, 3.3% stigmasterol, 66.1% sitosterol and 9.0% Δ5-

TABLE IV

The Total Fatty Acids and the Fatty Acids of Sterol Esters in Sunflower and Poppy Seed Oils[a]

Species and cultivar	Total fatty acids (%)					Fatty acids (%) of the sterol esters						
	16:0	18:0	18:1	18:2	18:3	16:0	16:1	18:0	18:1	18:2	20:0	22:0	24:0
Sunflower													
Sunbred[b]	6.3	3.1	12.7	76.7	0.2	6	1	2	10	35	12	22	9[d]
Sunbred[c]						7	tr	3	22	50	1	8	8[e]
Poppy seed													
Soma[b]	10.4	1.9	10.2	76.5	1.0	18	7	5	19	51			
Soma[c]						17	tr	9	30	44			
Indra[c]	10.1	1.7	10.4	77.4	0.5	33	tr	14	24	29			
Bialy Oleisty[c]	11.1	2.1	9.1	77.1	0.4	23	tr	12	27	38			

[a]The sterols were isolated by direct preparative TLC or by low temperature crystallization of the oil followed by preparative TLC.
[b]The sterols were isolated by direct preparative TLC of the oil.
[c]The sterols were isolated by low temperature crystallization of the oil followed by preparative TLC.
[d]Also 1% of 18:3, 1% of 20:1 and 1% of an unidentified fatty acid are present.
[e]Also 1% of 20:1 is present.

avenasterol.

A lower percentage of linoleic acid (51%) was obtained among the sterol-bound fatty acids than among the total fatty acids (77%), whereas fatty acids esterified to sterols contained a higher percentage of 16:0, 16:1 and 18:1 (Table IV). The pattern of the fatty acids esterified to sterols in the Soma cultivar was slightly different from those of Indra and Bialy Oleisty. The analyses of Soma were, however, performed on freshly harvested seeds while the others had been stored for about 10 months. Analyses of fatty acids esterified to sterols in Soma after the same period of storage showed the following composition: 27% 16:0, tr 16.1, 12% 18:0, 28% 18:1 and 33% 18:2, which agree with the patterns found for Indra and Bialy Oleisty. Enrichment of the sterol esters by the low temperature crystallization technique resulted in a loss of sterols esterified to 16:1 and 18:2, which tended to separate out in the precipitate (Table IV).

DISCUSSION

Concentration of minor lipid classes by low temperature crystallization of vegetable oils has previously proved selective towards some sterol esters in rapeseed oil (5), and recovery factors are needed in quantitative studies. Since the technique yielded different amounts of "liquid fraction" for various oils, it is likely that different recovery factors are also needed. This would make the technique unpractical for quantitative studies. In the present investigation, all quantitative determinations have been performed by direct preparative TLC of the original oils. However, we consider the crystallization technique still of interest in qualitative studies of minor components, and a comparison between this technique and the direct preparative TLC technique was made.

The content of sterols in the sunflower oil (0.62%) was higher than that previously reported for similar oils. A German study (8) on six varieties showed 0.42-0.48% and others have reported 0.25-0.35% (1,12,13). In these investigations, the sterols were determined in the nonsaponifiable fraction by the digitonin method. Two reports (1,13) show great differences on the quantities of free and esterified sterols. The free sterols account for three times as much as the esterified sterols in one report (1). In the second (13), approximately similar amounts were found and this agrees with the data in the present investigation. These differences in quantities of sterols might be due to the anaytical techniques used, plant cultivars or climatic influences.

Reports on the sterol composition of sunflower oils (8,14-22) show a great diversity, and ranges are: 0-0.7% cholesterol, 7.6-13.6% campesterol, 4.8-13.1% stigmasterol, 0-2.8% Δ7-campesterol, 53.6-69.0% sitosterol, 3.0-19.3% Δ5-avenasterol, 4.1-15% Δ7-stigmastenol and tr-7.2% Δ7-avenasterol. The results obtained in the present investigation were within the ranges reported in the literature. Small amounts of two Δ7-stigmastadienols and one Δ7-stigmastatrienol, not usually detected in nature, have been isolated from sunflower (11). It is, however, unlikely that the unidentified components in this investigation, having relative retention times shorter than that of campesterol (Table I), represent any of these sterols. GLC analyses of sterols on capillary columns show traces of other components which are usually not observed in analyses on packed columns (18,19,23).

Δ7-Stigmastenol and Δ7-avenasterol were found in smaller amounts in the free than in the esterified fraction (9% vs. 22%), while other workers have found that Δ7-sterols only occur in the esterified fraction and are absent in the free fraction (3). The differences could be due to a number of variables. However, it has been observed that Δ7-sterols in rapeseed occurred in lower amounts in seeds that had been stored for six months compared to freshly harvested seeds (24). This observation might be of importance also for Δ7-sterols in sunflower seeds. Δ7-Stigmastenol is of special interest, since a relatively high amount of this sterol has been suggested as characteristic of Compositae oils (25). The mass spectra of TMS-Δ7-stigmastenol had a very high relative intensity of the molecular ion (100% at m/e 486) whereas others (26) have found a relative intensity of 70% for the molecular ion and a base peak at m/e 255. This difference is probably due to instrumental parameters, which influence the mass spectra of trimethylsilyl ethers of sterols (27).

The fatty acids esterified to sterols in sunflower oil were characterized by relatively high percentages of very long chain fatty acids. It is probable, however, that these fatty acids might be bound to 4-monomethyl sterols or 4,4-dimethyl sterols, which appear in large amounts in sunflower oil (12,25), rather than to the desmethyl sterols, since such esters were not fully separated in the TLC system used. Attempts to separate des-, mono- and dimethyl sterol esters in citrus species by argentation TLC (28) indicated that very long chain fatty acids were esterified primarily to dimethyl sterols. A previous report on fatty acids in sunflower oil did not show any long chain fatty

acids (3). The investigators isolated the sterol esters by adsorption TLC with a somewhat less polar developing system than in the present investigation. From experience in our laboratory, this is, however, not sufficient to separate des-, mono- and dimethyl sterol esters.

The content of sterols in poppy seed oil (0.38%) was found to be slightly higher than previously found (0.25-0.28%) (9,12,13). Nothing seems to be reported on the quantitative composition of the separate free and esterified sterols, whereas the total sterol pattern in the present investigation agrees rather well with literature data (9,13). It is noteworthy that Δ5-avenasterol, which is of interest since it might be effective in retarding the oxidative polymerization of oils during heating (29-31), accounted for 26-29% of the esterified sterols.

The composition of sterols in the free form was remarkably different from the composition in the esterified form in the poppy seed oils. Thus, the total sterol patterns are dependent on the quantities of free and esterified sterols. Factors such as cultivar, place of cultivation, etc., that might influence these quantities may also effect the total sterol pattern.

Differences were observed in the sterol-bound fatty acids in the three cultivars of poppy seed. Caution must be taken, however, in drawing any conclusions about cultivar differences since the analytical procedure involves many steps, and analytical errors cannot be excluded. Whether any changes in the patterns of fatty acids of the sterol esters occur during storage is also uncertain.

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Metabolism of Naphthenic Hydrocarbons. Utilization of a Monocyclic Paraffin, Dodecylcyclohexane, by Rat

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ABSTRACT

^{3}H-Dodecylcyclohexane was incorporated in rat diet in order to study the metabolic utilization by mammals of a monocycloparaffin chosen as a typical naphthenic constituent of mineral oils. Dodecylcyclohexane was largely absorbed. No elimination of the hydrocarbon was observed in urine while an extended excretion of ^{3}H occurred via this route. About 7% of absorbed dodecylcyclohexane was stored in the carcass, while the rest was ω-oxidized to cyclohexyldodecanoic acid, which incorporated into neutral lipids and phospholipids. The aliphatic chain of this unusual fatty acid underwent the normal fatty acid degradation pathways, leading to even fatty acids, squalene, cholesterol and nonlipid resynthesis, while the cyclohexyl ring was eliminated as urinary metabolites. Incorporation of ω-cyclohexyl fatty acids in phospholipids may raise a point of toxicological significance which is to be investigated.

INTRODUCTION

Saturated hydrocarbons are widespread in the environment (1), and it is now well established that they are ubiquitous throughout the animal kingdom; their presence in mammalian tissues is not completely elucidated, but a dietary origin is commonly admitted.

Normal and branched paraffins are known to be synthesized (2-5) by marine and terrestrial plants, and thus are present in most mammal diets. Moreover, fossil hydrocarbons constitute the main source of naphthenic hydrocarbons, and petroleum industry derivatives, such as mineral oils, contain 70 to 85% of these compounds, distributed mainly in mono-, di-, tri- and tetracycloparaffins with alkyl side chains. Since 1945, an increasing accumulation of cyclic paraffins in human tissues has been mentioned (6), and correlated to an increasing use of mineral oil in the food processing industry. These contaminations, associated with dietetic and therapeutic utilizations, provide a human exposure of significant magnitude.

Until it has been recently established that dodecylcyclohexane was highly absorbed by rat (7) and stored in adipose tissue (8), then that the terminal oxidation and subsequent oxidation of the n-alkyl side chain occurred (9), no data were available concerning the metabolic fate of cycloparaffins. The joint FAO/OMS Expert Committee on Food Additives (1974) underlined the need of investigations on the metabolic fate of such compounds and the significance of their storage. The aim of this work was to complete these partial data in order to obtain a general view of the metabolic utilization of these uncommon carbon chains by mammals. Moreover, because fatty acids with cyclic structure result from the initial oxidation step, incorporation into the lipid compartment as well as the possible interactions with lipid metabolism were particularly pointed out.

Dodecylcyclohexane, already identified as a normal component of mineral oils (10), was chosen as a model of monocycloparaffin.

EXPERIMENTAL PROCEDURES

Phenyldodecane (Fluka) was tritiated in the Service of Labeled Molecules at the C.E.A. (Saclay, France) by direct contact of 2 g phenyldodecane with tritium gas (Wilzbach method). ^{3}H-dodecylcyclohexane was prepared in our laboratory by catalytic hydrogenation of ^{3}H-phenyldodecane; 500 mg dodecylbenzene in acetic acid (5 ml) were hydrogenated in an autoclave at 10 atm in the presence of Adam's catalyst (50 mg). After 24 hr, hydrocarbons were extracted with hexane, and the product obtained was freed from traces of unreacted phenyldodecane by preparative thin layer chromatography on silica gel, using hexane as developing solvent. Chemical and radiochemical purities were checked by gas chromatography and radiochromatography, respectively. Specific activity of ^{3}H-dodecylcyclohexane obtained was 116 mCi/mM.

Wistar male rats weighing about 200 g were fed "ad libitum" on a semisynthetic diet, the composition of which has been given previously (11). ^{3}H-dodecylcyclohexane diluted with analytical grade (99% min) dodecylcyclohexane (J.T. Baker Chem.) was incorporated in the peanut oil of the diet. As in previous experiments, a small amount of feed was distributed to the animals the day before administration of the labeled hydrocarbon, so that ^{3}H-dodecyl-

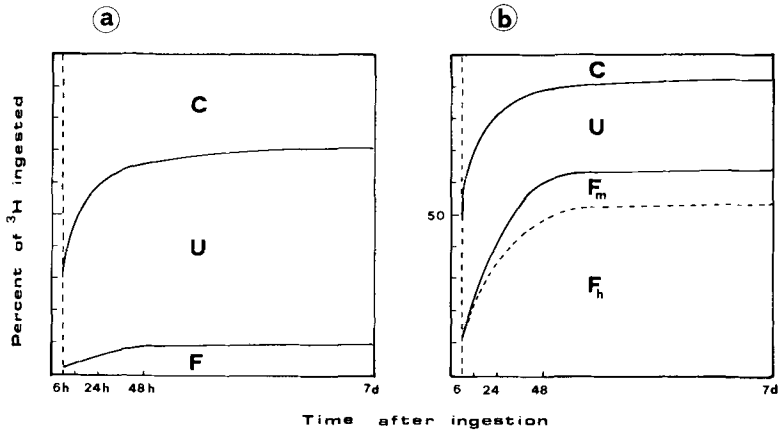

FIG. 1. Metabolic balance established after administration of one dose of dodecylcyclohexane. a = 20 mg; b = 200 mg; C = carcass; U = urine; F = feces; F_h = fecal hydrocarbon; F_m = fecal metabolites.

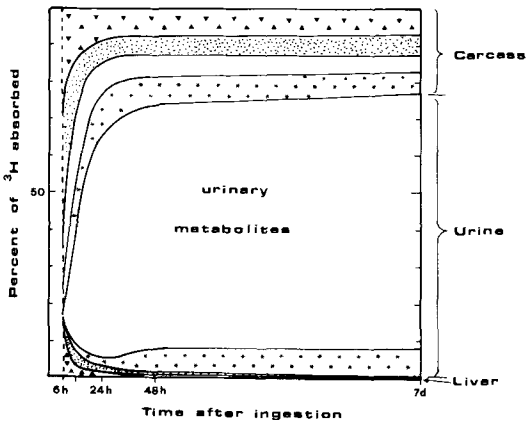

FIG. 2. Distribution of ^{3}H in rat liver, carcass and urine after oral administration of ^{3}H-dodecylcyclohexane (one 20 mg dose) ▲▲ hydrocarbon; ⁙ lipids; □ nonlipids; * * tritiated water.

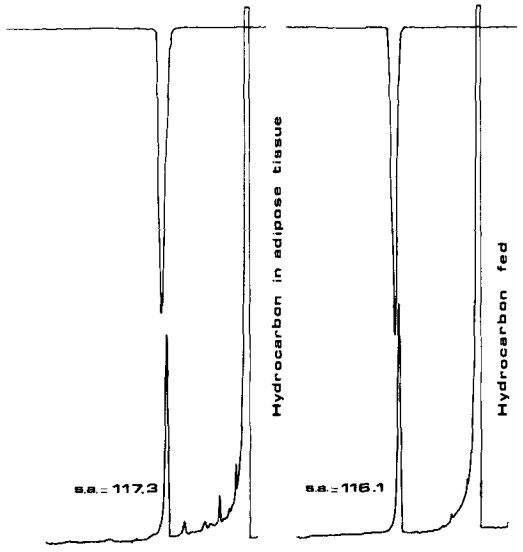

FIG. 3. Radio gas chromatography of hydrocarbon fed and of hydrocarbon in adipose tissue: upper trace = radioactivity; lower trace = mass; s.a. = specific activity (mCi/mM).

cyclohexane would be ingested within 15 min; then control diet was given "ad libitum."

Lipid incorporation kinetics were studied in animals killed 6 hr, 12 hr, 24 hr, 48 hr and on the 7th day, respectively, after ingestion of labeled dodecylcyclohexane.

The methods used for separation of total lipids into different classes as well as for separation of each class into its different components have been described (11). Urea adducts of straight chain fatty acid methyl esters were prepared (12) in order to enrich the fraction containing branched and cyclic fatty acids.

Total radioactivity of tissues, urine, and feces was measured after combustion in an oxidizer (Intertechnique, Oxymat). Estimation of tritiated water was obtained by comparing radioactivities in fresh and lyophilized samples, after it has been established that no loss of unchanged hydrocarbon or metabolites occurred during the freeze-drying process. Radioactivity on thin layer plates was localized by using a Berthold II thin layer scanner. Labeled fatty acids were identified by radio gas chromatography using a reactor (RGC 170 Perkin Elmer) connected to the gas chromatograph (Packard 7300). Tritiated water resulting from hydrogenative cracking was measured with a proportional counting tube.

TABLE I

Distribution of ^3H in Tissues 24 hr after Oral Administration of ^3H Dodecylcyclohexane

Tissue	dpm/mg
Adipose tissue	2846
Kidney	660
Liver	596
Lung	424
Spleen	390
Brain	380
Testes	343
Thymus	341
Heart	254
Blood	22

RESULTS

The metabolic balance was established after a single administration to a rat of two doses of ^3H-dodecylcyclohexane (20 and 200 mg), and was calculated from the radioactivity excreted in urine and feces for 6 days and from the residual radioactivity measured in the whole carcass at the end of the experiment (Fig. 1).

In the first 48 hr, 95% of total ^3H excretion occurred, but a major difference is observed in the extent of fecal radioactivity excretion. The nature of labeled substances in feces was not studied in detail. However, it was shown that the percentage of ^3H-dodecylcyclohexane (F_h) decreased with time. Hydrocarbon oxidation by the intestinal flora has been investigated by several authors (13), who concluded that no attack occurs at extended level. Moreover, no recirculation of unchanged hydrocarbon via the biliary route was noticed (13,14). Therefore, it may be stated that labeled hydrocarbon in feces corresponds to the nonabsorbed fraction of ingested dodecylcyclohexane. The absorption level is clearly related to the administered dose.

No trace of hydrocarbon was detected in urine when a high level of radioactivity was excreted by this route, indicating a metabolic transformation of dodecylcyclohexane. This fact is underlined by the continuous and slow decrease of carcass radioactivity over the 7 day period and concomitant increase of urinary excretion.

Kinetics of the distribution of the radioactivity in liver, remaining carcass without digestive tract, and urine, over the 7 day period, are reported in Figure 2. They show a biphasic phenomenon. As already mentioned, most of the urinary excretion occurs during the first 48 hr; at the same time, radioactivity in liver and carcass decreases rapidly. These observations underline the intensity of the metabolic transformations of dodecylcyclohexane. The analysis of the labeling of different compartments indicates that ^3H was largely incorporated into lipids as early a 6 hr after ingestion of the hydrocarbon; however, ^3H incorporation in the nonlipids and the tritiated water was delayed, reaching a maximum after 24 hr, which would suggest successive metabolic steps.

Specific activity of stored hydrocarbon was compared to that of hydrocarbon fed (Fig. 3); results are in agreement and establish that the distribution of radioactivity in compounds other than hydrocarbon was entirely due to metabolism and not exchange.

Phenomena occuring between 48 hr and 7 days are very slow. Radioactivity in the form of dodecylcyclohexane remained almost unchanged in the carcass, and the weak evolving of label in the nonlipid fraction must be related to the slow turnover of body constituents such as proteins. The distribution of ^3H among body tissues, as measured 24 hr after oral administration of ^3H dodecylcyclohexane, is shown in Table I; the main part of the radioactivity was associated with the lipidic fraction of the tissues.

NEUTRAL LIPIDS

Neutral lipids of liver and adipose tissue,

TABLE II

Distribution of ^3H in Liver Neutral Lipids 24 hr after Ingestion of ^3H Dodecylcyclohexane

Fraction	Percent of total ^3H[a]	Specific activity[b]
Monoglycerides	2.1	12050
Diglycerides + cholesterol	17.6	4390
Free fatty acids	11.2	39600
Triglycerides	35.7	2940
Cholesteryl esters + squalene	33.4[c]	19320

[a] ^3H in neutral lipids freed from saturated hydrocarbons.

[b] dpm/mg.

[c] Further separation indicated that ^3H was distributed in cholesterol 6.6, fatty acids 25.3 and squalene 1.5.

TABLE III

Distribution of ^3H in Fatty Acids from Liver Neutral Lipids

Fatty acid	Composition[a]	Radioactivity[b] After 6 hr	Radioactivity[b] After 24 hr
14:0	1.4	N.D.	2.5
16:0	22.8	1.8	18.4
Cyclohexyloctanoic	N.D.	N.D.	1.2
16:1 and/or Cyclohexyloctenoic	3.8[c]	0.6	1.1
18:0	2.4	1.2	12.1
Cyclohexyldecanoic	N.D.	11.1	20.2
18:1 and/or Cyclohexyldecenoic	53.2[c]	2.1	2.6
18:2	13.2	---	---
20:0 + 18:3	0.3	N.D.	N.D.
Cyclohexyldodecanoic	N.D.	80.9	40.6
20:1 and/or Cyclohexyldodecenoic	0.7[c]	2.3	1.3
20:4	1.2	---	---

[a]p.100 of total fatty acids determined by GLC on DEGS.

[b]p.100 of ^3H in fatty acids.

[c]Monounsaturated cyclohexyl fatty acids were undetectable, but their theoretical retention time on DEGS were the same as for monounsaturated even fatty acids.

freed from hydrocarbons, were separated into their different classes (Table II). If triglycerides were the most labeled fraction of adipose tissue (70% of total radioactivity), radioactivity was widespread in the lipid classes of the liver. Specific activity of free fatty acids was the highest one, due to the first step of hydrocarbon metabolism. Fractionation of the unresolved fraction squalene + cholesteryl esters into its components showed that squalene and cholesterol were tritiated and correspond to "de novo" synthesis from tritiated acetyl-CoA resulting from β-oxidation of the alkyl substituent of cyclohexyldodecanoic acid.

Analysis of liver fatty acids indicated that 6 hr as well as 24 hr after ingestion of ^3H-dodecylcyclohexane, 95% of the radioactivity was bound to saturated acids and the rest to monounsaturated acids. Distribution according to the chain length was studied at different times after hydrocarbon ingestion (Table III). After 6 hr, 97% of the labeled saturated fatty acids were ω-cyclohexyl-acids (Fig. 4), whereas 24 hr later these compounds represented no more than 65% of the labeling, and resynthesis of even fatty acids (stearic and palmitic) was important. Regarding labeled monounsaturated fatty acids, we were unable, due to their weak specific activity, to determine whether they were unsaturated ω-cyclohexyl-acids or resynthesized even fatty acids.

In adipose tissue, the radioactivity was

TABLE IV

Incorporation of ^3H in Tissue Phospholipids 24 hr after Ingestion of ^3H Dodecylcyclohexane

Tissue	Specific activity[a]
Liver	35400
Heart	2500
Brain	4500
Spleen	6000
Kidney	6000
Testes	1100

[a]dpm/mg phospholipids.

TABLE V

Distribution of ^3H in Liver Phospholipids 6 hr after Ingestion of ^3H Dodecylcyclohexane[a]

	Percent of total ^3H	Specific activity[b]
Phosphatidic acid	2.9	638
Phosphatidyl ethanolamine	37.1	842
Phosphatidyl serine	7.7	568
Phosphatidyl choline	48.4	406
Sphingomyelin	3.9	478

[a]10 μCi ^3H dodecylcyclohexane.

[b]dpm/mg.

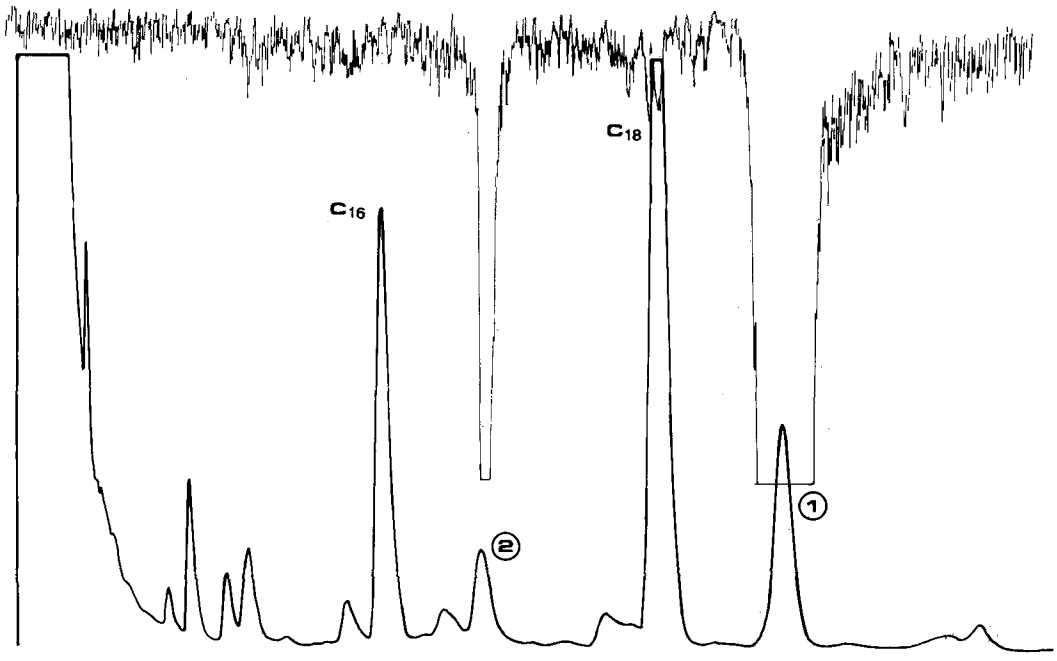

FIG. 4. Gas liquid radiochromatography of saturated fatty acid methyl esters derived from neutral lipids of rat liver 6 hr after ingestion of ^{3}H-dodecylcyclohexane; cyclohexyl fatty acids were concentrated by complexation with urea of straight chain fatty acids; 1 = cyclohexyldodecanoic acid; 2 = cyclohexyldecanoic acid; upper trace = radioactivity; lower trace = mass.

distributed between saturated and monounsaturated fatty acids, the same as in the liver; after 7 days, ω-cyclohexylacids represented 70% of the labeling.

PHOSPHOLIPIDS

All organs incorporated radioactivity into their phospholipids within the first 24 hr (Table IV), and the major labeling was observed in liver. Phospholipid fatty acids were found to contain 99% of total ^{3}H; when separated according to the degree of unsaturation, 90% of the radioactivity was associated with saturated acids and the rest with monounsaturated acids. Analysis of phospholipid fatty acid methyl esters indicated that 80% of the saturated fraction were ω-cyclohexyl acids.

Separation of the different classes of liver phospholipids (Table V) revealed that most of the radioactivity was in phosphatidylcholine. Determination of the specific activities indicating an homogeneous distribution, it can be stated that ω-cyclohexylacids are incorporated into all phospholipid classes at about the same rate.

DISCUSSION

The present work is the first investigation of the metabolic utilization of an alkyl-substituted cycloparaffin by mammals; it has been conducted with the same methodology as already used for the study of n-heptadecane metabolism (11). Our results clearly show that absorption, storage, and oxidation of the aliphatic chain of dodecylcyclohexane occur in the same way as for the n-paraffin. Among data available concerning hydrocarbon absorption, only one refers to naphthenes, namely pentadecylcyclohexane (13), which were given by stomach probe. In our study, a higher absorption level of dodecylcyclohexane is observed, owing to the incorporation of hydrocarbon in the animal diet that mimics food contamination conditions. It was pointed out that absorption was related to the administered dose, and from this point of view, no difference between cycloparaffins and n-paraffins can be noticed, at least when total number of carbon atoms are in the same range. As shown for n-heptadecane, dodecylcyclohexane accumulates in the carcass during the first hours, then levels off; distribution in the organism suggests that dodecylcyclohexane is likely transported via lymphatic route and reaches the tissues, especially adipose tissue, where it accumulates because of its lipophilic nature (14,15). Stored hydrocarbon

evolves little between 48 hr and 7 days after ingestion, and this is in agreement with what was observed previously (7).

Evidence for ω-oxidation of the aliphatic chain by the rat has been given (9). This fact is underlined by the presence of cyclohexyldodecanoic acid in liver and adipose tissue. It is noteworthy that conversion of paraffinic and naphthenic hydrocarbons to the corresponding acids occurs in the microsomal fraction of rat liver (Durand and Tulliez, unpublished results), but it is not excluded that the reaction might happen in the mucosa of the small intestine (16) during the absorption process. The alkyl chain undergoes the classical fatty acid degradation pathway, as shown by the presence of cyclohexyldecanoic acid. Metabolic utilization of the resulting labeled acetyl-CoA explains the extent of resynthesis of labeled fatty acids as soon as 6 hr after ingestion of the hydrocarbon. If incorporation of tritium in the nonlipids is noticeable in the carcass at 6 hr, it reaches a maximum at 24 hr. Moreover, it was not possible to know whether radioactivity in the tissues was bound to normal body components (carbohydrates, proteins) or associated to labeled metabolites with cyclohexane ring.

Metabolism of fatty acids produced by ω-oxidation of linear and branched paraffins has been described (11,17) and thus, if the metabolic behavior of the saturated linear chain of n-alkyl substituted cycloparaffins is clearly established, the fate of the residual cyclic structure is not elucidated until now. It may be supposed that the β-oxidation process leads to cyclohexylacetic acid and such an assumption is supported by the identification in the urine of a metabolite with cyclic structure, namely a glycine conjugate of cyclohexenylacetic acid (18).

It has been shown that oxidation of n-alkyl substituted cycloparaffins may occur in some microorganism species, mainly from soil (19), and that the resulting acids were incorporated predominantly in the structural lipids. The identification of minute quantities of cyclohexylundecanoic acid in butter (20) and sheep perinephric fat (21) has been related to the metabolic activity of the rumen microflora (22). It results that incorporation of ω-cyclohexyl substituted fatty acids into lipids and, more particularly in phospholipids, raises a point of toxicological significance. A preliminary study was carried out, the goal of which was to determine the extent of this phenomenon. Rats maintained on a diet containing 1% dodecylcyclohexane for two months exhibited ω-cyclohexyl fatty acid levels corresponding to 3% of the total fatty acids in whole body phospholipids. Thus, it will be of interest to investigate the possible interactions of such uncommon fatty acids with the biochemical mechanisms in which the structural lipids are involved.

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Distribution of Hydrocarbons in Bovine Tissues

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ABSTRACT

Liver, heart, kidneys, muscle and adipose (perirenal and subcutaneous) tissues were collected from six animals for analysis of their hydrocarbon composition. Qualitative and quantitative determinations were carried out by gas chromatography and combined gas chromatography-mass spectrometry. Although differing in the proportions, a homologous series of n-alkanes ranging from n-C_{12} to n-C_{31} was found in all the samples examined. The isoprenoid hydrocarbons phytane and phytene (phyt-1-ene and phyt-2-ene) were also identified.

INTRODUCTION

Several authors have reported the presence of different classes of hydrocarbons (linear, branched and isoprenoid) in animal tissue (1-4), human tissue (5-7), microorganisms (8-10), milk fat and seed oil (11-13), and wheat flour (14). Although the origin of hydrocarbons in animal tissues has not yet been elucidated, they are thought to be derived either from natural biosynthetic processes (15), environmental pollution, or the use of paraffins and mineral oils in food technology. Furthermore, the proposed utilization of single cell proteins (SCP) as partial protein source in cattle feed may contribute to the transferring of n-alkane residues to animal tissues and hence to the food chain.

Although the metabolic role and biological significance of hydrocarbons have not been clarified, their presence has been associated with several human pathological conditions, such as splenic follicular lipidosis (16), human atherosclerotic plaques (7,17) and neoplastic tissues (5). More recently, the cocarcinogenic and tumor-promoting activities of n-alkanes have been studied in relation to possible effects on membrane transport processes (18,19).

Consequently, because of the potential effects of n-alkanes on human health, a determination of levels and distribution of n-alkanes in various bovine tissues was carried out with the aim of establishing baseline values which would further allow comparison between tissue distribution and accumulation in traditionally and SCP fed cows.

n-Alkanes ranging from C_{12} to C_{32} and some isoprenoid hydrocarbons were identified and quantitatively determined in tissues from cows aged 8-12 yr. Liver, heart, kidney, muscle and adipose tissues were separately collected from six animals and analyzed for hydrocarbon content.

Variation in values in different tissues from the same animal was interpreted as due to differences in kinetics of deposition or metabolic pathways.

MATERIALS AND METHODS

All solvents (analytical grade reagents) used were redistilled using a 40 cm column packed with Raschig rings. The adsorbents for column chromatography and the anhydrous sodium sulfate were extracted with petroleum ether (40-60 C) in a Soxhlet apparatus. All glassware was washed with diethyl ether and n-pentane. Only separatory funnels with Teflon stopcocks were used and the samples were never allowed to come in contact with rubber or plastic. Complete procedure blanks were often made to ascertain the presence of any contamination.

The tissues were obtained from freshly butchered animals, wrapped in aluminum foil previously washed with diethyl ether and n-pentane, chilled and transported immediately to the laboratory for analysis. An outer layer of ca. 1 cm was always discarded using solvent-washed blades. Major arteries and veins were also removed from the organs.

Extraction and Isolation

The minced sample (25 g, wet weight) was saponified by refluxing for 2 hr with 100 ml of 95 ethanol containing 25 g of KOH and 50 ml of water. The lukewarm solution was then transferred to a separatory funnel and the saponification flask rinsed with small amounts of water, ethanol and n-pentane, in that order. The mixture was extracted with 3 x 50 ml portions of n-pentane. The combined pentane phases were eluted on a glass column (20 mm i.d. x 200 mm high) packed with (from bottom to top) a 2 cm layer of nonactivated silica gel followed by an 8 cm layer of anhydrous sodium sulfate. The separatory funnel and the column were washed with n-pentane (3 x 20 ml). The solution was carefully concentrated to a volume of ca. 1 ml under reduced pressure

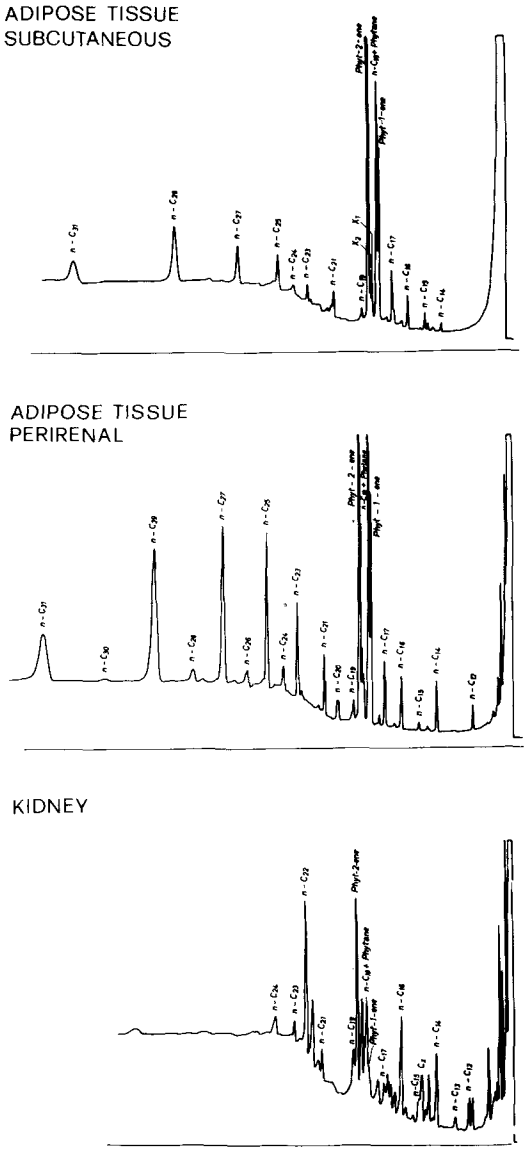

FIG. 1. Gas liquid chromatograms of the hydrocarbon fraction of three bovine tissues. For experimental conditions, see Materials and Methods.

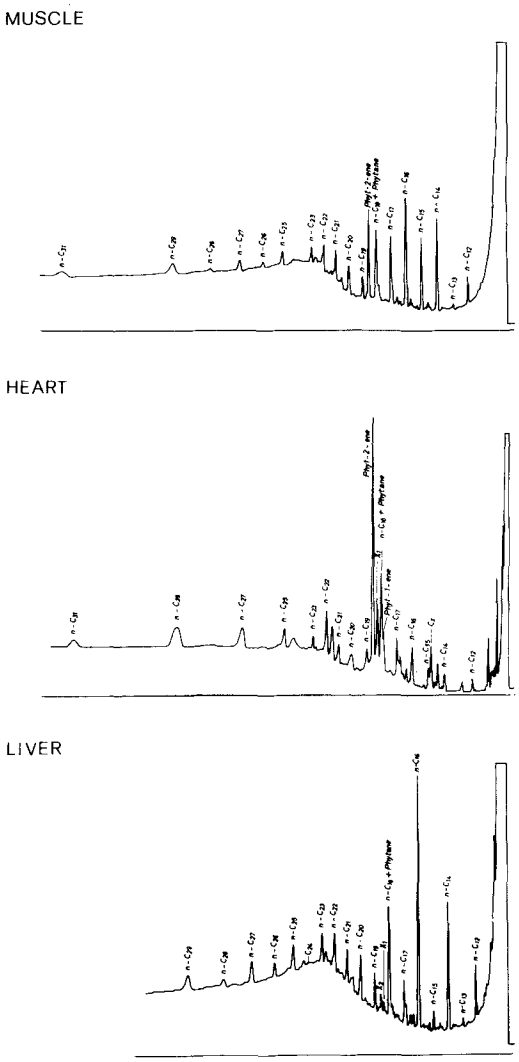

FIG. 2. Gas liquid chromatogram of the hydrocarbon fraction of three bovine tissues. For experimental conditions, see Materials and Methods.

on a rotary evaporator at 30 C, and the concentrate was applied as a narrow band, extending over a length of 18 cm, on a silica gel plate (0.5 mm thick, washed with diethyl ether and activated for 1 hr at 105 C).

The plate was developed with n-pentane and then sprayed with an 0.2% ethanol solution of 2′,7′ -dichlorofluorescein. The band corresponding to the R_f of a standard n-C_{16} (Applied Science Labs., Inc., State College, PA) was then scraped from the plate, transferred to a small glass column and eluted with 15 ml of a 1:1 mixture of diethyl ether and pentane. The eluate was collected in a graduated tube. A small amount of iso-octane (0.2-0.3 ml) was then added to the sample prior to concentration to a final volume of ~0.5 ml on a rotary evaporator.

Identification and Quantitative Determination

Hydrocarbons were analyzed by gas chromatography on a 3 m x 3 mm i.d. glass column packed with 5% OV-61 on 80/100 mesh Chromosorb W-HP programmed from 100 to 250 C, 6°/min, and then run at 250 C for a

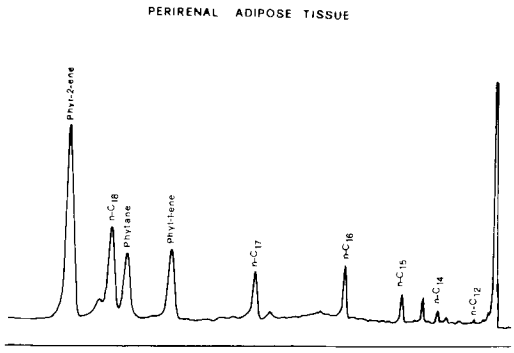

FIG. 3. Gas liquid chromatogram of the hydrocarbon fraction of perirenal subcutaneous tissue on a capillary column. For conditions, see Materials and Methods.

further 30 min. The gas chromatograph utilized was a Carlo Erba Fractovap 2200, equipped with a flame ionization detector. The areas of the peaks were quantified by an electronic integrating unit (Varian, model CDS 101) with the baseline technique.

The identification was achieved by comparison with a standard mixture of even-numbered n-alkanes from C_{12} to C_{32} (Applied Science Labs Inc., State College, PA). The identification was confirmed further by injecting a small amount of the standard with the sample and observing the enhancement of the corresponding peaks.

Quantitative determination was performed by adding to the final extract an appropriate amount of an n-alkane not present in the chromatogram or present in a small amount.

The samples were successively analyzed on a 90 m x 0.25 mm i.d. stainless steel capillary column coated with Apiezon L operated isothermally at 175 C, splitter ratio at the injection port 1/50, carrier gas helium.

Gas chromatography-mass spectrometry (GC-MS) was employed as a confirmatory technique using an LKB 9000S unit. The gas chromatographic conditions previously described were utilized for the GC/MS analysis, employing helium as the carrier gas. Mass spectra were recorded at the maximum of the most intense gas chromatographic peaks at an ionizing energy of 20 eV.

The extracts prepared according to the method described often contained considerable amounts of phytenes, which are predominant among the unsaturated hydrocarbons. To confirm their identity, the samples were submitted to catalytic hydrogenation and then reanalyzed. The hydrocarbon mixture, dissolved in iso-octane, was transferred to a Packard microhydrogenator to which 10-15 mg of PtO_2 had been previously added. After saturation of the chamber with pure hydrogen, the solution was kept under bubbling hydrogen for 30 min at room temperature and then filtered to remove the catalyst.

By the use of n-pentane for the extraction and purification of the hydrocarbon fraction, it was possible to minimize the losses of some of the most volatile n-alkanes. Percent recoveries, performed at the 2 ppm level, gave values of over 90% for all n-alkanes from n-C_{16} up, while for n-C_{14} and n-C_{12} the values were 72% and 60%, respectively.

RESULTS AND DISCUSSION

The analyses revealed a complete homologous series of n-alkanes from C_{12} to C_{31}. The chromatograms reported in Figures 1 and 2 show some complexity in the area corresponding to the n-C_{18}. When the same samples were analyzed on a capillary column, the area corresponding to n-C_{18} displayed two peaks (Fig. 3). The compound responsible for one of them was partially differentiated by successive GC/MS analysis on a packed column and identified as phytane (2,6,10,14-tetramethylhexadecane) by comparison with mass spectra reported in the literature for this compound. Further GC/MS analyses evidenced also phyt-1-ene (3,7,11,15-tetramethylhexadec-1-ene) and phyt-2-ene, as identified by comparison with mass spectra reported by Urbach and Stark (13). In agreement with these authors, the peak identified as phyt-1-ene showed a retention time shorter than that of phyt-2-ene when a nonpolar column (OV-101, OV-61, Apiezon L) was utilized. Furthermore, after hydrogenation, the two peaks resulted in a single peak with retention time and mass spectra corresponding to those of phytane.

In some cases, mass spectra recorded on the tail of the peak identified as phyt-2-ene showed a molecular ion at m/e 278, suggesting the presence of neo-phytadiene (3-methylene-7,11,15-trimethylhexadec-1-ene), which, according to Urbach and Stark (13), is not well separated from phyt-2-ene on packed columns.

In the area of n-C_{18}, two additional peaks were observed; these peaks were designated as x_1 and x_2. Because of their low concentration, no clear identification was possible. However, because of their retention time (not corresponding to any n-alkane), the fact that they do not undergo hydrogenation and are well separated from phytane, they can only be hypothesized to belong to the group of the branched n-alkanes and/or alkyl-substituted cyclohexanes

TABLE I

Range Values (ppm) for Each Hydrocarbon Identified in the Tissues of the Six Animals Examined

Hydrocarbon	Adipose tissues		Muscle	Liver	Heart	Kidney
	Subcutaneous	Perirenal				
C_{12}	0.03- 0.28	0.10- 0.23	0.02-0.09	0.08-0.35	0.04-0.10	0.02-0.08
C_{13}			0.01-0.02	0.02-0.03		
C_{14}	0.03- 0.78	0.27- 0.56	0.14-0.34	0.23-1.66	0.14-0.30	0.05-0.22
C_{15}	0.04- 0.45	0.16- 0.37	0.09-0.27	0.04-0.14	0.05-0.19	0.02-0.15
C_{16}	0.11- 0.76	0.44- 0.62	0.25-0.54	0.46-2.04	0.21-0.72	0.08-0.31
C_{17}	0.09- 1.18	0.16- 0.48	0.03-0.17	0.13-0.38	0.07-0.55	0.03-0.06
Phyt-1-ene	3.46- 6.83	1.88-15.57	0.22-3.09			
C_{18}	2.26- 5.00	2.58- 4.11	0.21-0.28	0.15-1.30	0.23-0.35	0.13-0.35
Phytane	2.03- 3.50	2.05- 4.89	0.10-0.25	0.11-0.40	0.15-0.22	tr-0.35
x_1	0.53- 0.76	0.37- 0.42	0.05-0.08	0.03-0.06		
x_2	0.98- 2.49	0.60- 1.30	0.07-0.23	tr-0.08	tr-0.92	
Phyt-2-ene	4.88-71.36	2.47-43.60	0.26-5.56		tr-2.76	tr-1.08
C_{19}	0.08- 0.33	0.15- 0.85	0.04-0.15	0.08-0.11	0.05-0.36	
C_{20}	0.25- 2.00	0.10- 0.38	0.04-0.22	0.08-0.78	0.07-0.24	0.08-0.18
C_{21}	0.29- 0.81	0.12- 2.02	0.06-0.35	0.07-0.08	0.08-0.20	0.02-0.08
C_{22}	0.06- 0.34	0.04- 1.06	0.05-0.26	0.06-0.43	tr-0.20	0.05-0.18
C_{23}	0.24- 0.81	0.12- 4.34	0.05-0.34	0.06-0.20	0.05-0.13	0.04-0.09
C_{24}	0.12- 0.26	0.05- 1.50	0.03-0.15	tr-0.14	0.06-0.07	0.04-0.09
C_{25}	0.38- 1.77	0.35- 4.00	0.05-5.00	0.06-0.07	0.03-0.25	tr-0.20
C_{26}	0.20- 0.40		0.04-0.27	tr-0.06		
C_{27}	0.29- 2.89	0.07- 1.58	0.05-0.86	0.10-0.18	tr-0.35	
C_{28}	0.30- 0.50	0.06- 3.00	0.04-0.25	tr-0.04		
C_{29}	1.15- 5.99	3.38-18.55	0.06-1.93	0.09-0.27	tr-0.50	tr-0.80
C_{30}	0.19- 0.33	0.20- 2.68	0.10-0.11			
C_{31}	0.49- 3.58	0.22-21.22	0.18-0.76		tr-0.30	tr-0.27

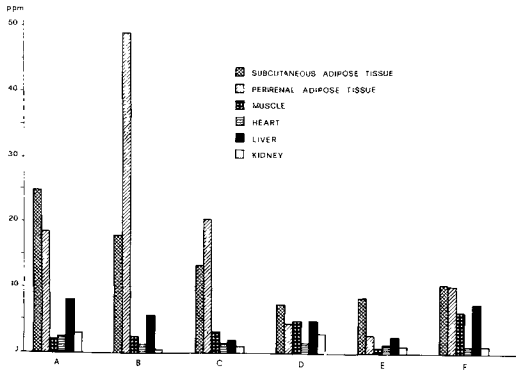

FIG. 4. Distribution of the total n-alkanes in the various tissues of the six bovines examined.

of the type described by Nagy et al. (2). Some other peaks, smaller than those relative to n-alkanes, can also be observed in the chromatograms. Such peaks were not identified but can be assumed to be unsaturated, branched and cyclic hydrocarbons, showing on thin layer chromatography (TLC) the same R_f as the n-alkane band.

The presence of squalene is not reported here, because by the method utilized, this compound was purposely eliminated during TLC to facilitate the quantitative determination of n-alkanes in the area of n-C_{28}. Furthermore, its determination was not relevant to the purpose of this work. All other polyunsaturated hydrocarbons and diunsaturated terpenoids, if present, were removed by TLC. Saturated (linear, branched, alicyclic) and monounsaturated hydrocarbons were recovered in the scraped band.

Under the gas chromatographic conditions utilized, squalane could be expected between n-C_{26} and n-C_{27}. Its presence cannot be ruled out, owing to a small peak in this zone which from time to time has been observed and was not amenable to identification.

The tissues analyzed were obtained from 6 cows, between 8 and 12 yr of age, stall-fed on hay and dried forage in winter and fresh forage for the rest of the year. The choice of aged animals was justified on the basis of expected equilibrium between ingested and metabolized hydrocarbons which should result in a steady state of tissue concentration.

Range-values in ppm (mg/kg, wet weight) for each hydrocarbon identified in the tissues examined are reported in Table I. The distribution of the total n-alkanes in the various tissues of the six animals is reported in Figure 4. It can be observed that the n-alkanes are concentrated preferentially in the adipose tissues. For the sake of brevity, the hydrocarbon distribution in the various tissues of only one animal is reported in Table II.

TABLE II

Hydrocarbon Distribution in the Various Tissues of a Single Animal (C)

Hydrocarbon	Adipose tissues		Muscle	Liver	Heart	Kidney
	Subcutaneous	Perirenal				
C_{12}		0.23	0.09	0.08	0.04	0.08
C_{13}			0.02	0.01		
C_{14}	0.17	0.56	0.34	0.23	0.14	0.35
C_{15}	0.06		0.27	0.04	0.05	0.15
C_{16}	0.71	0.62	0.54	0.46	0.21	0.30
C_{17}	0.39	0.65	0.27	0.13	0.11	0.07
Phyt-1-ene	6.82	2.14			0.07	
C_{18}	2.26	2.60	0.25	0.15	0.23	0.13
Phytane	2.03	2.18	0.12	0.11	0.17	0.05
x_1	1.60	0.40		0.03	0.06	
x_2	2.49	0.64		0.08		
Phyt-2-ene	71.36	18.45	0.33		0.43	tr
C_{19}	0.12	0.19	0.10	0.11	0.05	
C_{20}	0.77	0.44	0.20	0.08	0.07	0.08
C_{21}	0.29	0.59	0.17	0.08	0.09	0.02
C_{22}			0.11	0.06	tr	
C_{23}	0.41	1.05	0.07	0.10	0.09	0.04
C_{24}		0.32			0.07	0.04
C_{25}	1.01	2.22	0.10	0.07	0.06	
C_{26}		0.27	0.04	0.06		
C_{27}	1.78	3.74	0.12	0.10	0.05	
C_{28}		0.16	0.05	0.04		
C_{29}	3.07	5.72	0.19	0.09	0.09	
C_{30}	0.19					
C_{31}	2.19	3.27	0.18		tr	

In the adipose tissues (Fig. 1), a marked predominance of odd-numbered hydrocarbons in the upper part of the chromatogram, in particular of n-nonacosane, can be observed, while in the lower part, n-octadecane among the linear, phytane among the saturated branched, and phytene among unsaturated branched hydrocarbons predominate.

In the case of liver (Fig. 2), a homologous series of n-alkanes, both odd- and even-numbered, with a predominance of even-numbered n-alkanes, with a bell shaped distribution centered on n-C_{16}, can be noted. An analogous distribution was reported by Ferretti and Flanagan (20) in rabbit liver, but with the apex at n-C_{23}. A baseline hump, probably due to small amounts of several unresolved branched isomers can be similarly observed.

A predominance of short chain, odd- and even-numbered hydrocarbons (from C_{12} to C_{18}) can be observed in muscle tissue (Fig. 1). The hump attributed to unresolved, branched hydrocarbons can be observed again.

The hydrocarbon distribution in adipose tissue, liver and muscle appears to be quite uniform in all the animals examined. In kidney and heart (Fig. 1 and 2), on the other hand, the hydrocarbon distribution was not particularly characteristic and changed from animal to animal. In all the samples of liver examined, the constant absence of both phytenes was observed.

Results are available indicating that in rats and pigs fed SCP, even- and odd-numbered n-alkanes, between C_{12} and C_{20}, employed as substrates for yeast growth, are found in significant amount in muscle tissue (21). However, no data are available from the literature for muscle tissues from bovines fed SCP. The results reported in this study will contribute to establishing baseline values, particularly with respect to important edible portions of bovines.

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METHODS

Determination of Free Choline and Phosphorylcholine in Rat Liver

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ABSTRACT

A simple procedure for the determination of levels of free choline and phosphorylcholine in hepatic tissue is outlined. The method makes use of the enzyme acid phosphatase to liberate choline from phosphorylcholine and incorporates the ability of choline to react with potassium triiodide to yield choline periodide for the measurement of choline and phosphorylcholine in liver. The method is accurate for both entities (recovery of 97-100% for choline and 92-98% for phosphorylcholine). For phosphorylcholine, the method is markedly simpler than other methods previously described and the results for normally fed rats are of the same order of magnitude. The applicability of the method was shown when it was demonstrated that diets containing different amounts of choline influenced the level of choline and phosphorylcholine in liver.

INTRODUCTION

It has recently been established that choline is the key lipotropic substance in animals. Upon being taken up by the liver, this substance is subject to two main metabolic fates. First, choline is readily oxidized by choline oxidase to betaine aldehyde and betaine (1) and secondly, it is converted by choline phosphokinase to phosphorylcholine (2). Since phosphorylcholine, which is not oxidized by choline oxidase, composes 10% of the water soluble phosphate of the liver (3,4) and is reduced with the depletion of choline from the diet (4), this choline derivative may play a vital role in the lipotropic activity of choline.

Presently the methods used for the analysis of phosphorylcholine in tissues involve either paper chromatographic separation (3) or column chromatographic (5,6) separation from interfering substances before analyses can be carried out. This makes the measurement of the pool sizes of phosphorylcholine quite cumbersome even from an investigative standpoint.

The current procedure utilizes the features of the accepted method of Appleton et al. (7) for the sensitive analysis of free choline in plasma and adapts it for use in liver tissue. This method is based on the ability of choline, a quaternary ammonium compound, to react with potassium triiodide and form choline periodide, a colored complex measureable in ethylene dichloride. The method also incorporates the use of acid phosphatase, initially used by Wittenberg and Kornberg (8) to liberate free choline from esterified choline. In this way, the level of phosphorylcholine is arrived at by difference from the total choline and free choline contents in acid extracts of liver.

MATERIALS AND METHODS

Reagents

Potassium triiodide. 15.7 g of iodine reagent grade and 20 g of potassium iodide, reagent grade, were dissolve in 100 ml of water shaken for 45 min on a mechanical shaker to affect solution and stored at 4 C.

Standard choline solution. Choline chloride, C.P., was dried over concentrated sulfuric acid in vacuo. A standard solution was prepared containing 1 mg of base per ml of water. Working standards were prepared by suitable dilution of the stock solution with water.

Standard phosphorylcholine solution. The calcium salt of phosphorylcholine (crystalline with 5 H_2O from Sigma Chemical Co., St. Louis, MO,) was used as the standard for this method. A stock solution of phosphorylcholine containing 200 μg of choline base per 0.5 ml of water was prepared. Working standards were prepared by a suitable dilution of this stock solution.

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Acid phosphatase solution. Potato acid phosphatase (E.C. 3.1.3.2) from the Sigma Chemical Co., St. Louis, MO, was used for this determination. A fresh solution of this enzyme (0.4 IU/mg) was made up before each determination at a concentration of 50 mg/10 ml.

Other reagents: (a) 15% and 75% trichloroacetic acid solutions, (b) ethyl ether, (c) ethylene dichloride, (d) citrate buffer, 0.1 M, pH 4.8. Only reagent grade chemicals were used.

Choline Standard Curve

Varying amounts of pure choline in solution were added to proportional amounts of the incubating medium (citrate buffer and enzyme solution) as used in the procedure for total choline below. This mixture was carried through the incubation, protein precipitation, and ether extraction steps and choline (10 to 50 μg of base) was precipitated from 0.5 ml of this solution with 0.2 ml of the potassium triiodide reagent. When the precipitate was dissolved in 10 ml of ethylene dichloride and the absorbance measured at 365 nm, the standard curve shown in Figure 1 was obtained. Solutions of phosphorylcholine containing equivalent amounts of choline base yielded the same standard curve following enzyme hydrolysis. Since in the method esterified choline (phosphorylcholine) was determined as choline, the standard curve obtained was used for the determination of both free choline and total choline in the acid extracts.

Preparation of Liver Extract

Male Sprague-Dawley rats were placed under ether anesthesia and surgically opened and their livers freeze-clamped *in-situ*. These livers were then stored at -70 C until analyzed. Two grams of frozen livers were placed in 8 ml of 15% trichloroacetic acid and homogenized in a Potter homogenizer. This mixture was centrifuged at 12,000 rpm for 20 min and the supernatant was decanted and saved. The liver pellet was washed one time with 5 ml of 15% trichloroacetic acid, centrifuged at 12,000 rpm for 10 min, and the supernatant solution combined with the orignal extract in a 20-25 ml graduate test tube and the volume indicated. The extract was then extracted 3 times with 10 ml of ethyl ether to remove the trichloroacetic acid. The solution was then subjected to a stream of air for 60 min at room temperature to remove residual ether. Following this, the pH of the solution was adjusted to between 7 and 8 and the solution brought back to its original volume with water.

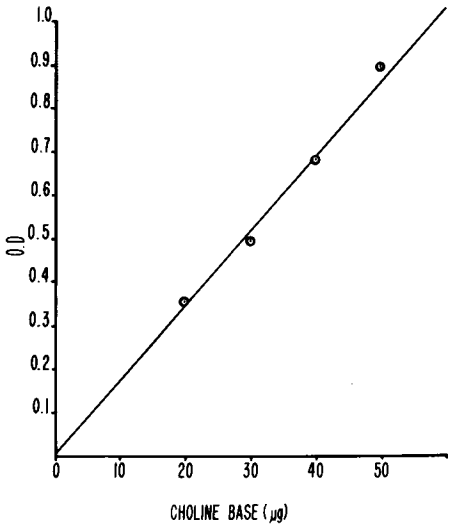

FIG. 1. Standard curve constructed from pure choline solutions that were carried through the various steps of the analytical procedure.

Determination of Free Choline

Two milliliters of the liver extract were added to a 15 ml graduated centrifuge tube with a vary narrow tip. After chilling in ice, 0.2 ml of the potassium triiodide solution was added, the sample mixed, covered and refrigerated overnight. The sample was then centrifuged at 3000 rpm for 15 min and the supernatant solution aspirated off with care being taken not to disturb the dark precipitate at the bottom of the test tube. The precipitate was then dissolved in 5 or 10 ml of ethylene dichloride depending on the amount of precipitated choline present and the absorption read in a spectrophotometer at 365 nm using ethylene dichloride as a blank. The free choline content of liver was estimated from the standard curve and the level expressed as μmoles of choline base per gram liver.

In the procedure following ether extraction of the trichloroacetic acid from the tissue extracts, the pH still remains at 2.6 to 3.0. As demonstrated by Wall et al. (9) if choline is precipitated as the periodide in this pH range, existing betaine would interfere and enhance the value for choline. In order to circumvent betaine interference, the procedure calls for the adjustment of the tissue extracts to between pH 7 and 8 where it has been shown (9) that betaine is not precipitated as the periodide. In our hands, we have found that failure to make the pH adjustment significantly enhances the value for choline in liver tissue. In further studies we have found that betaine interference

TABLE I

Recovery of Choline and Phosphorylcholine from Rat Liver[a]

Diet	Choline			Phosphorylcholine		
	Amount added μg	Mean amount recovered μg	Percent recovery	Amount added μg	Mean amount recovered μg	Percent recovery
Purina rat pellets	20	20.0 ± 2.4	100.0	20	18.4 ± 0.7	92.0
Purina rat pellets	40	39.0 ± 3.3	97.5	40	39.3 ± 3.0	98.3

[a]All recoveries represent 4 duplicate determinations on 4 different livers of animals fed Purina Commercial Rat Diet for 30 days.

TABLE II

Effect of Dietary Choline on Choline and Phosphorylcholine in Rat Liver

Diet	No. animals	Average daily intake MG	Choline level[a]		Phosphorylcholine level[a]	
			μMol/g	μMol/liver/ 100 g body weight	μMol/g	μMol/liver/ 100 g body weight
Choline deficient (5 days)	5	0	0.37 ± 0.07	1.95 ± 0.38	0.91 ± 0.26	4.73 ± 1.19
Mixed deficient and control (5 days)	5	57	0.70 ± 0.11[b]	3.64 ± 0.75[c]	1.57 ± 0.14[c]	8.04 ± 0.92[d]
Control (5 days)	5	114	0.92 ± 0.06[b]	4.65 ± 0.41[b]	2.79 ± 0.69[b]	14.0 ± 3.02[b]
Rat chow (30 days)	5	34	0.60 ± 0.06	3.07 ± 0.31	1.48 ± 0.38	7.57 ± 1.96

[a]Values are means ± S.D.

[b]When compared to choline deficient diet: $p < 0.001$; $c = < 0.01$; $d = < 0.002$.

can be eliminated by pH adjustment even when 50 and 100% of the level of betaine normally found in liver (10) was artificially added to liver extracts.

Determination of Free Phosphorylcholine in Liver

Two milliliters of the original extract was placed in a test tube to which was added 3.6 ml of 0.1 M citrate buffer (pH4.8) and 2.0 ml of acid phosphatase solution, in that order. This mixture was incubated in a Dubnoff water bath at 32 C for 16 hr (overnight). Following this, 0.4 ml of 75% trichloroacetic acid was added to the mixture and allowed to stand for 10 min. The solution was centrifuged at 2,500 rpm for 10 min to sediment the enzyme protein. The supernatant solution was then transferred to a graduated test tube and the the volume indicated. This solution was extracted 3 times with 10 ml of ethyl ether to remove the trichloroacetic acid and the residual ether was removed in the same manner described above. The pH of the solution was then adjusted to between 7 and 8 and the solution brought back to its original volume with water. Two milliliters of this solution were placed in a graduated centrifuge tube, the choline periodide precipitated as described above, and the total choline measured as described for free choline. Following the conversion of the level of total choline to μmoles per gram of liver, the level of phosphorylcholine was estimated by subtracting the free choline content from the total choline.

In the procedure for the determination of phosphorylcholine, it was found that two substances naturally occurring in liver, i.e., CDP-choline and α-glycerol-phosphorylcholine, did not interfere in the determination. Artifically adding to the liver extracts 100% more α-glycerophosphorylcholine and 20 times more CDP-choline as reported in liver by Sundler et al. (6) and Haines and Rose (11), respectively, did not cause enhancement in the estimation of phosphorylcholine.

EXPERIMENTAL

To demonstrate the influence of dietary choline on hepatic levels of choline and phosphorylcholine, male Sprague-Dawley rats (250 g) were divided into three groups. One group was fed the choline-deficient diet of French

(12) for five days and another group was fed the French (12) control diet for the same period. A third group was fed a 50:50 mixture by weight of the choline deficient diet and the control diet. The daily intake of choline of the animals on the deficient diet was negligible, where with the control diet the average daily intake was 114 mg and with the mixed diet 57 mg. The animals were killed in the nonfasting state and their livers promptly assayed for levels of choline and phosphorylcholine.

Also, in order to establish the levels of choline and phosphorylcholine in animals fed regular commercial rat diet, rats similar to those above were fed Purina rat pellets for 30 days and the nonfasted livers assayed for these metabolites.

In this study animals fed choline were compared to animals on a choline-deficient diet which is known to produce some fatty infiltration of the liver in five days. In addition to expressing results on the basis of μmoles per g of tissue, they were also expressed on the basis of μmoles per total liver per 100 g of body weight for proper comparison (13).

RESULTS

One of the main advantages of the present method for the analyses of choline and phosphorylcholine in livers is that it is markedly simpler than other methods previously described and the results obtained are of the same order of magnitude as those reported by other investigators (14,15). As shown in Figure 1, the standard curve is linear, and this is true whether the standard is choline or phosphorylcholine which has been hydrolyzed by acid phosphatase.

The reliability of the method lies in the recovery studies which were conducted on livers from animals fed a commercial rat chow (Purina). Table I shows that good recoveries were obtained at two different levels for both choline and phosphorylcholine. Table II demonstrates that hepatic pool sizes of both compounds vary with the availability of choline in the diet.

DISCUSSION

In the development of the present method, advantage was taken of the earlier finding (8) that semen acid phosphatase can be used to liberate choline from the "esterified choline" (phosphorylcholine). Since semen acid phosphatase was unavailable, acid phosphatase obtained from wheat germ (Sigma Chemical Co.) was tested but found to be ineffective in hydrolyzing phosphorylcholine to free choline. The use of acid phosphatase from a potato source (Sigma Chemical Co.), however, was found to effect total hydrolysis in a 0.1 M citrate buffer at pH 4.8 and hence afforded the analysis of phosphorylcholine by difference using the procedure of Appleton et al. (7) for free choline before and after enzyme hydrolysis.

The data listed in Table II show that an increased dietary level of choline does reflect itself in the hepatic choline and phosphorylcholine pool sizes. These data tend to confirm earlier work conducted in this laboratory (16) using the isolated perfused liver technique which demonstrated that the uptake of choline by the liver is directly related to the level of choline presented to it in the perfusate.

The data in Table II also tends to confirm the work reported by Thompson et al. (4) that a diet deficient in choline depresses the phosphorylcholine pool in the liver. Since, as stated above, the phosphorylcholine pool in liver may be highly important from a lipotropic standpoint, any agent which may impede the absorption of choline or the uptake of choline by the liver may be detrimental to liver integrity.

The procedure described herein for the convenient, specific, and accurate measurement of hepatic choline and phosphorylcholine is submitted to permit further studies into the roles played by choline and phosphorylcholine in the well-being of the liver and the disease states of this organ.

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COMMUNICATIONS

Substrate Selectivity of Diacylglycerol Kinase in Guinea Pig Brain[1]

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ABSTRACT

The present study was conducted to test the selectivity of the microsomal diacylglycerol kinase (ATP:1,2-diacyl-*sn*-glycerol phosphotransferase) from guinea pig brain towards different 1,2-diacyl-*sn*-glycerols. The molecular species added to the incubation medium as substrates were a mixture of the 1-[^{3}H] palmitoyl plus 1-[^{14}C] stearoyl homologs of either the monoenoic (2-oleoyl), dienoic (2-linoleoyl), tetraenoic (2-arachidonoyl), or hexaenoic (2-docosahexaenoyl) diacylglycerols. Rates of phosphatidic acid synthesis (1-palmitoy plus 1-stearoyl homologs) with each of the four classes of unsaturated diacylglycerols were not greatly different, although they were moderately higher in the case of the monoenes. No marked enzyme selectivity for either the 1-palmitoyl or 1-stearoyl homolog of the various 1-saturated 2-unsaturated diacylglycerols was apparent. Generally similar results were obtained with the diacylglycerol kinase in rat brain microsomes.

INTRODUCTION

The pioneering work of Hokin and Hokin (1) characterized the presence of diacylglycerol kinase (ATP:1,2-diacyl-*sn*-glycerol phosphotransferase) in microsomal preparations from guinea pig brain. The stimulatory effect of added diacylglycerol prepared from cabbage phosphatidic acid on phosphatidate synthesis was 20- and 82-fold greater than that found with diacylglycerol from brain phosphatidylcholine and 1-palmitoyl 2-oleoyl diacylglycerol, respectively (1). Previous experiments have revealed that the diacylglycerol kinase in rat brain microsomes shows only a moderate selectivity towards various diacylglycerols which contained saturated fatty acids in the 1-position (mixture of palmitate plus stearate) and different unsaturated acids in the 2-position (2). The work of Kanoh and Åkesson (3) indicated that the microsomal and soluble diacylglycerol kinase from rat liver did not possess marked selectivity in utilizing diacylglycerols of different unsaturation.

The purpose of the present investigation was to compare various 1-saturated 2-unsaturated diacylglycerols, as well as their 1-palmitoyl and 1-stearoyl homologs, as substrates for the diacylglycerol kinase in microsomes from guinea pig brain. The 1-saturated 2-oleoyl, 2-linoleoyl, 2-arachidonoyl, and 2-docosahexaenoyl diacylglycerols were tested as substrates since these molecular speices predominate in phosphatidic acid from guinea pig brain (4). For comparative purposes, parallel experiments were conducted on the diacylglycerol kinase in rat brain microsomes.

EXPERIMENTAL PROCEDURES

Microsomes were prepared from the pooled brains of male guinea pigs or rats according to the method of Eichberg et al. (5). Microsomal protein was determined as described by Lowry et al. (6). Assay conditions for measuring phosphatidic acid synthesis via the diacylglycerol kinase were based on the work of Holub and Piekarski (2) as modified from Lapetine and Hawthorne (7). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), 0.6 mM 1,2-diacyl-*sn*-glycerol, 8.0 mM $MgCl_2$, 10.0 mM NaF, 2.4 mM sodium deoxycholate, 5.6 mM ATP, and 0.6 mg of microsomal protein in a total volume of 0.5 ml. The radioactive diacylglycerols were prepared, characterized, and introduced into the incubation medium exactly as described (2,8). The molecular species added to the medium were a mixture of the 1-[^{3}H] palmitoyl and 1-[^{14}C]-stearoyl homogs of either the 2-oleoyl (monoenoic), 2-linoleoyl (dienoic), 2-arachidonoyl (tetraenoic), or 2-docosahexaenoyl (hexaenoic) diacylglycerols having (palmitate/stearate)

[1]Presented in part at the AOCS Annual Meeting, St. Louis, May 1978.

TABLE I

Utilization of Different Molecular Species of Diacylglycerols by Diacylglycerol Kinase in Guinea Pig and Rat Brain Microsomes[a]

Molecular species of diacylglycerols	Rate of phosphatidate synthesis (nmol/min / mg protein)					
	Guinea pig brain			Rat brain		
	1-Palmitoyl homolog	1-Stearoyl homolog	Total	1-Palmitoyl homolog	1-Stearoyl homolog	Total
Monoenes (1-saturated 2-oleoyl)	7.60 ± 0.55	1.86 ± 0.19	9.46 ± 0.75[b]	4.51 ± 0.18	1.20 ± 0.09	5.71 ± 0.26[b]
Dienes (1-saturated 2-linoleoyl)	3.35 ± 0.20	3.17 ± 0.42	6.52 ± 0.43[c]	1.81 ± 0.30	1.78 ± 0.29	3.59 ± 0.59[c]
Tetraenes (1-saturated 2-arachidonoyl)	2.29 ± 0.29	2.41 ± 0.26	4.70 ± 0.55[c]	1.34 ± 0.12	1.41 ± 0.05	2.75 ± 0.17[c]
Hexaenes (1-saturated 2-docosahexaenoyl)	2.54 ± 0.30	1.70 ± 0.16	4.24 ± 0.46[c]	2.11 ± 0.18	1.48 ± 0.09	3.60 ± 0.26[c]

[a]All values are given as means ± S.E. (n = 3). Means in the vertical columns for the total phosphatidate (1-palmitoyl plus 1-stearoyl homologs) having different superscript letters are significantly different from each other ($P<0.01$) as determined by analysis of variance and Duncan's multiple range test (9).

TABLE II

Relative Utilization of 1-Palmitoyl and 1-Stearoyl Homologs of Various Classes of Diacylglycerols by Diacylglycerol Kinase in Guinea Pig and Rat Brain Microsomes[a]

Molecular species of diacylglycerols	Selectivity index	
	Guinea pig brain	Rat brain
Monoenes (1-saturated 2-oleoyl)	1.19 ± 0.04	1.09 ± 0.03
Dienes (1-saturated 2-linoleoyl)	0.97 ± 0.14	0.90 ± 0.01
Tetraenes (1-saturated 2-arachidonoyl)	1.07 ± 0.02	1.08 ± 0.06
Hexaenes (1-saturated 2-docosahexaenoyl)	0.88 ± 0.02	0.84 ± 0.02

[a]All values are given as means ± S.E. (n = 3). The selectivity index was calculated for each unsaturation class as (rate of synthesis of 1-palmitoyl phosphatidate/rate of synthesis of 1-stearoyl phosphatidate) divided by molar ratio (1-palmitoyl homolog/1-stearoyl homolog) in corresponding diacylglycerol precursor.

molar ratios of 3.45, 1.13, 0.88, and 1.69, respectively, and isotope ratios ($^3H/^{14}C$) of ca. 1.00. After a preincubation period of 2 min, the reaction was initiated by the addition of enzyme. Incubations were conducted at 37 C for 5 min after which the reaction was terminated and product formation was measured as previously described (2).

RESULTS AND DISCUSSION

Table I gives the reaction rates for phosphatidic acid synthesis when the different diacylglycerols were tested as substrates. With the microsomes from guinea pig brain, rates for the total monoenes(1-palmitoyl plus 1-stearoyl homologs) were moderately greater than those for the dienes, tetraenes, or hexaenes in a manner which was statistically signficant ($P<0.01$). The rates for the latter three classes were not significantly different ($P>0.01$). In the case of rat brain microsomes, the rates for the total monoenes were moderately greater than those for the more unsaturated classes in agreement with previous findings (2).

The selectivity of the diacylglycerol kinase for either the 1-palmitoyl or 1-stearoyl homolog of the various 2-unsaturated diacylglycerols was calculated (Table II). A selectivity index of 1.00 indicates no enzyme selectivity, whereas a mean index which is significantly greater or less than 1.00 would represent a preference for the 1-palmitoyl and 1-stearoyl species, respectively. Regardless of the unsaturated fatty acid in the

2-position, the microsomal enzyme from guinea pig brain gave selectivity indices which were within 19% of unity and within 30% of each other. The diacylglycerol kinase from rat brain also exhibited very little selectivity for palmitate or stearate in the 1-position in accordance with an earlier study (2).

The present work was of particular interest because of an earlier report that the effectiveness of diacylglycerols from various sources as substrates for the microsomal diacylglycerol kinase from guinea pig brain differed by up to 82-fold (1). The 1-saturated 2-unsaturated diacylglycerols tested herein corresponded to those molecular species present in phosphatidic acid from guinea pig brain (4). Only a moderate preference of the kinase for total (1-palmitoyl plus 1-stearoyl homologus) monoenoic relative to other unsaturated diacylglycerol mixtures was indicated. The enzyme from guinea pig brain did not exhibit a significant preference for either palmitate or stearate in the 1-position.

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Fatty Acid Metabolism in the Calanoid Copepod *Paracalanus parvus:* 1. Polyunsaturated Fatty Acids

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ABSTRACT

The metabolic fate of radioactive linoleate and α-linolenate administered to the South Atlantic copepod *Paracalanus parvus* was studied. The wild copepod was able to incorporate the labeled acids dissolved in seawater. The radioactive linoleate was elongated to 20:2ω6 and 22:2ω6 and desaturated by a Δ6 desaturase to 18:3ω6. α-Linolenate was also desaturated by a Δ6 desaturase to 18:4ω3 and elongated to 20:3ω3. The copepod was able to convert α-18:3 to 20:5ω3 and 22:6ω3.

INTRODUCTION

Phytoplankton is the first link of the food chain in the sea. Some algae are able to synthesize de novo the polyunsaturated acids 20:5ω3 and 22:6ω3 (1,2) that are preferentially found in marine animals instead of 20:4ω6 acid that is found predominantly in land animals. We have shown in previous work (3) that the phytoplanktofagous mollusc *Mesodesma mactroides* is not only able to incorporate polyunsaturated acids of phytoplankton, but also to convert linoleic, and α-linolenic acid to higher members of the families. Therefore, these animals and others may contribute to modify the primary fatty acid composition synthesized by the phytoplankton.

Zooplankton is an important link in the marine food chain and calanoid copepods especially are crucial, since they are phytoplanktofagous and the major food of a variety of fish. For this reason, we have decided to study the capacity of *Paracalanus parvus* to convert linoleic and α-linolenic acids to highly polyunsaturated acids. *P. parvus* is a cosmopolitan calanoid copepod, abundant in Argentine sea waters. It largely modifies the amount and composition of the fatty acids with the ecological conditions (4).

MATERIALS AND METHODS

Materials

The acids [1-^{14}C] linoleic (56 mCi/mmol) and [1-^{14}C] α-linolenic (57 mCi/mmol) were provided by Amersham-Searle (Amersham, England). The radiochemical purity was higher than 99%.

Organisms

Calanoid copepods *P. parvus* of 0.75-0.80 mm were collected in September, in shore, in Mar del Plata, Buenos Aires Province, Argentina, with zooplankton nets (160-180 μ mesh). In the laboratory they were suspended in fresh, sterile sea water and filtered through a thicker sieve to eliminate larger organisms than this copepod. They were concentrated on the surface of the water by light attraction, filtered through a 160-180 μ mesh and washed with sterile sea water. The sample of *P. parvus* so obtained was more than 90% pure. The organisms were then transferred to the incubation solution.

Incubation

The incubation solution was prepared with sterile synthetic sea water and the ammonium salt of the radioactive fatty acids. The concentration of the fatty acids in the medium was 0.06-0.10 μM.

One gram (wet weight) of the wild *P. parvus* was incubated in 2400 ml of incubation solution at 15 ± 2 C. A stream of air was gently passed through the solution. After determined periods of time, the copepods were collected in a net and repeatedly washed with fresh synthetic sea water until the washings were free of radioactive material. The copepods were then dried on filter paper and the lipids extracted with chloroform-methanol (2:1 v/v) (5). The lipids were weighed and the radioactivity counted in a Packard scintillation counter. They were saponified, the nonsaponifiable extracted, the aqueous-alcoholic solution acidified and the free acids were taken in petroleum ether. The free acids were esterified with

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TABLE I

Radioactivity Incorporated in the Lipids of *P. parvus* after Incubation with [1-^{14}C] Linoleic and [1-^{14}C] α-Linolenic Acids[a]

Substrate	Radioactivity			
	Fatty acids	Nonsaponifiable	Water soluble products	Nonidentified
[1-^{14}C] 18:2ω6	74.8	18.2	1.0	6.0
[1-^{14}C] 18:3ω3	73.2	18.5	1.2	7.1

[a]The copepods were incubated during 5 hr. Results represent the percent distribution of the radioactivity in the total lipids extracted with $CHCl_3$-CH_3OH (2:1 v/v). They are the mean of two incubations of ca. 1 g copepods.

TABLE II

Radioactivity Distribution in *P. parvus* Fatty Acids after Incubation with [1-^{14}C] Linoleic Acid[a]

Fatty acids	Time of incubation (hr)		
	1 hr	5 hr	12 hr
14:0	---	0.6 ± 0	---
16:0 + 16:1	0.8 ± 0.1	4.0 ± 0.6	3.3 ± 0.1
18:0 + 18:1	2.3 ± 0.2	0.8 ± 0.2	1.5 ± 0.1
18:2ω6	86.5 ± 0.5	85.3 ± 1.0	86.1 ± 0.9
18:3ω6	1.5 ± 0.1	1.8 ± 0.4	1.6 ± 0.3
20:0	1.3 ± 0.1	---	---
20:1	0.9 ± 0.1	---	1.7 ± 0.2
X	1.9 ± 0.1	---	---
20:2ω6	1.0 ± 0.1	4.6 ± 0.8	4.1 ± 0.1
20:3ω6	1.5 ± 0.1	0.7 ± 0.1	---
22:2ω6	2.3 ± 0.5	2.1 ± 0.3	1.7 ± 0.2
Total incorporation in the lipids (%)	4.1 ± 0.5[b]	9.3 ± 0.6	12.2 ± 1.8

[a]Results represent the present distribution of the radioactivity in the fatty acids. They are the mean of the analysis of two samples of 1 g copepods ± extreme values. The chromatogram was run until 22:6 ω3 peak.

[b]The radioactivity measured in the medium before incubation was considered 100%.

methanol and sublimated (6).

The distribution of radioactivity in the fatty acid methyl esters was determined by gas liquid radiochromatography in a Pye apparatus equipped with a proportional counter as described previously (3).

RESULTS AND DISCUSSION

Radioactivity Incorporation in the Lipids

The incorporation of the radioactivity of the fatty acids in the lipids of *P. parvus* is summarized from Tables I to III. After 1 hr incubation, 4.1 to 4.2% of the radioactivity of [1-^{14}C] linoleic and [1-^{14}C] α-linolenic acids was incorporated in the lipids of *P. parvus*. These figures increased with time, reaching 12.2% and 13.2%, respectively, after 12 hr. Therefore, the wild copepod is able to absorb fatty acids dissolved in the sea water and incorporate them to the lipids. Table I shows that after 5 hr, more than 70% of the incorporated radioactivity was present in the fatty acids and the rest in the nonsaponifiable, water soluble compounds and unidentified products. Therefore, although the majority of the labeling remained in the substrate and the fatty acids derived from the substrate, part of the linoleic and α-linolenic acids was metabolized. A substantial proportion of the [^{14}C] of both substrates went to the nonsaponifiable fraction. Unfortunately, this fraction was lost, and we could not determine if the radioactivity was present in wax esters, alcohols or other similar products that have been found in copepods (7). However, previously we found that samples of *P. parvus* collected in July and October contained little wax esters (4). Therefore, the labeled nonsaponifiable might represent another lipid, but this is improbable.

Biosynthesis of Fatty Acids of Linoleic Acid Family

The distribution of the radioactivity in the

TABLE III

Labeling Distribution in *P. parvus* Fatty Acids after Incubation with [1-^{14}C]α-Linolenic Acid[a]

Fatty acids	Time of incubation (hr)	
	1 hr	5 hr
14:0	---	0.9 ± 0
16:0 + 16:1	0.7 ± 0.3	5.9 ± 0.5
18:0	0.3 ± 0.1	0.9 ± 0.1
18:1	0.2 ± 0.1	1.6 ± 0.2
18:2ω9	0.2 ± 0.1	---
18:3ω3	90.2 ± 1.1	78.2 ± 1.2
18:4ω3	2.4 ± 0.1	2.1 ± 0.1
X	1.7 ± 0.1	---
20:3ω3	3.4 ± 0.5	5.7 ± 0.3
20:4ω3	0.9 ± 0.1	0.6 ± 0.1
20:5ω3	---	1.9 ± 0.3
22:3ω3	---	0.8 ± 0.2
22:4ω3	---	0.5 ± 0.1
22:5ω3	---	0.3 ± 0
22:6ω3	---	0.6 ± 0.1
Total incorporation in the lipids (%)	4.2 ± 0.2[b]	10.4 ± 1.5

[a]Results represent the percent distribution of the radioactivity in the fatty acids. They are the mean of the analysis of two samples of 1 g copepods ± extreme values. The chromatogram was run until 22:6ω3 peak.

[b]The radioactivity measured in the medium before incubation was considered 100%.

fatty acids of *P. parvus* after the incubation of the copepod with [1-^{14}C]linoleic acid is illustrated in Table II. The total radioactivity incorporated in lipids increased with the incubation time, but most of this increase occurred between 1 and 5 hr. Therefore, since the percent distribution in the fatty acids remained rather constant from 1 to 12 hr incubation, it means that the radioactivity increased steadily in all the fatty acids of Table II. The wild organism was able to elongate and desaturate the fatty acids, but since small amounts of labeling were also found in the fatty acids: 14:0, 16:0, 16:1, 18:0, 18:1, 20:0 and 20:1, we may admit that part of the substrate was oxidized and labeled acetate so formed was used in de novo synthesis of fatty acids.

Labeled linoleic acid was converted to 18:3ω6. Therefore, we must conclude that a Δ6 desaturase is present. In addition, 20:2ω6, 20:3ω6 and 22:2ω6 acids were also labeled, suggesting the existence of elongating enzymes. Both types of enzymes are present in land animals and, together with a Δ5 desaturase that converts 20:3ω6 to 20:4ω6, contribute to the synthesis of arachidonic acid (8). However, even after 12 hr incubation, no labeling in arachidonic acid was detected. Similar results were also obtained by us when [1-^{14}C]linoleic acid was administered to the marine mollusc *Mesodesma mactroides* (3). The difficulty in synthesizing arachidonic acid in both marine animals could be attributed to the absence or very low activity of the Δ5 desaturase, since the desaturation does not go farther than 20:3ω6. However, the absence of the enzyme is apparently not admissible, since [1-^{14}C]α-linolenic acid was converted by the copepod to 20:5ω3 acid (Table III). Besides, it is known (9) that the same Δ5 desaturase converts 20:3ω6 to 20:4ω6 and 20:4ω3 to 20:5ω3. Therefore, it would be easier to admit that the absence or very low synthesis of arachidonic acid, when compared to the synthesis of 20:5ω3 shown in Table III, is apparently the consequence of low Δ5 desaturase activity and preferential inhibition of the Δ5 desaturation of fatty acid of linoleic acid family. Note that the activity of the Δ5 desaturase is lower in the fish *Pimelodus maculatus* than in the rat, whereas the activity of the Δ6 desaturase is higher (9).

Biosynthesis of Fatty Acids of α-Linolenic Family

The distribution of the radioactivity of [1-^{14}C]α-linolenic acid in the fatty acids of *P. parvus* is shown in Table III. Again the amount of the labeled acid incorporated increased with the incubation time. Some oxidation of the substrate and de novo synthesis of fatty acids took place apparently, since the radioactivity was also detected in 14:0, 16:0, 16:1, 18:0, 18:1 and 18:2ω9 peaks.

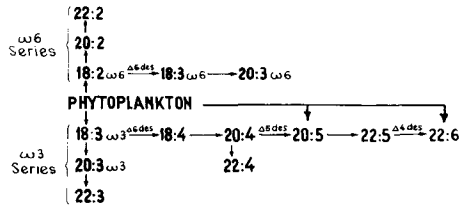

FIG. 1. Possible biosynthetic chains of the fatty acids of ω6 and ω3 families in *P. parvus*.

During 1 hr incubation, the 18:3ω3 acid apparently followed two independent biosynthetic pathways. On one side, it was elongated to 20:3ω3 and, on the other, it was converted to 18:4ω3 by a Δ6 desaturase. After this time, the biosynthetic route only reached 18:4ω3 acid step. After 5 hr incubation, the biosynthesis went farther on synthesizing the acids of ω3 family: 20:5, 22:3, 22:4, 22:5 and 22:6. Therefore, it is possible to deduce that the wild *P. parvus* has the full array of enzymes, Δ6, Δ5 and Δ4 desaturases as well as the elongating systems, recognized in the mammals to synthesize 20:5ω3 and 22:6 ω3 from 18:3ω3 (Figure 1). However, it was apparently some catabolism of administered [1-^{14}C]α-linolenic acid to [1-^{14}C]acetate which, in turn, was used to synthesize 14:0, 16:0, 18:0, and 18:1 acids. Therefore, it could be expected that labeling of polyunsaturated fatty acids of 20 and 22 carbons could be produced by biosynthesis from this [^{14}C]acetate. The same reasoning could be applied to the polyunsaturated acids 20:2ω6, 20:3ω6, and 22:2ω6 biosynthesized when [1-^{14}C]linoleate was administered. However, the different labeling patterns produced from [1-^{14}C]linoleate compared to [1-^{14}C]α-linolenate seem to indicate the desaturation and elongation of exogenous linoleate and α-linolenate. This would confirm the lack of label in the polyunsaturated fatty acids of 20 and 22 carbons following administration of [1-^{14}C]acetate (10).

The biosynthetic route outlined in Figure 1 has been also suggested for other marine animals. Morris et al. (11) showed the biosynthesis of 20:5ω3 and 22:6ω3 in *Neomysis integer* after the administration of labeled 18:3ω3 acid. Moreno et al. (3) also showed that the clam *M. mactroides* converts 18:3ω3 to 18:4ω3 and 20:3ω3 acids. Kayama et al. (12), after [1-^{14}C]18:3ω3 injection to the fish *Paralabrax clathratus* suggested that 20:5ω3 and 22:6ω3 biosynthesis was produced by the same pathways found in the rat (8,13,14). Besides, the properties of the Δ6 desaturase in the fish *P. maculatus* and the conversion of 18:3ω3 to 18:4ω3 and 18:2ω6 to 18:3ω6 acids have been carefully studied (9,15). Therefore, in spite of the scarce information available and the large variety of marine life, it is possible to consider that at least in many sea animals the biosynthetic chain of polyunsaturated fatty acids is the same recognized in mammals.

Since all the experiments shown in this work were not performed with axenic cultures, but with wild copepods with less than 100% purity, it is possible to discuss if the biosynthetic pathways outlined in Figure 1 correspond to *P. parvus* or if they are the joined contribution of *P. parvus* and other microorganisms. This last possibility is rather unlikely since the purity of the sample was higher than 90%, and the biosynthetic routes found did not deviate from the "normal" pathways typical of animals. Therefore, if some other microorganisms also contributed to the results, this contribution would be very small, and it would not invalidate the conclusions.

The presence of 18:3ω3 in diatoms and other components of phytoplankton constituting *P. parvus* food is not very high, whereas 20:5ω3 and 22:6ω3 acids are rather abundant in the same food sources (1,2,4). Therefore, although the copepod has the capacity to synthesize its own 20:5ω3 and 22:6ω3 acids from α-linolenate, the predominance of these fatty acids in the copepod composition (4) is apparently mainly due to the food composition (Fig. 1). In the last instance, some unicellular components of phytoplankton for still unknown reasons, require and synthesize de novo 20:5ω3 and 22:6ω3 acids.

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Fatty Acid Metabolism of the Calanoid Copepod *Paracalanus parvus:* 2. Palmitate, Stearate, Oleate and Acetate

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ABSTRACT

The de novo biosynthesis of fatty acids in the wild, calanoid copepod *Paracalanus parvus* was studied. The incubation of labeled acetate proved the de novo biosynthesis of saturated and monounsaturated even fatty acids from 14 to 20 carbons and the 22:1 acid. Saturated and monounsaturated uneven fatty acids from 15 to 21 carbons were also synthesized. The copepod could not synthesize linoleic and α-linolenic acids. By administration of [1-^{14}C]palmitate, [1-^{14}C] stearate and [1-^{14}C] oleate, it was possible to elucidate the general pattern of the de novo biosynthesis of fatty acids in the wild *P. parvus*.

INTRODUCTION

In a previous paper (1), the lipid and the fatty acid compositions of the wild copepod *Paracalanus parvus* collected in Mar del Plata shores, Argentina, were reported. It was also reported (2) that either α-linolenate or linoleate dissolved in the sea water were incorporated into the lipids of the copepod and converted to higher homologs. The crustacean had a Δ6 desaturase that converted 18:2ω 6 to 18:3ω6 and 18:3ω3 to 18:4ω3. It was also able to elongate both substrates to fatty acids of 20 and 22 carbons.

Eicosapentenoic (20:5ω3) and 22:6ω3 acids were synthesized by the copepod from α-linolenate apparently following the normal route found in mammals (3).

In the present work, these reports are completed by the incubation of the copepod with labeled acetate, palmitate, stearate and oleate. In this way, the de novo biosynthesis of fatty acids was investigated.

MATERIALS AND METHODS

Materials

[C^{14}] Labeled stearic, palmitic, and oleic acids as well as sodium [1^{14}C] acetate were provided by Amersham-Searle (Amersham, England). The radiochemical purity was higher than 99%.

Organisms

The calanoid copepods *P. parvus* were collected in September (in the South Atlantic coast, in Mar del Plata, Argentina) with a zooplankton net of 160/180 mesh. They were purified and concentrated by the procedure already described (2).

Procedure

The same method described in the previous paper (2) was followed, Approximately 1 g of copepods was incubated in 2400 ml sterile sea water in the presence of either 0.06-0.10 μM [1-^{14}C] acids or 0.3 μM sodium [1-^{14}C]-acetate. The temperature of incubation was 15 ± 2 C. After determined periods of time, the incubation was stopped and the copepods were collected and thoroughly washed. Lipids were extracted with Cl_3CH-CH_3OH (2:1 v/v) and then saponified. The distribution of the radioactivity among fatty acids, nonsaponifiable, and water soluble products was measured in a Packard scintillation counter. The distribution of the labeling among the fatty acids was determined by gas liquid radiochromatography of the methyl esters in a Pye apparatus with a proportional counter. The column, experimental conditions, and procedure used to identify and quantitate the labeled esters were as described (2).

The specific radioactivity of the synthesized fatty acid was determined in some cases. The fatty acid methyl esters were separated by gas liquid chromatography in a F and M apparatus equipped with a column of 15% diethylene-glycol-succinate in Chromosorb W (80/100 mesh). They were collected from the effluent in counting solutions of 4 g of 2,5-diphenyloxazol (PPO) and 100 mg of 1,4-bis-2(5 phenyloxazolyl)bencene (POPOP) per liter in toluene. The radioactivity was assayed in a Packard scintill-

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TABLE I

Radioactivity Incorporated in the Lipids of *P. parvus* after the Incubation with [1-^{14}C]16:0 and [1-^{14}C]18:0 during 5 hr[a]

Radioactive substrates	Radioactivity, %[b]			
	Fatty acids	Nonsaponifiable	Water soluble products	Not determined
16:0	73.5	20.2	0.2	6.1
18:0	75.4	18.4	0.2	6.0

[a]Results are the mean of two samples of 1 g of wild copepods incubated at 15 ± 2 C.
[b]Calculated considering that the radioactivity of total lipids was 100%.

ation counter. The total radioactivity recovered was 70-72%. The relative specific radioactivity of each methyl ester was calculated dividing the d.p.m. counted in each peak by the area of the peak in the chromatogram.

RESULTS AND DISCUSSION

Incorporation of [1-^{14}C] Acetate and of [1-^{14}C] Fatty Acids dissolved in the Sea Water

Results in Tables I to V show that wild *P. parvus* is able to absorb and incorporate either labeled acetate or stearate, palmitate and oleate into the lipids. The saturated and monoenoic acids were absorbed similarly to linoleic and linolenic acids (2). Testerman (4) has also shown that the poliquetous *Stauronereis rudolphi* absorbed fatty acids dissolved in sea water and Moreno et al. (5,6) found similar incorporations in *Mesodesma mactroides.* However, since the *P. parvus* studied were not axenic and represented samples of wild organisms more than 90% pure, the incorporation and metabolic fate of the substrates studied may show the effect evoked by the copepod and other possible microorganisms present in the sample. However, this possibility, as it was previously discussed (2), does not invalidate the results here. They may show the density of the tested substrates in the wild copepod in conditions rather similar to the natural ones.

When [1-^{14}C]16:0 and [1-^{14}C]18:0 acids were incubated, the label of the substrates was mainly incorporated in the fatty acids of the *P. parvus,* but some radioactivity also appeared in the nonsaponifiable and in unidentified compounds (Table I). The proportion of the total [^{14}C] absorbed which was found in the nonsaponifiable was remarkably constant, regardless of the source of exogenous label; it was 18.4% and 20.2% for 18:0 and 16:0 or 18.2% and 18.5% for 18:2 and 18:3 acids, respectively (2). As discussed in part I of this series (2), it is possible that this nonsaponifiable corresponds to wax esters that are common constituents of crustaceans, although these lipids were minor constituents of our samples of *P. parvus* (1). Also it is unlikely that this copepod biosynthesized sterols de novo from the breakdown products of labeled acids since it has been shown that other crustaceans fail to utilize acetate to synthesize cholesterol (7,8).

De Novo Biosynthesis of Fatty Acids

The incubation of *P. parvus* with [1-^{14}C]-acetate during increasing periods of time showed, as expected, that the copepod was able to synthesize de novo saturated and monoenoic acids (Table II), but it was unable to form linoleic and α-linolenic acids. The crustacea synthesized all the saturated and monoenoic acids of even number of carbons from 14 to 20 carbons as well as 22:1 acid. However, it is

TABLE II

Labeling Distribution in the Fatty Acids of the Copepod after Administration of [1-^{14}C]Acetate[a]

Fatty acids	Time of incubation (hr)	
	7	15
X_1	0.5 ± 0.1	0.5 ± 0.0
14:0	1.1 ± 0.2	0.9 ± 0.2
14:1	0.3 ± 0.1	0.2 ± 0.1
15:0	5.5 ± 0.6	3.4 ± 0.4
15:1	2.4 ± 0.4	1.6 ± 0.3
16:0	24.0 ± 2.1	17.1 ± 1.4
16:1	27.9 ± 2.8	29.8 ± 2.1
17:0	5.9 ± 0.7	7.2 ± 0.7
17:1	14.8 ± 0.9	13.2 ± 1.0
18:0	2.2 ± 0.5	2.2 ± 0.4
18:1	13.3 ± 0.9	17.9 ± 1.6
19:1	1.0 ± 0.1	1.7 ± 0.3
20:0	0.6 ± 0.2	0.9 ± 0.1
20:1	0.5 ± 0.1	0.7 ± 0.1
21:0	---	0.6 ± 0.2
21:1	---	1.3 ± 0.4
22:1	---	0.6 ± 0.1
X_2	---	0.2 ± 0.1

[a]Results are expressed as percent of total recovered [^{14}C]. They are the mean of two samples ± extreme deviation of the mean. The chromatogram was run until the 22:6ω3 peak was eluted.

TABLE III

Labeling Distribution in *P. parvus* Fatty Acids after Administration of [1-^{14}C] Palmitate Dissolved in Sea Water[a]

Fatty acids	Time of incubation (hr)		
	1	5	
	Percent	Percent	dpm/peak area
14:0	---	1.6 ± 0	10 ± 0
16:0	95.0 ± 1.1	90.5 ± 1.0	131 ± 10
16:1	2.2 ± 0.6	2.6 ± 0.2	17 ± 2
16:2	0.6 ± 0.2	0.8 ± 0.1	13 ± 1
18:0	1.1 ± 0.2	3.2 ± 0.1	13 ± 2
18:1	0.6 ± 0.1	1.3 ± 0.1	6 ± 1
Total incorporation in the lipids %[b]	3.1 ± 0.2	9.3 ± 1.2	

[a]Results are the mean of the analysis of two samples ± extreme deviations from the mean. The chromatogram was run until 22:6ω 3 peak.

[b]The radioactivity measured in the medium before incubation was considered 100%.

remarkable that the odd chain fatty acids of 15 to 21 carbons saturated and monounsaturated were also synthesized. The highest radioactivity found in the fatty acids of uneven number of carbons was restricted to 15:0, 17:0 and 17:1 acids. The presence of the uneven fatty acids 13:0, 15:0, 15:1 and 17:0 was already recognized in the fatty acid composition of *P. parvus* (1) and are normal components of the copepod. The high yield of the de novo biosynthesis of whole series of uneven fatty acids suggests that an uneven acid of short chain has been used as a primer for successive additions of the label of the acetate. This primer could be propionate. However, while exogenous [^{14}C] acetate labeled 17:1 and 18:1 about equally, there was presumably a complete absence of 17:1 biosynthesis from endogenous [^{14}C] acetate derived from catabolism of 16:0, 18:0, 18:1, 18:2 and 18:3 (Tables III to V) (2). Different pools of acetate could explain these findings.

Data collected in Table II also show that after the incubation labeled acetate was mainly incorporated in 16:0 and the monoenoic acids, 16:1, 17:1 and 18:1. By increasing the time of incubation to 15 hr, the pattern of labeling was similar, but a shift of the radioactivity to the acids of higher chain length is easily recognizable. The amount of synthesized monoenoic acids was remarkably high. They constituted 60.2% of all labeled acids after 7 hr administration and increased to 67.0% after 15 hr. These results would apparently indicate a special requirement of unsaturated fatty acids in the copepod probably related with the synthesis of lipids of appropriate "fluidity."

TABLE IV

Labeling Distribution in *P. parvus* Fatty Acids after Administration of [1-^{14}C] Stearate Dissolved in Sea Water During 5 hr[a]

Fatty acids	Radioactivity, %
14:0	0.6 ± 0.2
16:0 + 16:1	6.0 ± 0.2
16:2	---
18:0	82.7 ± 0.3
18:1ω9	6.2 ± 0
18:2ω9	1.4 ± 0.4
20:0	0.9 ± 0
20:1ω9	1.3 ± 0
20:2	0.9 ± 0.1
Total incorporation in the lipids, %[a]	9.3 ± 1.2

[a]Results are the mean of the analysis of two samples ± extreme deviations from the mean. The chromatogram was run until 22:6ω3 peak.

[b]The radioactivity measured in the medium before incubation was considered 100%.

Fate of Administered Palmitic, Stearic and Oleic Acids

The fate of [1-^{14}C] palmitic acid administered to *P. parvus* is shown in Table III. The acid followed two routes. On the one hand, it was presumably desaturated by a Δ9 desaturase to 16:1 and then desaturated again by a Δ6 desaturase to 16:2 acid. On the other hand, palmitic acid was elongated to 18:0 and then desaturated to 18:1 acid. The 18:1 acid so synthesized is oleic acid. However, it is not unlikely that some 18:1ω7 would be also produced by elongation of 16:1ω7.

Similar sequences of reactions were found when [1-^{14}C] 18:0 acid was incubated. Stearic acid was converted by the Δ9 desaturase to 18:1ω9 that was presumably again desaturated by the Δ6 desaturase to 18:2ω9 acid. The existence of a Δ6 desaturase in *P. parvus* was shown in a previous work (2). The enzyme

TABLE V

Labeling Distribution in *P. parvus* Fatty Acids after Administration of [1-^{14}C-Oleate Dissolved in Sea Water[a]

Fatty acids	Radioactivity	
	Percent	dpm/peak area
14:0	0.4 ± 0	1 ± 0
16:0 + 16:1ω7	4.9 ± 0.1	3 ± 0
18:0	0.1 ± 0.1	0.3 ± 0
18:1ω9	81.7 ± 0.7	277 ± 9
18:2ω9	1.9 ± 0	59 ± 1
20:1ω9	9.1 ± 0.3	33 ± 1
20:2ω9	1.9 ± 0.6	55 ± 7

[a]Results are the mean of the analysis of two samples of 1 g copepods each fed during 5 hr ± extreme deviation from the mean.

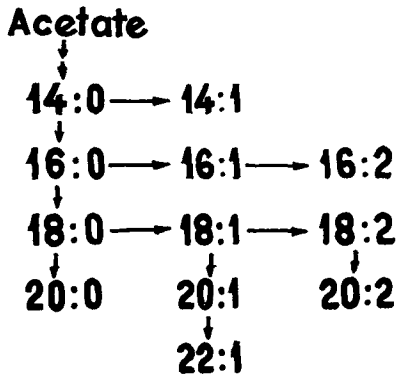

FIG. 1. Suggested pathways in the de novo biosynthesis of fatty acids in *P. parvus*.

desaturated 18:2ω6 to 18:3ω6 and 18:3ω3 to 18:4ω3 acids.

Since 18:0 acid was elongated to 20:0 and since the label was also found in 20:1 and 20:2 acids, it is possible that 20:0 was converted to 20:1 and then desaturated again to 20:2. However, it is also possible that 20:1 was the product of 18:1 elongation and 20:2 the elongation product of 18:2 acid. This uncertainty could be resolved by determining the structure of the fatty acids. However, the scarcity of labeled 20:1 and 20:2 acids impeded this determination.

This puzzle might be resolved by incubation of the copepod with [1-^{14}C] oleic acid (Table V). In Table V, it is shown that oleic acid was desaturated by the Δ6 desaturase to 18:2ω9 acid and also elongated to 20:1ω9. In addition, the analysis of the relative specific radioactivities of the different fatty acids suggests that 20:2ω9 acid is produced by elongation of 18:2ω9 and not by desaturation of 20:1ω9 acid.

Thus, we can suggest that the following sequence of reactions takes place in the de novo biosynthesis of fatty acids of even number of carbons in *P. parvus* (Fig. 1). This scheme is very probable since it results after comparing the present data with the biosynthetic routes of other animals, although the structure of the formed acids was not unequivocally proved. Therefore, this synthesis would evoke the formation of 20:1Δ^{11} and 20:2$\Delta^{8,11}$ acids. Reduced amounts of these acids have been recognized in the fatty acid composition of *P. parvus* (1). In addition, 20:1 acid has been identified in marine crustaceae (9-11) and other salt water organisms (12,13). The Δ^{11} isomer was reported for the first time in menhaden oil (14), and it is a major component of the blubber of the sea whale *Balaenoptera borealis* (15).

A monoenoic acid of 22 carbons was also synthesized by *P. parvus* when labeled acetate was administered (Table II). This acid would be produced very probably by elongation of 20:1 acid and not by desaturation of 22:0 acid since no radioactive 22:0 acid was detected when labeled acetate was incubated. Therefore, one can speculate that the synthesized 22:1 acid would correspond to a Δ^{13} structure, but it was not reported in the fatty acid composition of *P. parvus* (1). Therefore, the contribution of the de novo biosynthesis of 20:1ω9 and 22:1ω9 acids to the composition of the lipids of this copepod is conceivable but low.

By means of a whole survey of the results obtained in part I and II of these series (2), we may conclude that *P. parvus* is able to synthesize fatty acids following the typical animal pathways. It would possess fatty acid synthetase, Δ9, Δ6, Δ5 and Δ4 desaturases and elongating enzymes. However, for some still unknown reasons, the polyunsaturated fatty acids prefereably synthesized correspond to the ω3 series.

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Lipid Composition of Yoshida Ascites Hepatoma and of Livers and Blood Plasma from Host and Normal Rats

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ABSTRACT

The lipid composition of Yoshida ascites hepatoma cells was analyzed together with that of ascitic plasma and of livers and blood plasma from host and normal rats. In comparison to normal livers, host livers showed no significant differences in the content of the various lipid classes, but contained a higher percentage of palmitic acid and a lower proportion of arachidonic acid in the major phospholipid classes. In addition, tumor growth induced a marked hypertriglyceridemia in host animals; changes in the concentration of other plasma lipid classes were not statistically significant. The ascitic plasma contained small amounts of lipids mainly constituted by cholesteryl esters and phospholipids. Yoshida hepatoma cells contained less phospholipids in comparison to both host and normal liver, while the increased level of triglycerides and the decrease of free fatty acids were not statistically significant. Hepatoma cells showed appreciable amounts of ether-linked lipids associated in part to neutral lipids (as glyceryl ether diesters) and, in part, to ethanolamine and choline phosphoglycerides. The alkyl groups in GEDE as well as in ethanolamine and choline phosphoglycerides were mainly constituted by $C_{16:0}$ and $C_{18:0}$ followed by $C_{18:1}$. The alk-1-enyl groups in ethanolamine and choline phosphoglycerides were $C_{16:0}$ and $C_{18:0}$ with only a minor proportion of $C_{18:1}$. In comparison to both host and normal liver, Yoshida hepatoma cells showed significant changes in the fatty acid composition of neutral lipids and phospholipids. Some of the major changes consisted of an increase of monoenoic acids associated with a decrease of arachidonic and docosahexaenoic acids in phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol.

INTRODUCTION

It is well known that the lipid composition of animal tissues, even within a given species, varies considerably from one tissue to another (1) and is affected by several factors such as composition of diet (2), sex (3), age (4), circadian rhythm (5), feeding state (6) and environmental conditions (7). In the case of tumors, other factors may possibly influence their lipid composition, e.g., degree of differentiation, form of neoplasia (solid or ascitic, primary or transplantable) and period of growth. Tumor lipids may also be influenced by the tumor-host relationships (8). In fact, changes in host fluids induced by the presence of tumors may affect the lipids of host tissues, including the tumor itself. In this regard, it should also be mentioned that use of host tissues as term of comparison in the study of tumor lipids may be misleading if the possible changes induced in the host tissues by the presence of tumors are not taken into consideration. Therefore, the "neoplastic" relevance of the lipid composition of tumors may be assessed only after taking into account all the above mentioned variables.

Numerous laboratories have studied the lipid composition of tumors (9-11). However, only in a few cases have tumor lipids been analyzed concomitantly with lipids of both host and normal animal tissues (12-14), although there are specific studies on the lipid changes induced by tumor growth on host tissues (15-21) and fluids (22-29).

In the present investigation, the lipids of Yoshida hepatoma cells and ascitic plasma have been studied together with the lipids of both blood plasma and livers from normal and tumor-bearing rats. The aim of this study was to discriminate the lipid changes typical of neoplasia per se from those dependent on tumor-host relationships.

MATERIALS AND METHODS

Animals and Tumors

The Yoshida ascites hepatoma AH130 was maintained in two-month old male Wistar rats by inoculating ca. 10^7 cells. Animals were fed a commercial stock diet with the same lipid composition as described in a previous paper (30).

Extraction of Lipids

Seven to ten days after tumor inoculation, host rats were fasted overnight and exsanguinated under diethyl ether anaesthesia by heart puncture using heparinized syringes. The blood was centrifuged at room temperature and the separated plasma was extracted by the method of Folch et al. (31). Tumors were collected from the exsanguinated rats and centrifuged for 15 min in the cold at 1000 x g to separate the ascitic plasma from the hepatoma cells. The ascitic plasma was immediately extracted by the method of Bligh and Dyer (32) and the hepatoma cells were washed twice

in a 0.14 M NaCl solution and extracted (32) after homogenization in 3 vol of 0.04% $CaCl_2$. The livers of host rats were pooled, homogenized in 3 vol of the 0.04% $CaCl_2$, and extracted according to Folch et al. (32).

In parallel experiments, three normal rats of the same age as the host rats were fasted overnight and then killed by exsanguination; blood and livers were processed as in host rats.

Analytical Procedures

Total lipids were determined gravimetrically on portions of lipid extracts evaporated at room temperature under a flux of nitrogen. Lipid-free dry weight, calculated as the difference between total lipids and constant dry weight, was used as a basis to express quantitative results in this study. The percentage of water, calculated as the difference between constant dry weight and wet weight, was 70, 72 and 82 in normal liver, host liver and Yoshida hepatoma cells, respectively. Before calculating the water content of hepatoma cells, the wet weight was corrected by a 17% extracellular water which was trapped in the pellet of packed cells.

Total lipids were fractionated into neutral lipids and phospholipids (PL) by silicic acid column chromatography. Neutral lipids were sequentially separated into cholesteryl esters, triglycerides (TG), diglycerides (DG), free cholesterol, monoglycerides (MG) and free fatty acids (FFA) on Florisil columns (Florisil 60/100 mesh, Fisher Scientific Co., Fair Lawn, NJ) (33). TG, DG and MG were estimated by glycerol determination (34) and by using molecular weights derived from the fatty acid composition of the individual glycerides in the various tissues examined. Free and esterified cholesterol was determined according to Cramer and Isaksson (35) and FFA following the method of Duncombe (36).

The hapatoma cell TG fraction eluted from Florisil columns was found to contain appreciable amounts of glyceryl ether diesters (GEDE). These were separated from TG by preparative thin layer chromatography (TLC) on Silica Gel H with hexanes/diethyl ether/acetic acid (85:15:1, v/v) and submitted to hydrogenolysis with 65% Sodium-bis-(2-methoxyethoxy)-aluminum hydride in toluene following the method of Su and Schmid (37). The released alkyl glycerols were then determined by the quantitative gas chromatographic analysis of the isopropylidene derivatives (38) using a pentadecyl glycerol internal standard.

PL were separated into diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) plus phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), sphingomyelin (SP) and lysolecithin (LPC) by silicic acid (Unisil 200/325 mesh, Clarkson Chemical Co. Inc., Williamsport, PA), column chromatography using a stepwise elution system reported in a previous paper (30). Phospholipids were determined by multiplying x 25 the phospholipid-phosphorus assayed following the method of Martin and Doty (39) after digestion with sulfuric acid-perchloric acid (3:2, v/v).

The ether-linked moieties present in PE and in PC of Yoshida hepatoma cells were analyzed in detail on the basis of the indications from previous work by this laboratory on the same cells (40). For this study, tumor phospholipids were fractionated by bidimensional TLC with chloroform/methanol/ammonia (90:45:11, v/v) and chloroform/methanol/acetic acid/water (90:40:12:2, v/v), and PE and PC were recovered from the absorbent by elution with chloroform/methanol (3:2, v/v) followed by methanol. PE and PC were then submitted to the procedure of Su and Schmid which transformed the alk-1-enyl and alkyl groups into alkyl substituted dioxanes and alkyl glycerols, respectively (37). The alkyl substituted dioxanes and alkyl glycerols were then separated from the other reaction products by TLC (37) and recovered from the absorbent by exhaustive elution with diethyl ether/methanol (90:10, v/v). The alkyl glycerols converted into isopropylidene derivatives (38) and the alkyl substituted dioxanes were quantitatively analyzed by gas liquid chromatography using pentadecyl glycerol and 1-1-dimethoxytetradecane, respectively, as internal standards added to the reaction mixture at the beginning of the procedure.

Gas Liquid Chromatography

The acyl group composition of individual lipid classes was determined by the gas chromatographic analysis of the methyl esters obtained by H_2SO_4-methanolysis. Methyl esters were analyzed at 185 C on an Aerograph model 1520 gas chromatograph using 6 ft x 1/8 in. (i.d.) stainless steel columns packed with 15% EGSS-X on Gas Chrom P 100/120 mesh, (Applied Science Lab., State College, PA) and identified on the basis of several parameters (30). Quantitative response was routinely checked by using the calibrated KF mixture from Applied Science. The unsaturated fatty acids were designated by the trivial name of the more common isomer, although the location of the double bonds in the hydrocarbon chains was not determined.

TABLE I

Content of Neutral Lipid Classes in Normal and Host Rat Livers, and Yoshida Hepatoma Cells (AH130)[a]

Lipid class	Normal liver	Host liver	Hepatoma cells
Esterified cholesterol	1.0 ± 0.2 (5)	1.3 ± 0.2 (11)	2.1 ± 0.4 (10)
Free cholesterol	7.5 ± 0.1 (5)	8.3 ± 1.5 (11)	9.4 ± 0.7 (10)
Glyceryl ether diesters[b]	ND[c] (5)	ND[c] (11)	0.4 ± 0.0 (5)
Triglycerides[d]	38.8 ± 7.2 (5)	51.5 ± 11.7 (11)	63.3 ± 10.9 (10)
Diglycerides[d]	1.8 ± 0.1 (5)	3.9 ± 1.0 (11)	2.8 ± 0.8 (10)
Monoglycerides[d]	0.1 ± 0.1 (5)	0.3 ± 0.9 (11)	0.2 ± 0.0 (10)
Free fatty acids	2.4 ± 0.3 (5)	4.2 ± 0.9 (11)	1.9 ± 0.5 (10)

[a]The values, expressed as mg/g lipid-free dry weight, are the mean ± SE of the number of experiments listed in parentheses.

[b]Amounts of glyceryl ether diesters were obtained by multiplying the alkyl glycerol values x 2.5 (m.w. 830).

[c]ND = not detectable under the analytical conditions used.

[d]818, 546 and 267 were the molecular weights used to calculate the amounts of tri-, di- and monoglycerides, respectively, in livers and hepatoma cells.

The alkyl substituted dioxanes and isopropylidene derivatives of alkyl glycerols were both analyzed at 195 C on 6 ft x 1/8 in. glass columns packed with 10% EGSS-X in a Perkin-Elmer model 3920 gas chromatograph. Quantitative response of the apparatus to alkyl glycerols was checked by using a known mixture of pentadecyl, hexadecyl, and octadecyl glycerols, which were prepared in our laboratory following the method of Oswald et al. (41).

Statistical Analyses

Statistical significance of the differences between normal and host rat livers, normal and host blood plasma and tumor cells and host or normal rat livers was assessed by using the Student's t test. The minimum level of significance was considered to be the 5% level.

RESULTS AND DISCUSSION

Table I shows that tumor growth did not influence the concentration of free and esterified cholesterol in the liver of rats bearing Yoshida ascites hepatoma. In these livers, the levels of TG, DG, MG and FFA were increased as compared to normal livers, but also these differences were not statistically significant. In this regard, it should be mentioned that in glioma-bearing mice (17) as well as in Ehrlich carcinoma-bearing mice (20) the sequential determination of liver TG revealed, after an initial fall, a sharp increase of TG followed by a subsequent decrease which persisted over the remaining period of observation. Moreover, the liver of rats bearing a 4-5 week-old 7288 CTC hepatoma has been found to contain lower amounts of TG and cholesterol than normal rat liver (12).

From the examination of Table I, it is apparent that, in comparison to both normal and host rat livers, Yoshida hepatoma cells contained higher amounts of esterified and free cholesterol and of TG, and lower amounts of FFA, but these differences were not statistically significant. Higher concentrations of cholesteryl esters, free cholesterol and FFA have been found by Snyder et al. (42) in both the fast growing 7777 and slow growing 7794A Morris hepatomas compared with normal rat liver. The same study (42) also showed that TG were reduced in the 7777 hepatoma but unchanged in the 7794A hepatoma in respect to normal rat liver. A lower concentration of TG associated with a higher level of cholesterol was also found by Wood et al. (12) in the 7288 CTC hepatoma when compared with either normal or host rat livers.

Table I also shows that in Yoshida hepatoma cells the level of GEDE was lower than that reported for 7777 Morris hepatoma (42) and for Ehrlich carcinoma (43). In addition, Yoshida hepatoma cells contained small amounts of DG and MG, at variance with the considerable quantity of MG noticed by Gray (44) in the Landschutz ascites carcinoma cells.

The concentration of total PL and of the individual PL classes in the livers of both host and normal rats and in Yoshida hepatoma cells are reported in Table II. In agreement with the observation by Wood concerning 7288 CTC hepatoma (13), no significant difference was found in the level of either total PL or individual PL classes between normal and host livers. These results are at variance with those of a study by Carruthers and Kim (18) revealing an increase of PL in the liver of rats bearing mammary carcinomas (when the tumors had

TABLE II

Content of Phospholipid Classes in Normal and Host Rat Livers, and Yoshida Hepatoma Cells (AH130)[a]

Lipid class[b]	Normal liver n = 2		Host liver n = 7		Hepatoma cells n = 7	
TPL	120.0 ± 5.4		125.0 ± 2.8		85.0 ± 4.5	
DPG	6.5 ± 0.3	(5.4)	7.1 ± 0.8	(5.7)	4.4 ± 0.6	(5.1)
PE (plus PS)[c]	36.8 ± 4.2	(30.6)	41.5 ± 0.9	(33.4)	27.0 ± 0.8	(31.7)
PI	9.1 ± 0.5	(7.6)	4.1 ± 0.8	(3.2)	4.3 ± 0.7	(5.1)
PC	59.2 ± 2.2	(49.4)	64.8 ± 1.9	(51.4)	43.4 ± 0.9	(51.1)
SP	6.6 ± 0.8	(5.5)	5.7 ± 0.7	(4.8)	5.3 ± 0.4	(6.3)
LPC	1.7 ± 0.2	(1.4)	1.6 ± 0.3	(1.4)	0.6 ± 0.1	(0.7)

[a]The values are means ± SE and are expressed as mg/g lipid-free dry weight. n = number of experiments. Figures in parentheses represent percentages of total lipid phosphorus.

[b]TPL = Total phospholipids; DPG = diphosphatidylglycerol; PE (plus PS) = phosphatidylethanolamine plus phosphatidylserine; PI = phosphatidylinositol; PC = phosphatidylcholine; SP = sphingomyelin; LPC = lysophosphatidylcholine.

[c]When in a few experiments, PE (plus PS) was further analyzed (45), PS was found to account for 2.5-5.8% of total fraction in both livers and hepatoma cells.

TABLE III

Percentages of Ether-linked Subfractions of Phosphatidylethanolamine and Phosphatidylcholine from Yoshida Hepatoma Cells (AH130)[a]

Class	Percentage of the total phospholipid class	
Diacylphosphatidylethanolamine	73.4	(71.9-75.1)
Alkyl-acylphosphatidylethanolamine	12.7	(12.8-12.6)
Alk-1-enyl-acylphosphatidylethanolamine	14.3	(16.3-12.3)
Diacylphosphatidylcholine	86.9	(86.1-87.8)
Alkyl-acylphosphatidylcholine	10.0	(11.0-9.0)
Alk-1-enyl-acylphosphatidylcholine	3.1	(2.9-3.2)

[a]Means of the values of two experiments reported in parentheses. Percentage of each individual subfraction was evaluated in the phospholipid class isolated by TLC (see Materials and Methods). Values were calculated from the amounts of alkyl and alk-1-enyl phospholipids and the amount of the phospholipid class determined on the basis of phosphorus content. The alkyl and alk-1-enyl phospholipids were evaluated from the alkyl glycerol and alk-1-enyl group contents multiplied by appropriate factors. 2.4 and 3.5 were the factors used to calculate the alkylPE (m.w. 739) and alk-1-enylPE (m.w. 738), respectively; 2.5 and 3.7 were the factors used to calculate the alkylPC (m.w. 773) and alk-1-enylPC (m.w. 786), respectively.

reached 47% of the body weight) as well as with the results by Nakazawa and Mead (21) showing an increase of PL in the liver of mice transplanted with human ovarian carcinoma.

In comparison to both normal and host rat livers, Yoshida hepatoma cells showed the reduction of PL concentration that was repeatedly found in different tumors when compared with normal tissues at the level of whole homogenate (12,42,46-48). and of microsomal fraction (14,30,49,50). Moreover, different ascites tumors (44,47,51) showed phospholipid concentrations similar to that found in Yoshida hepatoma cells, provided that the results were expressed on the same basis. From Table II it is also apparent that, with the exception of minor variations in the percentage of PI, SP and LPC, the phospholipid pattern was almost the same in livers and hepatoma cells, indicating that the reduction of tumoral PL interested all the major phospholipid classes.

The analyses of ether-linked forms of PE and PC in hepatoma cells revealed that alkyl and alk-1-enyl species represent 13 and 14% of PE and 10 and 3% of PC, respectively (Table III). In comparison to the results on Yoshida ascites hepatoma, Snyder et al. (42) found lower percentages of alkyl and alk-1-enyl species in both PE and PC from 7794A and 7777 Morris hepatomas, while Wood and Snyder (52) observed higher proportions of alkyl, but similar percentages of alk-1-enyl species in PE and PC of Ehrlich carcinoma. On the other hand, Su and Schmid (37) found lower levels of

TABLE IV

Lipid Concentrations in Blood Plasma from Normal Rats and in Blood and Ascitic Plasma from Rats Bearing Yoshida Hepatoma (AH130)[a]

Lipid class	Normal rat blood plasma	Host rat blood plasma	Ascitic plasma
Total phospholipids	125.0 ± 23.4 (5)	152.5 ± 23.3 (7)	42.5 ± 5.3 (5)
Esterified cholesterol	36.2 ± 3.7 (5)	35.3 ± 5.9 (6)	22.4 ± 0.1 (5)
Free cholesterol	15.9 ± 2.0 (5)	30.0 ± 9.1 (6)	6.0 ± 0.8 (5)
Triglycerides[b]	68.4 ± 16.1 (3)	196.3 ± 36.4 (3)[c]	4.9 ± 0.5 (3)
Free fatty acids	28.5 ± 9.4 (5)	18.6 ± 3.2 (5)	2.2 ± 0.0 (5)

[a]The values, expressed as mg/100 ml blood or ascitic plasma, are the means ± SE of the number of experiments listed in parentheses.

[b]824, 820 and 817 were the molecular weights used to calculate the amounts of triglycerides in normal and host rat blood plasma and in ascitic plasma, respectively.

[c]Significantly different from normal rat blood plasma at $P<0.05$.

total alkyl glycerols and higher concentrations of total alk-1-enyl glycerols in a series of transplantable tumors including Novikoff hepatoma.

It is worth noting that, taken altogether, the different ether-linked moieties of Yoshida hepatoma cells represented 3.3% of total lipids. This value falls in the range reported by Wood and Snyder in different rat and mouse transplantable tumors (53) and by Howard et al. in hepatomas with a high growth rate (54).

Table IV shows the lipid class content of blood plasma from normal rats and of blood and ascitic plasma from tumor-bearing rats. In comparison to the normal rats, tumor-bearing animals showed in their blood plasma higher levels of PL, free cholesterol and TG and lower concentrations of FFA.

However, when all these differences were submitted to the Student's *t* test, only the increase of plasma TG in host rats was significant. An increased level of plasma TG was also found in rats bearing Walker carcinoma 256 by Barklay et al. (23) and in mice bearing Ehrlich carcinoma by Brenneman et al. (26) and by Kannan and Baker (27). A recent investigation by Kannan and Baker (29) on this topic revealed that the magnitude of tumor-induced hypertriglyceridemia seems to be affected by the duration of tumor growth, variation in animal strain and feeding condition. However, hypertriglyceridemia cannot be considered a constant change associated with the growth of tumor. In fact, in the blood plasma of host rats bearing 7777 Morris hepatoma, Grigor et al. (22) found a decrease of TG associated with a rise of free and esterified cholesterol, and Narayan and Morris (25) reported an increase of cholesterol-rich high density lipoproteins. As far as plasma FFA are concerned, Bizzi et al. (24) found an increased level of plasma FFA in fed animals bearing different tumors, including Yoshida hepatoma AH130, when compared to the controls under the same feeding conditions. These authors also noted (24) that overnight fasting induced a marked increase of the plasma FFA of the controls, that was progressively less evident, however, in host animals as the tumor continued to grow. This effect may explain why our tumor-bearing animals, that were fasted overnight before blood sampling, contained a reduced level of plasma FFA as compared to the controls.

Ascitic plasma contained very low amounts of TG and FFA in comparison to the host blood plasma, while PL and esterified cholesterol were still present in appreciable amounts. The ratio of esterified to free cholesterol was much higher in ascitic plasma than in host blood plasma. Differences of the lipid pattern between the ascitic and the blood plasma of hepatoma-bearing rats would seem to indicate a selective permeability of the peritoneal wall towards plasma lipoproteins and/or a preferential utilization of certain lipid classes in ascitic plasma by the tumor cells. The ascitic plasma of Ehrlich carcinoma-bearing mice studied by Yamakawa and Ueta (55) also contained small concentrations of lipids mainly constituted by neutral lipids with a relatively high contribution of cholesteryl esters. In addition, the TG concentration found by Kannan and Baker (27) in the ascitic plasma of Ehrlich carcinoma-bearing mice was much similar to that reported in the ascitic fluid of our tumor-bearing rats. However, when CBA mice were used to grow Ehrlich carcinoma, an elevated amount of TG was found in their ascitic plasma by Spector's group (26,28), a result possibly accounted for by the remarkably high tumor-induced hypertriglyceridemia in mice of this strain.

The fatty acid compositions of neutral lipid classes, glycerophospholipids and sphingomyelin from host and normal rat livers and Yoshida hepatoma cells are reported in tables V, VI and VII.

As shown in Table V, no significant differences were found in the fatty acid composition of cholesteryl esters, TG and FFA of host and normal rat livers. In comparison to the livers, Yoshida hepatoma cells had a significantly higher proportion of myristic and a lower percentage of palmitic acids in cholesteryl esters; furthermore, hepatoma cholesteryl esters contained a higher proportion of stearic acid in comparison to host liver and a lower level of linoleic acid in respect to normal rat liver. TG of Yoshida hepatoma cells contained a lower proportion of palmitic and a higher level of stearic acid than TG of liver of both normal and host rats; in addition, tumor TG had a higher percentage of palmitoleic acid as compared to host liver TG and a higher percentage of oleic acid in comparison to normal liver TG. The finding that Yoshida hepatoma cells compared with the rat liver contain a lower proportion of palmitic and a higher percentage of stearic acid in TG is consistent with previous results concerning TG of 7777 Morris hepatoma (42) and of 7288 CTC hepatoma (12). In TG and cholesteryl esters of these tumors, however, there were elevated quantities of C_{22} polyunsaturated acids that were not found in the present study. Table V also shows that FFA of Yoshida hepatoma cells contained a lower proportion of palmitic acid and higher percentages of palmitoleic and oleic acids than FFA of both host and normal livers.

Table VI shows that, in comparison to the normal liver, the liver of the host exhibited a higher percentage of palmitic acid in PC and PI and a lower proportion of arachidonic in PE (plus PS), PC and PI. In the DPG, the host liver had significantly lower proportions of palmitoleic and oleic acids and a higher level of linoleic acid than the normal liver. In other laboratories, an increase of palmitic acid associated with a decrease of arachidonic acid was observed in the liver PL of rats bearing Walker carcinoma 256 (19) and in the liver PE and PC of rats bearing 7288 CTC hepatoma (13). Moreover, Ueta et al. (15) reported an increase of stearic and a decrease of oleic acid in total lipids of mouse liver after intraperitoneal inoculation of both Ehrlich carcinoma and MH-134 hepatoma cells. However, no significant variations were found in the fatty acid composition of the liver PL from mice bearing a transplantable glioma (16) and of the PC and PE of liver organelles from rats bearing Morris hepatoma 7777 (14).

In Yoshida hepatoma cells, diacylPE and -PC contained lower proportions of stearic, arachidonic, and docosahexaenoic acids and higher percentages of palmitoleic, oleic, and linoleic acids in comparison to PE (plus PS) and PC of either host or normal liver. An increase of oleic acid associated with a decrease of arachidonic and docosahexaenoic acids was also observed in the hepatoma PI in comparison to the PI of normal and host livers. In DPG, hepatoma cells revealed an unchanged fatty acid composition when compared to the normal liver, while showing a higher level of oleic acid and a lower proportion of linoleic acid with respect to host liver. These differences are consistent with those observed by Wood (13) in DPG of 7288 CTC hepatoma.

In SP (Table VII), host livers compared to normal livers showed higher levels of palmitic and nervonic acids and lower proportions of tricosanoic and lignoceric acids in agreement with the results reported by Wood (13) in rats bearing 7288 CTC hepatoma. When compared with normal liver, Yoshida hepatoma cells showed in SP higher levels of palmitic, arachidic and behenic acids and a lower percentage of tricosanoic and nervonic acids. In comparison to the host liver, tumor SP showed an increase of arachidic and behenic acids associated with a decrease of tricosanoic and lignoceric acids. In SP of Yoshida hepatoma cells, we did not find the relevant amount of $C_{24:2}$ observed by Wood in SP of either Ehrlich carcinoma (58) or several other transplantable tumors (59).

It should be mentioned that the fatty acid profiles of the various lipid classes from Yoshida hepatoma cells reported in this study confirm our earlier data on this hepatoma studied at the level of subcellular fractions (30). They are also similar, with only minor differences, to those found when Yoshida hepatoma cells were grown in control animals kept on a semisynthetic diet during a study of the effects of essential fatty acid deficiency on the lipids of tumor cells (60). In particular, the high levels of palmitoleic and oleic acids associated with low percentages of arachidonic and/or C_{22} polyunsaturated acids of Yoshida hepatoma cells are consistent with similar changes found in other hepatomas compared with liver (13,14,42,46,61-63). Recent studies by Wood et al. (64-66) indicate that vaccenic acid may partly account for the "oleification" observed in tumor cells.

Table VIII gives the composition of ether-

TABLE V

Fatty Acid Composition of Cholesteryl Esters, Triglycerides and Free Fatty Acids of Livers from Normal and Host Rats, and Yoshida Hepatoma Cells (AH130)[a]

Tissue	% (by wt) of total fatty acids[b]								
	14:0	16:0	16:1	18:0	18:1	18:2	18:3[c]	20:3[d]	20:4
Cholesteryl esters									
Normal rat liver (5)	1.5 ± 0.3	29.9 ± 3.2	5.8 ± 1.0	6.6 ± 2.2	27.8 ± 2.5	17.4 ± 1.3	1.2 ± 0.4	0.4 ± 0.1	9.8 ± 2.1
Host rat liver (7)	0.9 ± 0.3	34.5 ± 4.3	4.3 ± 0.4	5.3 ± 0.3	29.1 ± 2.5	15.3 ± 0.9	1.7 ± 0.5	1.1 ± 0.5	7.8 ± 0.5
Hepatoma cells[e] (7)	7.6 ± 1.0	18.4 ± 0.5	5.8 ± 0.4	10.9 ± 0.8	33.5 ± 0.9	12.2 ± 1.2	3.6 ± 0.8	1.7 ± 0.3	6.3 ± 0.7
Triglycerides									
Normal rat liver (5)	0.9 ± 0.0	28.8 ± 1.7	3.4 ± 0.6	3.4 ± 0.6	30.1 ± 2.6	29.0 ± 2.0	2.6 ± 0.3	—	2.4 ± 0.4
Host rat liver (7)	0.6 ± 0.1	28.5 ± 0.9	1.9 ± 0.4	3.8 ± 0.4	32.4 ± 1.2	27.1 ± 1.6	2.1 ± 0.2	0.7 ± 0.2	2.9 ± 0.4
Hepatoma cells[e] (7)	1.3 ± 0.1	17.1 ± 0.5	3.6 ± 0.3	11.3 ± 0.9	35.3 ± 0.9	25.5 ± 1.5	2.1 ± 0.5	0.6 ± 0.2	2.2 ± 0.4
Free fatty acids									
Normal rat liver (5)	3.8 ± 2.0	49.5 ± 1.9	2.9 ± 0.4	12.1 ± 1.0	15.1 ± 1.8	11.9 ± 2.4	1.2 ± 0.4	—	3.5 ± 0.2
Host rat liver (7)	4.9 ± 0.9	42.1 ± 1.7	3.0 ± 0.4	11.1 ± 1.0	19.9 ± 2.1	13.8 ± 1.4	0.9 ± 0.3	0.8 ± 0.2	3.5 ± 0.6
Hepatoma cells[e] (7)	4.9 ± 0.3	23.4 ± 1.0	5.9 ± 0.7	17.9 ± 2.1	26.4 ± 2.4	13.3 ± 1.3	6.2 ± 1.4	—	2.0 ± 0.5

[a]All the fractions indicated in this table were prepared by Florisil column chromatography. In the case of hepatoma cells, values given under the heading Triglycerides also include minor amounts of fatty acids derived from glyceryl ether diesters.

[b]Percentages represent means ± SE of the number of experiments listed in parentheses.

[c]Percentage of 18:3 also includes small amounts of eicosaenoic acid.

[d]Percentage of eicosatrienoic acid includes either ω6 or ω9 isomer, this latter being present only in trace amounts.

[e]Significant differences between hepatoma cells and host or normal livers are reported in the text.

TABLE VI

Fatty Acid Composition of Phosphatidylethanolamine (plus Phosphatidylserine), Phosphatidylcholine, Phosphatidylinositol and Diphosphatidylglycerol of Normal and Host Rat Livers, and Yoshida Hepatoma Cells (AH130)[a]

Tissue	% (by wt) of total fatty acids[b]											
	14:0	16:0	16:1	18:0	18:1	18:2	18:3[c]	20:3[d]	20:4	20:5	22:5	22:6
Phosphatidylethanolamine (plus Phosphatidylserine)												
Normal rat liver (2)	0.1 ± 0.0	15.8 ± 1.7	0.4 ± 0.1	25.0 ± 0.1	6.7 ± 0.2	9.0 ± 0.7	---	0.4 ± 0.0	29.1 ± 0.7	0.5 ± 0.1	1.8 ± 0.7	11.2 ± 2.2
Host rat liver[e] (6)	0.2 ± 0.0	21.9 ± 1.3	0.2 ± 0.1	30.2 ± 1.2	4.4 ± 0.1	7.7 ± 0.4	0.3 ± 0.1	0.4 ± 0.1	18.5 ± 1.0***	---	1.5 ± 0.4	14.6 ± 1.5
Hepatoma Cells[f,g] (3)	1.1 ± 0.5	14.5 ± 2.6	1.3 ± 0.1	19.8 ± 1.0	22.5 ± 0.1	22.4 ± 0.8	---	0.3 ± 0.3	14.3 ± 1.7	1.2 ± 0.0	0.7 ± 0.3	1.9 ± 0.3
Phosphatidylcholine												
Normal rat liver (3)	0.3 ± 0.0	25.2 ± 1.5	0.9 ± 0.1	20.9 ± 0.8	9.1 ± 0.2	14.3 ± 0.6	---	0.5 ± 0.2	22.2 ± 1.4	---	0.8 ± 0.1	5.6 ± 0.6
Host rat liver[e] (6)	0.3 ± 0.0	32.0 ± 1.0**	0.4 ± 0.0	19.9 ± 1.1	8.0 ± 0.2	17.5 ± 0.9	0.2 ± 0.1	0.7 ± 0.1	11.9 ± 0.9***	0.5 ± 0.1	0.6 ± 0.2	8.0 ± 1.1
Hepatoma cells[f,g] (3)	1.0 ± 0.0	27.4 ± 0.6	1.8 ± 0.1	13.8 ± 1.1	25.7 ± 0.8	22.6 ± 1.3	---	0.9 ± 0.4	4.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	1.3 ± 0.3
Phosphatidylinositol												
Normal rat liver (2)	0.3 ± 0.1	9.8 ± 2.7	0.8 ± 0.2	38.8 ± 5.4	5.5 ± 2.5	8.7 ± 5.5	---	0.8 ± 0.6	31.3 ± 4.8	0.5 ± 0.0	0.9 ± 0.1	2.7 ± 0.9
Host rat liver[e] (6)	1.2 ± 0.4	19.7 ± 1.4**	0.5 ± 0.2	35.8 ± 1.9	4.5 ± 0.4	9.0 ± 0.6	---	4.0 ± 1.5	19.0 ± 1.8*	1.7 ± 0.7	0.2 ± 0.1	4.4 ± 0.7
Hepatoma cells[f,g] (9)	0.8 ± 0.2	10.5 ± 1.1	1.7 ± 0.3	45.0 ± 2.9	17.2 ± 1.5	13.6 ± 1.2	0.3 ± 0.1	1.3 ± 0.3	8.8 ± 1.3	0.1 ± 0.1	0.5 ± 0.2	0.2 ± 0.1
Diphosphatidylglycerol												
Normal rat liver (2)	0.8 ± 0.0	7.8 ± 1.6	4.6 ± 1.0	5.9 ± 2.6	24.0 ± 1.6	54.0 ± 1.2	---	0.7 ± 0.5	2.2 ± 0.3	---	---	---
Host rat liver[e] (6)	0.5 ± 0.1	6.8 ± 1.0	1.8 ± 0.3**	4.3 ± 1.2	17.7 ± 1.3*	66.2 ± 1.7**	---	1.1 ± 0.9	1.5 ± 0.5	---	---	---
Hepatoma cells[f] (9)	1.2 ± 0.2	6.8 ± 0.9	4.2 ± 0.4	7.2 ± 1.1	24.9 ± 0.9	48.8 ± 2.9	0.8 ± 0.1	1.6 ± 0.7	3.4 ± 0.6	0.1 ± 0.1	---	1.0 ± 0.6

[a]All the fractions indicated in this table were prepared by silicic acid column chromatography, except phosphatidylethanolamine and phosphatidylcholine from hepatoma cells which were prepared by bidimensional TLC in order to be used for further analyses (see Materials and Methods). Consequently, in the case of hepatoma cells, values given under the heading Phosphatidylethanolamine (plus Phosphatidylserine) actually correspond to phosphatidylethanolamine alone.

[b,c,d]As in Table V.

[e]Levels of significant differences between host and normal rat liver are 5 (*), 1 (**) and 0.1% (***).

[f]Significant differences between hepatoma cells and host or normal rat livers are reported in the text.

[g]Fatty acid analyses were performed on the isolated diacyl fraction after TLC (56) of diglyceride acetate derivatives of the phosphoglyceride obtained by acetolysis (57).

TABLE VII

Fatty Acid Composition of Sphingomyelin of Normal and Host Rat Livers, and Yoshida Hepatoma Cells (AH130)

Tissue	% (by wt) of total fatty acids[a]												
	14:0	16:0	16:1	18:0	18:1	18:2	20:0	22:0	22:1	23:0	23:1	24:0	24:1
Normal rat liver (4)	3.4 ± 1.4	23.7 ± 1.7	1.2 ± 0.1	14.3 ± 1.5	4.8 ± 1.1	5.8 ± 3.2	1.4 ± 0.5	7.3 ± 0.5	1.7 ± 0.5	6.3 ± 1.1	1.5 ± 0.4	18.8 ± 2.0	9.8 ± 1.8
Host rat liver[b] (5)	0.5 ± 0.2	39.1 ± 1.7***	0.4 ± 0.1	12.6 ± 1.6	3.9 ± 0.6	6.3 ± 1.3	1.1 ± 0.2	7.6 ± 1.7	2.7 ± 0.5	1.6 ± 0.4**	---	8.4 ± 0.9**	15.8 ± 0.9*
Hepatoma cells[c] (7)	1.7 ± 0.3	36.9 ± 1.8	1.0 ± 0.2	13.3 ± 1.2	6.6 ± 1.7	5.1 ± 1.7	7.4 ± 0.8	9.0 ± 0.8	0.9 ± 0.3	0.2 ± 0.2	0.3 ± 0.1	9.9 ± 1.1	7.4 ± 0.9

[a]As in Table V.

[b]Levels of significant differences between host and normal rat liver are 5 (*), 1 (**) and 0.1% (***).

[c]Significant differences between hepatoma cells and host or normal rat liver are reported in the text.

TABLE VIII

Composition of Ether-linked Groups in Glyceryl Ether Diesters (GEDE) and Phosphoglycerides from Yoshida Hepatoma Cells (AH130)

Side chain	GEDE	Phosphatidylethanolamine[a]		Phosphatidylcholine[a]	
	Alkyl[b] (5)	Alkyl (3)[b]	Alk-1-enyl[c] (3)	Alkyl (3)[b]	Alk-1-enyl[c] (2)
14:0	1.6 ± 1.2[d]	---	---	---	---
16:0	39.6 ± 2.8	35.6 ± 4.2	47.1 ± 0.7	41.0 ± 0.9	47.2 ± 0.8
16:1	tr	tr	tr	tr	tr
18:0	38.5 ± 2.9	49.1 ± 5.3	45.0 ± 1.1	38.8 ± 2.3	45.7 ± 0.5
18:1	19.0 ± 2.4	15.3 ± 1.6	8.1 ± 1.0	20.3 ± 1.6	7.2 ± 0.3
18:2	1.3 ± 0.5	---	---	---	---

[a]Isolated by TLC (see Materials and Methods).

[b]Alkyl groups were analyzed by gas liquid chromatography as alkyl glycerol isopropylidene derivatives (see Materials and Methods).

[c]Alk-1-enyl groups were analyzed by gas liquid chromatography as alkyl substituted dioxanes (see Materials and Methods).

[d]Values represent weight percentages and are the means ± SE of the number of experiments listed in parentheses.

linked moieties in GEDE, PE and PC of Yoshida hepatoma cells. GEDE as well as PE and PC contained similar elevated amounts of alkyl $C_{16:0}$ and $C_{18:0}$ with lower proportions of $C_{18:1}$ and minor percentages of $C_{14:0}$ and $C_{18.2}$. Alk-1-enyl moieties in PE and PC were made up mostly of $C_{16:0}$and $C_{18:0}$ chains, while $C_{18:1}$ represent a minor component. The alkyl and alk-1-enyl moieties of Morris hepatoma 7777 studied by Snyder et al. (42) were made up of a lower proportion of $C_{18:0}$ and a higher proportion of $C_{18:1}$ chains in comparison to the values reported in the present study. Moreover, in the detailed study by Wood and Snyder (52) of ether-linked lipids of Ehrlich carcinoma, the alkyl chain composition of GEDE and PE was similar to that found in Yoshida hepatoma cells, while, in comparison to these latter, Ehrlich carcinoma cells contained lower proportions of $C_{16:0}$ and $C_{18:0}$ and a higher percentage of $C_{18:1}$ in alkyl PC. The prevalence of $C_{16:0}$ over $C_{18:0}$ alk-1-enyl chains found in PE of Ehrlich carcinoma (52) was not observed in the present study. The profiles of alkyl and alk-1-enyl moieties found by Su and Schmid (37) in Novikoff hepatoma were approximately the same as those found in Yoshida hepatoma.

Table IX shows that the presence of the tumor did not appreciably affect the fatty acid composition of cholesteryl esters, TG, FFA and PL, with the exception of a higher level of stearic acid in PL. The fatty acid compositions of the various lipid classes in ascitic plasma approximately reflect those found in host blood plasma, with the exception of a higher proportion of stearic acid associated with a lower level of oleic acid in FFA and of a lower proportion of stearic acid and a higher level of docosahexaenoic acid in PL.

In conclusion, this study revealed that growth of Yoshida ascites hepatoma did not significantly affect the lipid composition of host rat liver with the exception of moderate changes in the phospholipid fatty acid pattern. Therefore, the low level of phospholipids, the occurrence of relevant amounts of ether-linked lipids and the variations of the fatty acid profiles found in several lipid classes of Yoshida hepatoma cells do not seem to be related to host-tumor relationships but rather to metabolic derangements in those cells. Moreover, this study revealed that the major tumor-induced change in blood plasma consisted in a noticeable hypertriglyceridemia, the relevance of which in determining the pecularities found in tumor lipids is still to be clarified.

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TABLE IX

Fatty Acid Composition of Cholesteryl Esters, Triglycerides, Free Fatty Acids and Phospholipids of Blood Plasma from Normal Rats and of Blood and Ascitic Plasma from Rats Bearing Yoshida Hepatoma (AH130)

Tissue		% (by wt) of total fatty acids[a]											
		14:0	16:0	16:1	18:0	18:1	18:2	18:3[b]	20:3[c]	20:4	20:5	22:5	22:6
Cholesteryl esters													
Normal rat blood plasma	(5)	0.5 ± 0.0	11.6 ± 0.5	6.5 ± 1.2	0.5 ± 0.1	12.5 ± 1.3	22.7 ± 1.3	0.8 ± 0.5	0.5 ± 0.4	43.7 ± 3.5	0.5 ± 0.5	---	---
Host rat blood plasma	(10)	0.8 ± 0.1	13.7 ± 1.0	4.2 ± 0.3	1.7 ± 0.2	14.6 ± 0.6	23.7 ± 1.3	1.9 ± 0.8	1.6 ± 0.5	37.8 ± 1.5	---	---	---
Host rat ascitic plasma[d]	(5)	0.7 ± 0.2	12.9 ± 2.7	3.3 ± 0.9	1.4 ± 1.1	11.5 ± 0.9	26.6 ± 3.1	1.6 ± 0.1	1.3 ± 0.2	40.6 ± 0.3	---	---	---
Triglycerides													
Normal rat blood plasma	(5)	0.7 ± 0.1	26.2 ± 2.6	4.2 ± 1.0	2.8 ± 0.2	28.6 ± 2.3	24.7 ± 3.2	3.8 ± 1.5	2.4 ± 1.6	4.6 ± 1.1	2.0 ± 1.3	---	---
Host rat blood plasma	(8)	0.5 ± 0.0	31.6 ± 1.0	2.6 ± 0.4	4.0 ± 0.2	31.8 ± 0.9	24.6 ± 0.9	1.3 ± 0.3	0.4 ± 0.1	2.6 ± 0.5	0.4 ± 0.2	---	---
Host rat ascitic plasma[d]	(5)	1.1 ± 0.0	28.4 ± 0.8	3.7 ± 0.3	4.9 ± 0.5	31.1 ± 1.7	19.8 ± 1.9	2.7 ± 1.4	5.2 ± 1.7	3.1 ± 0.3	---	---	---
Free fatty acids													
Normal rat blood plasma	(5)	2.1 ± 0.6	31.7 ± 3.7	6.4 ± 0.3	6.6 ± 1.1	25.1 ± 0.2	22.8 ± 2.4	2.1 ± 0.7	0.7 ± 0.3	2.5 ± 0.5	---	---	---
Host rat blood plasma	(8)	3.0 ± 0.8	33.5 ± 1.4	5.3 ± 0.6	8.7 ± 0.8	25.9 ± 0.9	16.5 ± 1.5	1.7 ± 0.2	3.0 ± 1.7	2.4 ± 0.3	---	---	---
Host rat ascitic plasma[d]	(5)	4.3 ± 0.9	33.6 ± 1.2	7.7 ± 0.5	12.9 ± 1.5	23.3 ± 0.8	8.4 ± 0.9	---	3.9 ± 1.0	5.9 ± 1.2	---	---	---
Phospholipids													
Normal rat blood plasma	(3)	0.3 ± 0.0	25.4 ± 0.9	0.9 ± 0.2	21.2 ± 0.4	8.1 ± 0.8	20.0 ± 2.1	0.6 ± 0.0	1.0 ± 0.1	15.4 ± 3.9	1.3 ± 0.5	0.9 ± 0.3	4.9 ± 0.4
Host rat blood plasma	(8)	0.3 ± 0.1	28.4 ± 1.2	0.2 ± 0.0	28.3 ± 2.3[e]	7.7 ± 0.8	17.0 ± 0.7	0.4 ± 0.0	0.9 ± 0.3	11.8 ± 4.0	0.5 ± 0.1	0.7 ± 0.0	3.8 ± 0.8
Host rat ascitic plasma[d]	(3)	0.4 ± 0.0	32.4 ± 1.6	1.0 ± 0.2	19.8 ± 0.8	12.5 ± 1.8	11.4 ± 0.7	---	2.1 ± 0.2	11.5 ± 0.9	---	0.8 ± 0.1	8.0 ± 0.3

[a]See note [b] in Table V.

[b]See note [c] in Table V.

[c]See note [d] in Table V.

[d]Significant differences between ascitic plasma and host rat blood plasma are reported in the text.

[e]Significantly different from normal rat blood plasma at $P<0.05$.

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Identification of 15-Keto-9,11-Peroxidoprosta-5,13-Dienoic Acid as a Hematin-Catalyzed Decomposition Product of 15-Hydroperoxy-9,11-Peroxidoprosta-5,13-Dienoic Acid

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ABSTRACT

A labile prostaglandin was isolated as one of the products generated from [1-^{14}C] eicosatetraenoic acid incubated with sheep vesicular gland microsomes. The eicosatetraenoic acid metabolite amounted to ca. 16% of the total radiolabeled products. Formation of this new prostaglandin was prevented when heat-denatured microsomes were employed or when incubation mixtures were supplemented with indomethacin or phenol. However, incubation of prostaglandin G_2 (PGG_2) with hematin in the presence or absence of catalytically active or heat-inactivated microsomes led to production of approximately the same quantity of the new prostaglandin. These results indicated that the new prostaglandin can be formed nonenzymically. The new prostaglandin was conclusively identified by gas liquid chromatography-mass spectrometry analysis as 15-keto-9,11-peroxidoprosta-5,13-dienoic acid (15-keto-PGG_2) after chemical conversion to known prostaglandins. The effects of 15-keto-PGG_2 and PGG_2 were similar on canine lateral saphenous vein; both promoted contraction followed by prolonged relaxation, but 15-keto-PGG_2 appeared to be 1/50 as potent as PGG_2.

INTRODUCTION

In a recent investigation (1) that involved extensive purification of prostaglandin (PG) endoperoxides PGG_2 and PGH_2 generated by lipid-depleted sheep vesicular microsomes, a radioactive eicosatetraenoic acid-derived product was discovered that copurified with PGG_2 in previously described isolation systems (1), but was distinguishable from PGG_2 by more rigorous purification and analytical procedures. In the present communication, we described the purification of this eicosatetraenoic acid-derived product, its nonenzymic generation, its chemical modification, and its identification as 15-keto-9,11-peroxidoprosta-5,13-dienoic acid (15-keto-PGG_2).

MATERIALS AND METHODS

5,8,11,14-Eicosatetraenoic acid and other fatty acids were obtained from NuChek Prep, Elysian, MN. [1-^{14}C] 5,8,11,14-Eicosatetraenoic acid was purchased from Rosechem, Los Angeles, CA; its purity was established to be greater than 99% as judged by gas liquid and thin layer chromatography. Triphenylphosphine was a product of Pierce Chemical, Rockford, IL, Hematin, sodium borohydride and *p*-hydroxymercuribenzoate were obtained from Sigma, St. Louis, MO. Methoxyamine-HCl and *N,O-bis*-(trimethylsilyl) trifluoroacetamide (BSTFA) were products from Supelco, Bellefonte, PA. Petroleum ether (bp. 30-60 C) was treated with concentrated sulfuric acid, washed with water, and distilled over lithium aluminum hydride prior to use. Ethyl acetate (spectral grade) was obtained from Matheson, Coleman & Bell, Norwood, OH, and used without further purification. Silicic acid (100 mesh) (Mallinckrodt, St. Louis, MO) was exhaustively washed with distilled water and activated at 150 C overnight prior to use. All other chemicals used were of analytical grade and were products of either Mallinckrodt, J.T. Baker, Cleveland, OH, or Fisher Scientific, Chicago, IL. Vesicular glands from noncastrated sheep were obtained from a local slaughter house and were stored at -70 prior to use.

Thin layer chromatography (TLC) of cyclooxygenase reaction products before or after purification on silicic acid columns was carried out with TLC plates precoated with 0.25 mm Silica Gel G (Mallinckrodt) and developed with one of the following solvent systems: Solvent A: ethyl acetate/2'2,4trimethylpentane/petroleum ether/acetic acid (50:50:20:0.3, by vol); Solvent B: ethyl acetate/2,2,4trimethylpentane/acetic acid (50:50:0.3, by vol) (2); Solvent C: isopropyl ether/2-butanone/acetic acid (50:40:1, by vol) (3); Solvent D: chloroform/methanol/acetic acid/water (90:8:1:0.8, by vol) (4); Solvent E: ethyl acetate/acetic acid (100:1, by vol). Chromatography with solvents A and B was carried out at 4 C in TLC-tanks lined with filter paper (10 x 55 cm) that were preequilibrated for 20 min with the developing solvent. Chromatography with solvent systems C, D and E was performed similarly at room temperature. The location of radioactivity on TLC plates was determined with a Berthold Dünnschicht scanner. In addition to radioisotope scanning, cyclooxygenase reaction products were localized on TLC plates by exposure to I_2

vapor or by spraying with 50% sulfuric acid and charring. Radioactive zones on silica gel plates were scraped off with a razor blade, and radioactivity was determined by liquid scintillation counting using Aquasol-2 (New England Nuclear, Boston, MA) containing 5% water. Hydroperoxy and/or endoperoxy containing cyclooxygenase reaction products were made visible after TLC analysis by spraying with a solution containing 0.1% *N, N*-dimethyl-*p*-phenylenediamine in chloroform/acetic acid/water (5:5:1, by vol) (5). Purification of cyclooxygenase reaction products of [1-^{14}C] eicosatetraenoic acid was carried out as previously described (1) in a -20 cold room by chromatography on silicic acid (1.5 g in a 1.0 cm diameter glass column) and elution with solvent mixtures containing various proportions of ethyl acetate in purified petroleum ether. Gas liquid chromatography (GLC) was carried out isothermally at 220 C with a Varian gas liquid chromatograph (model 3700) equipped with a 2 ft x 0.125 in column and a flame ionization detector. The column support used was 5% OV-101 on 80/100 mesh chromosorb WAW. GLC-mass spectrometry (MS) was performed with a LKB-9000 and a column (4 ft x 0.125 in) packed with 3% OV-17 on 80/100 mesh Supelcoport. Chromatography was carried out with constant temperature of 220 C. Mass spectra were recorded at 20 eV.

Cyclooxygenase Preparation

Sheep vesicular gland microsomes were prepared as follows: excess fat was removed from glands which were then homogenized in ice cold 0.25 M sucrose (pH 7.4) containing 1 mM EDTA (sucrose-EDTA solution) (1.3 ml sucrose-EDTA solution per g tissue). The homogenate was filtered through cheese cloth and centrifuged for 20 min at 12,000 x g. The supernatant was collected and the pellet reextracted by homogenization with the sucrose-EDTA solution (1.0 ml per g of starting material) with a Potter-Elvehjem homogenizer. The homogenate was transferred into a round bottom flask, frozen (-70 C) and lyophilized overnight. The freeze-dried microsomes were lipid-depleted as described previously (1). Microsomal preparations that were not subjected to lipid depletion were resuspended in ice cold 100 mM phosphate buffer (pH 7.4) (0.3 ml per g starting material), kept on ice and used within 60 min.

Cyclooxygenase Reaction

[1-^{14}C] 5,8,11,14-Eicosatetraenoic acid was diluted with unlabeled fatty acid to a final specific activity of 2000 cpm/nmol. The fatty acid was converted to the ammonium salt and dissolved in either 4.0 ml 0.1 M Tris-HCl buffer pH 8.0, or in 250 μl of 0.1 M phosphate buffer pH 7.4 when reactions were conducted with lipid-depleted or freshly prepared sheep vesicular gland microsomes, respectively. When lipid-depleted microsomal preparations were used, 300 mg (dry weight) of microsomal powder was homogenized in 4.0 ml ice-cold 0.1 M Tris-HCl buffer, pH 8.0, containing 2 mM *p*-hydroxymercuribenzoate, 2 μM hematin, 0.75% Tween-40 and 10% glycerol. The microsomal suspension was incubated for 3 min at 37 C before transfer of 1.0 ml of the suspension to a reaction vessel containing [1-^{14}C] eicosatetraenoic acid. After an initial incubation for 15 sec at 37 C carried out with constant stirring and oxygenation (30 ml O_2/min), the remaining 3.0 ml of the microsomal suspension was added and the incubation was continued for 30 sec under the same conditions. The final concentration of [1-^{14}C] eicosatetraenoic acid was 625 μM.

When freshly prepared sheep vesicular gland microsomes were used, the experiment was conducted as follows: to 18.0 ml of 0.1 M phosphate buffer, pH 7.4, containing 1 mM *p*-hydroxymercuribenzoate and [1-^{14}C] eicosatetraenoic acid (preequilibrated to 37 C), 0.5 ml of microsomal homogenate (17.4 mg protein/ml) (preequilibrated to 37 C) was added. After an initial 15 sec incubation at 37 C with constant stirring, a second aliquot of 1.5 ml of microsomal homogenate was added and the reaction incubated for an additional 30 sec. The final concentration of [1-^{14}C] eicosatetraenoic acid was 190 μM.

Cyclooxygenase reactions were terminated by pouring the reaction mixture into a separatory funnel containing 100 ml of ethyl acetate/petroleum ether (1:1, by vol) precooled to -40 C followed by the addition of 4.0 ml 1 M citric acid. After vigorous shaking, the phases were allowed to separate and the organic phase was collected. The aqueous phase was reextracted with 100 ml of the ethyl acetate/petroleum ether mixture (-40 C). The combined organic extract was dried over sodium sulfate, filtered, and concentrated on a rotary evaporator under high vacuum. This extraction procedure resulted in an overall isotope recovery of 91.5 ± 9.8% (mean ± SEM, n = 12) with 36.0 ± 16.6% distributed in the aqueous phase and 55.7 ± 7.3% in the organic phase when reactions were conducted without oxygenation. When reactions were conducted with oxygenation, the overall isotope recovery was 90.1 ± 9.1% (mean ± SEM, n = 12) with

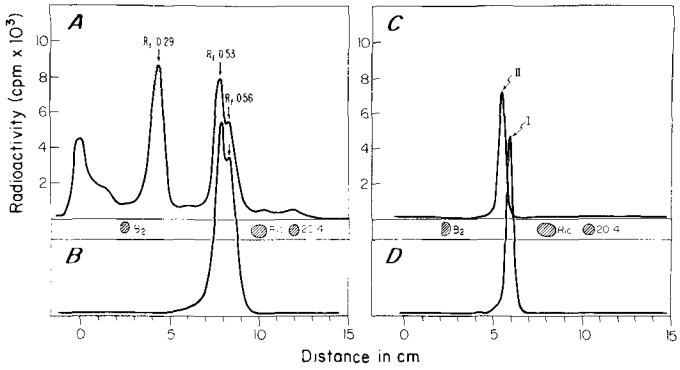

FIG. 1. Thin layer chromatography in solvent A of: (A) reaction products isolated from incubation of [1-^{14}C]eicosatetraenoic acid with lipid-depleted sheep vesicular gland microsomes as described in Methods; (B) the radioactive material isolated by the initial silicic acid column chromatography upon elution with 15% ethyl acetate in petroleum ether; (C) the component eluted from silicic acid with 20% ethyl acetate (component II, fractions 51-80 in Fig. 2) upon rechromatography of the peak eluted with 15% ethyl acetate from the initial silicic acid chromatography; and (D) the component eluted from silicic acid with 10% ethyl acetate (component I, fractions 21-40 in Fig. 2) upon re-chromatography of the material eluted with 15% ethyl acetate from the initial silicic acid column chromatography. Abbreviations used: 20:4 = 5,8,11,14eicosatetraenoic acid; Ric = ricinoleic acid (12-hydroxy-9-octadecenoic acid); B_2 = Prostaglandin B_2.

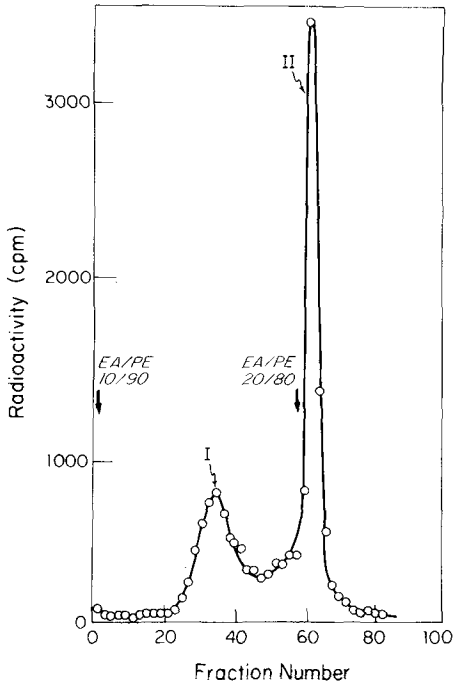

FIG. 2. Rechromatography on silicic acid of the radiolabeled material eluted with 15% ethyl acetate during the initial purification of the reaction products from an incubation of [1-^{14}C]eicosatetraenoic acid with lipid-depleted microsomes. Chromatography (1.5 g silicic acid) was carried out at -20 C with a 1 x 1.5 cm column. Fractions of 5 ml were collected and 50 μl aliquots were removed for liquid scintillation counting. The different proportions of ethyl acetate (EA) to petroleum ether (PE) in the developing solvent are indicated.

16.5 ± 9.5% of the radioactivity distributed in the aqueous phase and 73.5 ± 9.8% in the organic phase.

Vasoactivity and Platelet Aggregation

Canine lateral saphenous vein (LSV) strips were prepared for measurement of changes in contractility as described previously (6) except that the strips were suspended in a 2 ml muscle bath and washed continuously by overflow with Krebs solution containing indomethacin (2.8 μM) and phenylephrine-HCl (0.1-0.4 μM). Washed human platelets were prepared and their aggregation determined as described previously (7).

RESULTS

TLC analysis of the reaction products that were generated by incubating [1-^{14}C]eicosatetraenoic acid with lipid-depleted microsomes in the presence of molecular oxygen (Fig. 1A) showed that 4% of the radioactivity was represented by unreacted fatty acid, 39% by a fraction comprised of two components (R_f 0.56 and 0.53) that migrated below a monohydroxy fatty acid standard (ricinoleic acid), 34% by a component with an R_f value of 0.29 and 23% by more polar components that remained at or near the origin in the chromatographic system used. Initial purification of the products of the reaction mixture was achieved by silicic acid column chromatography (-20 C) as described previously (1), employing ethyl acetate (EA) and purified petroleum ether (PE) as the eluting solvent. Under these

TABLE I

Component I formation from 15-hydroperoxy-9,11-peroxidoprosta-5,13-enoic acid (PGG_2). The complete reaction mixture contained 292 μM [1-^{14}C]PGG_2 (spec. activity = 2,000 cpm/nmole) 7.5 mg (dry weight) lipid depleted microsomes, 1 μM hematin, 0.1 M Tris-HCl, 1mM *p*-hydroxymercuribenzoate, 0.75% Tween-40 and 10% glycerol, pH 8.0. Reactions were carried out at 37 C for 45 sec in a final volume of 0.4 ml. Reaction products were isolated as described in Methods. Component I formation was determined by reduction with triphenylphosphine of component I, PGG_2 and PGH_2 after their isolation from a silicic acid column upon elution with 20% EA in PA

	No. microsomes	Active microsomes	Heat denatured microsomes
Peak I	22.3	22.0	19.2
PGG_2	51.1	4.5	40.2
PGH_2	7.3	45.4	12.3
Other more polar products	19.3	28.1	28.3

conditions, three radioactive components were resolved. Unreacted eicosatetraenoic acid (5%) was eluted with 10% EA, a major radioactive peak (42%) was eluted with 15% EA, and a third component (33%) eluted with 20% EA. The radioactivity remaining on the column (19%) could be recovered upon elution with 100% EA. Very similar results were obtained in ten replicate experiments. Silicic acid column chromatography apparently causes a minimum or no breakdown of the original reaction products identified as indicated by the quantitative recovery of the total radioactivity applied and the qualitative and quantitative correspondence of the silicic acid column-derived components with the original reaction products identified on the basis of their relative migration on TLC. The component from the silicic acid column eluted with 20% EA that corresponded to the component with R_f value of 0.29 was conclusively identified as protaglandin H_2 by procedures previously described (1,2). TLC analysis of the peak eluted with 15% EA showed that it was comprised of two components with R_f values of 0.56 and 0.53 (Fig. 1B). Rechromatography of the components comprising this peak on silicic acid (-20 C) employing stepwise elution with 10 and 20% EA in PE resulted in the resolution of two constituents represented by fractions 21-47 (component I) and 48-80 (component II) (Fig. 2). TLC analysis of components I (Fig. 1D) and II (Fig. 1C) showed single separable substances by radioisotope scanning, or after exposure to I_2 vapor. Both components showed positive reaction with *N,N*-dimethyl-*p*-phenylenediamine indicating the presence of either a hydroperoxy and/or endoperoxy functional group in both substances. Component II was conclusively identified as previously described (1,2) by TLC and GLC-MS analysis as prostaglandin G_2 (PGG_2).

Examination of component I formation with freshly prepared microsomes under conditions similar to those described by Hamberg et al. (2), with the exception of adding microsomes in two stages (see METHODS), showed the formation of 16 ± 3% (mean and range of two experiments) component I and 84 ± 3% PGG_2 in the composite peak from the silicic acid column that was eluted with 15% EA. In identical experiments conducted in the presence of exogenous oxygen, the production of component I increased to 23 ± 3% (n = 2) of the composite peak. When lipid-depleted microsomes were used and the incubation conducted with exogenously supplied oxygen and 1 μM hematin, component I comprised 42 ± 2% (n = 2) and PGG_2 58 ± 2%, respectively, of the products present in the composite peak.

The possibility of nonenzymic formation of component I was examined by determining if this product was generated from [1-^{14}C]eicosatetraenoic acid with heat-inactivated, lipid-depleted microsomes or with microsomal reaction mixtures supplemented with a cyclooxygenase inhibitor, indomethacin (5μM). With either heat-inactivated microsomes or indomethacin-containing reactions, only unreacted [1-^{14}C]eicosatetraenoic acid was detected (not shown). Conducting the cyclooxygenase reaction with lipid-depleted microsomes under standard conditions in the presence of 0.5 mM phenol (a peroxidase cosubstrate, which promotes PGH_2 biosynthesis with minimal accumulation of PGG_2 (8)) resulted in the formation of only PGH_2; there was no detectable formation of the component I (not shown). The apparent lack of component I formation in reaction mixtures containing either indomethacin, phenol, or heat-inactivated microsomes suggested its formation required the accumulation of PGG_2 in the reaction mixture. This possibility was examined by determining if

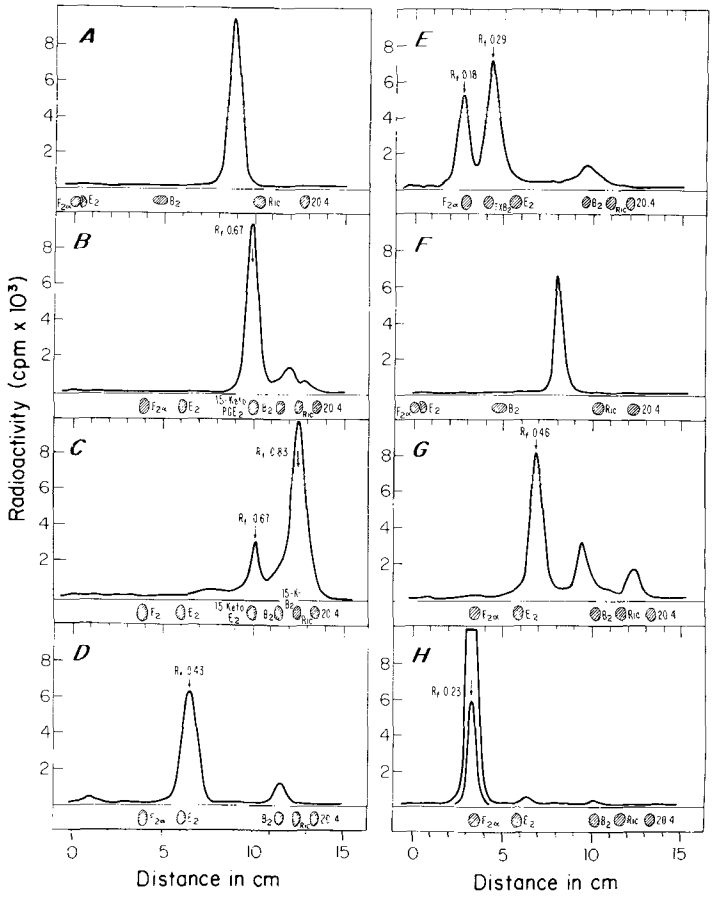

FIG. 3. Thin layer chromatography of component I represented by fractions 21-40 in Fig. 2 and PGG_2 ([component II] represented by fractions 50-80 in Fig. 2) and their respective reaction products formed after various chemical treatments. Thin layer chromatography of: (A) purified component I; (B) component I after 1 hr of reaction in 100 mM phosphate buffer, pH 7.4; (C) base treatment (1N KOH in methanol, 15 min) of the purified reaction product obtained from treatment of component I with phosphate buffer (as in (B)); (D) component I after reduction with triphenylphosphine in diethyl ether or stannous chloride in buffered ethanolic solution; (E) component I after reduction with sodium borohydride in methanol; (F) purified PGG_2 (component II represented by fractions 51-80 in Fig. 2); (G) PGG_2 after 1 hr in 100 mM phosphate buffer, pH 7.4; (H) PGG_2 after reduction with triphenylphosphine in diethyl ether or with stannous chloride in buffered ethanolic solution. Chromatography was carried out with solvent A (panels A and F) or solvent D (panels B,C,D,E,G,H) as described in Methods. Abbreviations used: 15-k-B_2 = 15-keto-prostaglandin B_2, 15-keto-PGE_2 = 15-keto-prostaglandin E_2, E_2 = prostaglandin E_2, $F_{2\alpha}$ = prostaglandin $F_{2\alpha}$; for others see Fig. 1.

component I could be generated from [1-^{14}C]-PGG_2 in reaction mixtures containing catalytically active microsomes, heat-inactivated microsomes or reaction buffer alone with the same hematin concentration (1 μM) used with the microsome-containing reactions. As shown in Table I, incubation of PGG_2 in reaction mixtures containing microsomes, heat-inactivated microsomes, or reaction buffer alone, resulted in the formation of approximately the same quantities of component I (19 to 22%). These observations confirm that PGG_2 probably represents the precursor of component I. The data also established that component I production can be catalyzed nonenzymically from PGG_2 since heat-inactivated microsomes as well as reaction buffer alone generated virtually the same quantities of component I.

Chemical Characterization of Component I

Component I, like PGG_2(2), was found to be labile (T ½ 4 to 5 min) in aqueous solution (100 mM phosphate buffer, pH 7.4, 30 C); however, the major aqueous rearrangement products from these two components were different. The major product resulting from

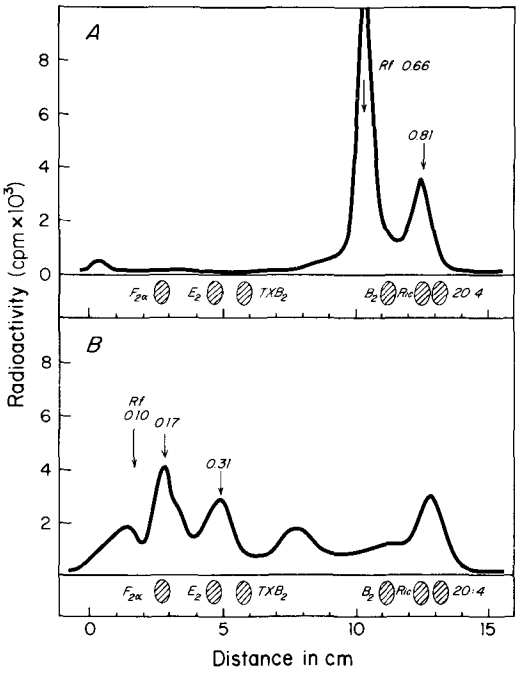

FIG. 4. Thin layer chromatography of: (A) component I after 1 hr of reaction in 100 mM phosphate buffer, pH 7.4, (B) sodium borohydride reaction products obtained from treatment of component I with phosphate buffer (as in (A)). Chromatography was carried out with solvent E as described in Methods. Abbreviations used are as in Fig. 1 and Fig. 3.

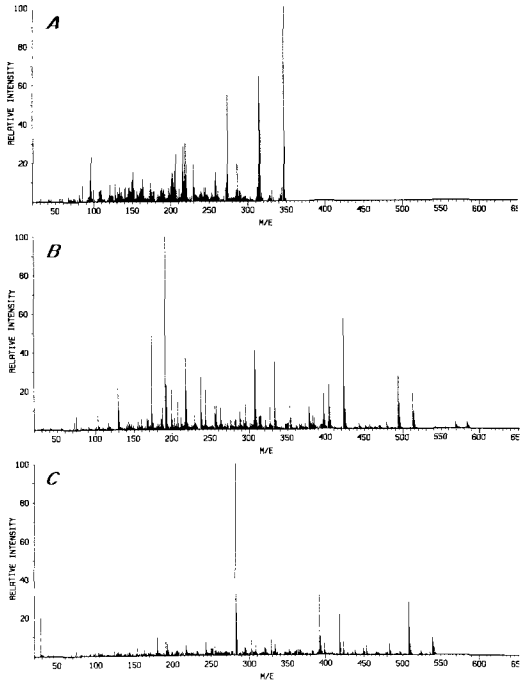

FIG. 5. Mass spectrum of the (A) methyl ester of the base treated buffer rearrangement product of component I (see Fig. 3C); (B) methyl ester trimethylsilyl ether derivatives of component I after sodium borohydride reduction; (G) methyl ester methyloxime trimethylsilyl ether derivative of component I after stannous chloride reduction.

buffer rearrangement of component I (77%) co-migrated with an authentic standard of 15-keto-PGE$_2$ (R_f 0.67, Fig. 3B) and showed UV absorbtivity. In constrast, the major buffer rearrangement product of PGG$_2$ exhibited an R_f value of 0.46 (Fig. 3G). This product was conclusively identified as 15-OOH-PGE$_2$ as previously described (1,2). Base treatment of the buffer rearrangement product of component I (in 0.2 ml 1N KOH in methanol for 15 min) resulted in the formation of a UV-absorbing, less polar product (Fig. 3C) with an R_f value of 0.83 which is identical to the product obtained from base treatment of authentic 15-keto-PGE$_2$ (e.g., 15-keto-PGB$_2$). GLC analysis of the methyl ester of the base-treated, buffer rearrangement product of component I showed a peak with an equivalent chain length (ECL) value of 24.32, which was identical to that of the methyl ester of a standard of 15-keto-PGE$_2$ that underwent base treatment (Me-15-keto-PGB$_2$). The mass spectra of the methyl esters of these two components were found to be identical showing a molecular ion (M) at m/e 346 (Fig. 5A) and ions at m/e 331 (M-15, loss of $\cdot CH_3$), 315 (M-31, loss of $\cdot OCH_3$), 314 (M-32, loss of CH_3OH), 286 (M-60, loss of CH_3COOH), 273 (M-73, loss of $\cdot CH_2$-$COOCH_3$), 258 (M-60 + 28), loss of CH_3COOH plus CO), 229 (M-(60 + 57), loss of CH_3COOH plus $\cdot(CH_2)_3CH_3$), 206, 205 (M-141, loss of $\cdot CH_2$-CH=CH-$(CH_2)_3$ -$COOCH_3$).

When the buffer rearrangement product of component I was reduced with either triphenylphosphine in diethyl ether or with stannous chloride in methanol, no further modification of this component was observed (not shown); this indicates that hydroperoxy or endoperoxy functional groups are absent in the rearrangement product. In contrast, 15-OOH-PGE$_2$, the major buffer rearrangement product of PGG$_2$ is further reduced with either triphenylphosphine or with stannous chloride (1,2) to PGE$_2$.

Direct reduction of component I with either triphenylphosphine in diethyl ether or with stannous chloride in buffered ethanolic solution led to the formation of a major product (ca. 80%) that migrated slightly ahead of PGE$_2$ in

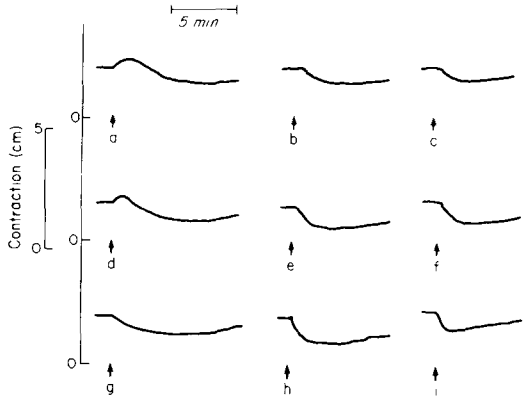

FIG. 6. Isotonic responses of canine lateral saphenous vein to prostaglandins. The vein strips were continually washed by overflow (1 ml/min) in a 2 ml bath. The Krebs solution contained phenylephrine-HCl (0.1-0.4 μM) and indomethacin (2.8 μM) which submaximally contracted the strips from their passive length (0). The prostaglandins were introduced (arrows) while momentarily stopping the wash (15 sec). Representative responses of vein strips from 3 separate dogs (top, middle and bottom panels) are shown. (A) 2 nmol 15-keto-PGG_2; (B) 0.5 nmol buffer rearranged 15-keto-PGG_2; (C) 0.6 pmol PGE_2; (D) 40 pmol PGG_2; (E) 5 pmol buffer rearranged PGG_2; (F) 1.2 pmol PGE_2; (G) 2 nmol 15-keto-PGE_2; (H) 1 nmol buffer rearranged 15-keto-PGG_2; and (I) 1.4 pmol PGE_2.

solvent system C (not shown) or in solvent system D (R_f 0.43) (Fig. 3D). On the other hand, treatment of PGG_2 with either triphenylphosphine or with stannous chloride resulted in the formation of $PGF_{2\alpha}$ (R_f 0.23) as shown in Fig. 3H. Reduction of component I with sodium borohydride in methanol resulted in the formation of approximately equal quantities of two products; one migrated between PGE_2 and thromboxane B_2 (R_f 0.29) and the other comigrated with $PGF_{2\alpha}$ (R_f 0.18, Fig. 3E). Purification of the sodium borohydride reduction products by silicic acid column chromatography (components R_f 0.29 and R_f 0.18, Fig. 3E) and derivatization of the isolated components into their respective methyl ester trimethyl silyl ethers (Me-OTMSi) showed peaks with ECL values of 24.23 (component with R_f 0.29, Fig. 3E) and 24.12 (component with R_f 0.18, Fig. 3E), respectively. GLC-MS analysis of these respective Me-OTMSi derivatives showed an identical cleavage pattern upon ion impact with minor differences in the relative intensities of the ions formed. The mass spectra showed a molecular ion (M) at m/e 584 (Fig. 5B) and a major ion of m/e 191 ((TMSiO-=CH-OTMSi) +). Less intense ions were present at m/e 569 (M-15, loss of $\cdot CH_3$); 513 (M-71, loss of $\cdot(CH_2)_4CH_3$); 494 (M-90, loss of TMSiOH); 404 (M-(2 x 90)); 397, 333 (M-(2 x 90 + 71)); 307, 217 [TMSiO-CH=CH-CH=OTMSi$^+$)$^+$] and 173 ((CH(=OTMSi)$(CH_2)_4CH_3)^+$). The mass spectrum obtained conclusively identifies the sodium borohydride reaction products generated from component I as 9,11,15 trihydroxyprosta-5,13-dienoic acid stereo isomers. On the basis of the TLC migration of the sodium borohydride reduction products of component I and their respective ECL values determined upon derivatization and GLC analysis compared to those reported for $PGF_{2\alpha}$, $PGF_{2\beta}$ (9,10) and 15-epi $PGF_{2\alpha}$ (11), the product with R_f of 0.18 and ECL-value of 24.12 most probably represents 9α,11α,15(S) prosta-5,13-dienoic acid ($PGF_{2\alpha}$) and that with Rf of 0.29 and EC1 of 24.23 represents 9α,11α, 15(R) prosta-5,13-dienoic acid (15-epi-$PGF_{2\alpha}$). Sodium borohydride reduction of the products of component I obtained from buffer rearrangement resulted in the formation of several products (Fig. 4). Although none of the products was further characterized, the components with Rf values of 0.10, 0.17 and 0.30 were tentatively identified as $PGF_{2\beta}$, a mixture of $PGF_{2\alpha}$ plus 15-epi-$PGF_{2\beta}$, and 15 epi-PGF_2 α, respectively. These assignments are based on (a) the TLC migration observed for the products generated compared to those reported by others (9-11) using a similar eluting solvent and (b) the relative radioactivity ratio of 1.0:1.7:1.1 determined for the components with Rf values of 0.10, 0.17 and 0.30 as compared to the expected theoretical ratio of 1:2:1 assuming that $PGF_{2\alpha}$ and 15-epi-$PGF_{2\beta}$ comigrate, which is anticipated upon reduction of 15-keto-PGE_2.

GLC analysis of the methyl ester methyloxime trimethylsilyl ether (Me-MeO-TMSi) derivative of the purified stannous chloride reduction product of component I showed a peak with an ECL-value of 24.75. The mass spectrum of the Me-MeO-TMSi derivative showed a molecular ion (M) at m/e 539 (Fig. 3C) and ions at m/e 524 (M-15, loss of $\cdot CH_3$), 508 (M-31, loss of $\cdot OCH_3$), 483 (M-56, loss of CH_2=CH-CH_2-CH_3), 418 (M-(90 + 31)), loss of TMSiOH plus $\cdot OCH_3$), 398 (M-141, loss of $\cdot$CH-CH=CH-$(CH_2)_3COOCH_3$), 392 (M-(90 + 57), loss of TMSiOH plus $\cdot(CH_2)_3CH_3$), 328 (M-(2 x 90 + 31), 302 (M-(2x 90 + 57)), 282 ((TMSiO=CH-CH=CH-C=(N-OCH_3)-$(CH_2)_4$-CH_3^+). The mass spectrum conclusively identifies the stannous chloride reduction product of component I as 15-keto-9,11-dihydroxyprosta 5,13-dienoic acid (15-keto-$PGF_{2\alpha}$).

Biological Activity

The biological activities of component I were compared to those of PGG_2 on canine lateral saphenous vein (LSV) strips and washed human platelets. Component I, added to the muscle bath within 15 sec after mixing with Krebs solution, elicited a biphasic response of vein strips (Fig. 6A) qualitatively similar to that observed with PGG_2 (Fig. 6D). The response observed with both component I and PGG_2 was an immediate contraction which rapidly proceeded into a prolonged relaxation; this was distinguishable from the response to PGE_2 which produced relaxation only (Fig. 6C, F, I). Qualitatively and quantitatively similar activity of component I was observed in eight experiments on LSV strips from separate animals and employing three different preparations representative of component I. Following incubation (37 C 30 min) of component I or PGG_2 in Krebs solution, the rearrangement products of both substances failed to produce contraction, but elicited a prolonged relaxation (Fig. 6B, E, H) similar to that produced by PGE_2(Fig. 6C, F, I). Although the vasoactivity of component I, PGG_2 and their aqueous rearrangement products were qualitatively similar, PGG_2 and its aqueous rearrangement products appeared to be ca. 50 to 100 times more potent than component I and its derived products under the conditions employed. Authentic 15-keto-PGE_2 consistently appeared less potent with respect to the onset and extent of relaxation (Fig. 6G, H) than the product(s) resulting from rearrangement of component I in Krebs solution. The difference in potency could conceivably result from potentiating effects of minor amounts of nonrearranged material of component I and/or other side products which remain even after 1 hr of buffer rearrangement.

Component I was clearly different from PGG_2 as an effector of human platelet function since it (0.1 to 1.0 μM) failed to mimic, promote or prevent eicosatetraenoic acid (3.3 μM) or PGG_2-induced (0.1 μM) platelet aggregation.

DISCUSSION

In this report, evidence is presented that sheep vesicular gland microsomes in the presence of hematin generate a labile oxygenated product from 5,8,11,14-eicosatetraenoic acid in addition to the previously described cyclooxygenase products, $PGG_2$and PGH_2 (2). The newly isolated prostaglandin was conclusively identified as 15-keto-9,11-peroxidoprosta-5,13-dienoic acid (15-keto-PGG_2). This structure assignment was based on several lines of evidence which are outlined below and in Figure

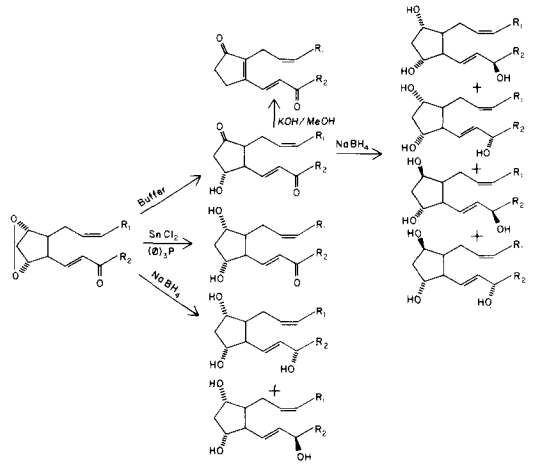

FIG. 7. Reactions performed on component I (fractions 21-40 in (Fig. 2), (15-keto-PGG_2). R_1 = $(CH_2)_3$-COOH; R_2 = $(CH_2)_4$-CH_3.

7. Reduction of 15-keto-PGG_2 with sodium borohydride resulted in the formation of $PGF_{2\alpha}$ and 15-epi-$PGF_{2\alpha}$ (Fig. 3E and 4B). These results do not permit distinction among keto, hydroperoxy and endoperoxy functional groups in the parent molecule. However, evidence for the presence of a C_9-C_{11} endoperoxy-bridge was obtained by treatment of 15-keto-PGG_2 with a mild reducing agent (i.e., stannous chloride or triphenylphosphine) (2) which reduced the 9,11-endoperoxy bridge to the 9,11-diol without altering the 15-keto group; this yielded the product 15-keto-$PGF_{2\alpha}$ (Fig. 3D, 5C). Exposure of 15-keto-PGG_2 to aqueous medium yielded a product that was chromatographically identical to an authentic standard of 15-keto-PGE_2 (Fig. 3B), which indicates an isomerization of the 9,11-endoperoxide functional group (2) in the parent compound. Further evidence for the formation of 15-keto-PGE_2 after aqueous isomerization of 15-keto-PGG_2 was provided by demonstrating that the aqueous isomerization product of both 15-keto-PGG_2 and an authentic standard of 15-keto-PGE_2 underwent base-catalyzed water elimination (12) to yield identical products identified chromatographically (Fig. 3C) as well as by GLC-MS analysis (Fig. 5A) as 15-keto-PGB_2. The similar half-lives of 4 to 5 min determined for 15-keto-PGG_2, PGG_2 and PGH_2 in aqueous medium is also consistent with the presence of an endoperoxy functional group on the newly discovered component.

Although 15-keto-PGG_2 was detectable as a product generated from eicosatetraenoic acid by sheep vesicular microsomes under incuba-

tion conditions approximating those employed by Hamberg et al. (2), it represented a relatively minor constituent (i.e. 4.5% of the total products formed under those conditions). In the present investigation in which reactions were conducted in the presence of hematin with a constant supply of exogenous O_2 and with lipid depleted microsomes, 15-keto-PGG_2 constituted 16% of the total products formed. A more detailed examination of 15-keto-PGG_2 production revealed that its generation occurred nonenzymically from PGG_2 presumably by a hematin-catalyzed reaction. This was indicated by the observation that reaction buffer containing 1 μM hematin, but no microsomes, or reaction buffer containing heat-inactivated microsomes promoted formation of equal quantities of 15-keto-PGG_2 (19-22%) from PGG_2, whereas incubation of PGG_2 in aqueous medium (phosphate buffer, pH 7.4) has been shown (1,2) to lead to the formation of 15-OOH-PGE_2.

Hamberg (13) reported a rapid decomposition of 13-hydroperoxi-9,11-octadecadienoic acid in phosphate buffer at 37 C in the presence of 3 to 80 mM hemoglobin over a 5 min incubation period. Under these conditions, over 90% of the starting material was converted to five identifiable products of which 11% was represented by 13-keto-9,11-octadecadienoic acid. Similar results were reported by Gardner et al. (14) using an isomeric mixture of linoleic acid hydroperoxide with an Fe(III)-cysteine couple. Miyamoto et al. (15), however, did not report any generation of 15-keto-PGG_1 in reaction mixtures containing purified cyclooxygenase, 8,11,14-eicosatrienoic acid and hematin. Under these conditions, we observed a relatively substantial production of 15-keto-PGG_2 in reaction mixtures supplemented with eicosatetraenoic acid, sheep vesicular microsomes, and hematin.

Numerous reports have appeared showing that 15-keto-analogs are less potent than their hydroxy-containing counterparts especially with respect to vasodilator activity (16). Our results comparing the vasoactivity of PGG_2 and 15-keto-PGG_2 on dog LSV preparations extend these findings with respect to hydroperoxy vs. keto functions on prostaglandin endoperoxides. The 15-keto substitution of the hydroperoxy function on PGG_2 diminishes the biological activity of the prostaglandin endoperoxide by ca. 50-fold. These strikingly different potencies of PGG_2 and 15-keto-PGG_2 provide a means of distinguishing between these two prostaglandin endoperoxides which are isolated only with relative difficulty. These findings underscore the importance of rigorous purification and identification of prostaglandin products generated in cyclooxygenase reaction mixtures, especially when they are supplemented with hematin.

ACKNOWLEDGMENTS

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Apoproteins in Association with Intralipid Incubations in Rat and Human Plasma

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ABSTRACT

Intralipid was incubated with rat and human plasma and examined for changes in lipid and apoprotein composition. Upon incubation in rat plasma, Intralipid acquired an apoprotein complement similar to that found in chylomicrons following plasma incubation or in chylomicrons after alimentary lipemia. Since the apoproteins of lipoproteins probably govern their metabolism, these results suggest that Intralipid and chylomicrons undergo similar metabolic fates. This pattern is characterized by a predominance of Apo E (the arginine-rich apoprotein) and Apo C. Incubation of Intralipid with human plasma showed the uptake of Apo A-I and Apo A-IV as well. Density fractionation of the plasma into separate lipoprotein classes facilitated identification of high density lipoprotein as the major apoprotein donor to the Intralipid. When rat lipoprotein-free plasma ($\delta > 1.21$) was incubated with Intralipid, a different apoprotein pattern appeared in the particles of $S_f > 400$ depending on whether the entire Intralipid preparation or only the $S_f > 400$ fraction alone was incubated. The difference consisted of a virtual total absence of the arginine-rich protein on the $S_f > 400$ particles in whole Intralipid incubations. Density fractionation of the $S_f < 400$ particles of Intralipid and recombination of these fractions with the $S_f > 400$ fraction before incubation revealed the major inhibitory fraction to be $\delta < 1.006$ (S_f 20-400).

INTRODUCTION

Ten percent Intralipid, an emulsion of soybean oil in egg phospholipid, has been frequently used as a calorie source in human total parenteral alimentation. The rationale has been to provide a nutritional source resembling chylomicrons, both in physical characteristics and behavior. Though the kinetic behavior of the infused Intralipid has been described (1), and intravenous fat tolerance tests have been developed (2), little is known about the changes that occur upon initial exposure to plasma.

The purpose of this study is to examine the analogy between Intralipid and chylomicrons. It is known that chylomicrons, when transported to plasma from lymph, undergo a major change in apoproteins to a characteristic apoprotein pattern (3). Since these apoproteins probably dictate the subsequent metabolic fate of the chylomicrons, it becomes important as well to document the apoprotein changes in Intralipid upon its entering plasma. This study finds that after plasma incubation, Intralipid acquires an apoprotein complement similar to that of chylomicrons after initial plasma exposure. The transfer of apoproteins onto Intralipid or chylomicrons does not depend on any previous complement of apoproteins. The results also suggest that Intralipid and chylomicrons subsequently undergo similar metabolic fates.

PROCEDURES

Preparation of Intralipid

The soybean oil emulsion, Intralipid (Cutter Laboratories, Berkeley, CA), was used for the incubations in the experiment. In addition to the unspun 10% emulsion, the Intralipid was divided into two fractions by ultracentrifugation (Beckman Spinco, Model LB-65, Palo Alto, CA) at 6 x 10^5 g/min. Under these conditions, most of the emulsion was concentrated in a semisolid layer at the top of the centrifuge tube, and was easily separated from the remaining suspension. This top fraction was twice resuspended in 0.15 M NaCl and 0.001 M EDTA and recentrifuged at 6 x 10^5 g/min before storage at 4 C until use. The particles in the top fraction had a flotation greater than S_f 400, analogous to chylomicrons isolated in a similar manner. The bottom fraction of the initial centrifugation was also respun twice to eliminate all particles of flotation greater than S_f 400.

The Intralipid infranate was further separated into four density fractions by ultracentrifugal flotation: $\delta > 1.006$, $\delta < 1.006$, δ 1.006-1.063, and $\delta > 1.063$. This was accomplished by adjusting the density with KBr and centrifugation according to a standard procedure (4).

Preparation of Serum and Plasma Density Fractions

After an overnight fast, adult male Sprague-Dawley rats were exsanguinated with 0.4 M EDTA (0.01 ml:10 ml blood) used as the anticoagulant. The cells were separated by low-speed centrifugation (Sorvall RC centrifuge, Norwalk, CT; 5000 rpm for 15 min), and the resulting plasma was spun at 6 x 10^7 g/min to separate the lipoproteins of flotation greater

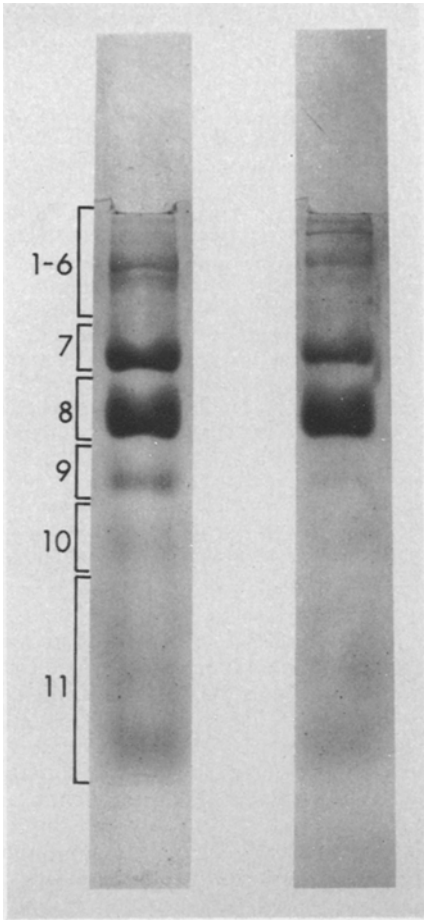

FIG. 1. SDS-polyacrylamide gel electrophoresis of apoproteins associated with Intralipid (left) and with chylomicrons (right) after plasma incubation. Band 7 is Apo A-66-IV; Band 8, ARP; Band 9, Apo A-I; and Band 11, Apo C.

than S_f 100, which were discarded. The infranate fractions with lipoproteins less than S_f 100 were pooled for use in the experiments.

Whole blood, with EDTA used as the anticoagulant, was also obtained from a normolipemic human donor according to guidelines approved by the Duke University Medical Center Committee for clinical investigations. This human blood was handled similarly to the rat blood. Portions of both the rat and human plasma were further separated by ultracentrifugation flotation into three density fractions. The rat plasma was separated into $\delta<1.070$ containing primarily very low density lipoprotein (VLDL) and low density lipoprotein (LDL); δ1.070-1.21 containing pure high density lipoprotein (HDL), and $\delta>1.21$ or lipoprotein-free plasma. The human plasma was separated into corresponding fractions of $\delta<1.063$, δ1.063-1.21, and $\delta>1.21$. The relative purity of the respective density fractions was assessed by lipoprotein agar gel electrophoresis.

Preparation of Chylomicrons

Mesenteric lymphatic chylomicrons were harvested from adult male Sprague-Dawley rats after intraduodenal administration of corn oil. The mesenteric lymphatic had been cannulated as previously described (3), and the tip of the lymphatic catheter placed under 1 ml of a solution of 0.4 M EDTA and 0.15 M NaCl at the bottom of a glass centrifuge tube. This tube was in ice for the duration of the collection, and the lymph was stored at 4 C until further use.

Incubation

Aliquots of the 10% Intralipid emulsion and of the top fraction ($S_f<400$) of the spun Intralipid were incubated with both the rat and human plasma density fractions in a Gyrotary Water Bath Shaker, Model G76 (New Brunswick Scientific, New Brunswick, NJ) at 37 C for one hour. The triglyceride concentrations of the incubation mixtures varied from 1500 to 3000 mg per dl. The top fraction ($S_f>400$) of the spun Intralipid was also recombined with the bottom fraction ($S_f<400$) and with the various Intralipid density fractions for incubation with rat lipoprotein-free plasma ($\delta>1.21$). In addition, incubations of chylous lymph with plasma were done at similar triglyceride concentrations.

After incubation, the mixtures were spun to reisolate only the lipoproteins of $S_f<400$ (6×10^5 g/min), and then these were washed twice by resuspension and recentrifugation as in the initial preparation procedures. The reisolated emulsions were then suspended in 5 ml of distilled water, and an aliquot was removed for quantitative total protein determination. The remaining suspension was lyophilized and subsequently delipidated at 4 C with two washes of ethanol/diethyl ether (3:1 v/v). The protein residue was dried under nitrogen and stored at 4 C prior to analysis.

Polyacrylamide Electrophoresis

The Intralipid-associated proteins were analyzed by polyacrylamide electrophoresis according to the procedure of Weber and Osborne (5), using the apparatus of Hoefer Co. (San Francisco, CA). Gels of 10% acrylamide were prepared in a 0.05 M phosphate buffer, pH 7.4, and 0.2% sodium dodecyl sulfate. The gels were stained with Amido Black (Millipore

TABLE I

Lipid Changes after Incubation of Intralipid and Whole Plasma and Plasma Lipoprotein Fractions for One Hour at 37 C

Intralipid incubation (number)	Triglyceride (mg) (% ± SE)[a]	Phospholipid (mg) (% ± SE)[a]	Total cholesterol (mg) (% ± SE)[a]	Protein (mg) (% ± SE)[a]
Saline (4)	148.6	6.23	0.427	0.117
	(95.68 ± 0.17)	(4.04 ± 0.15)	(0.28 ± 0.015)	(0.06)
Rat plasma				
Plasma (3)	115.7	3.22	0.473	0.544
	(96.93 ± 0.26)[b]	(2.70 ± 0.24)[c]	(0.36 ± 0.02)[b]	(0.70)
δ<1.070 (1)	195.8	6.63	0.640	0.117
	(96.42)	(3.27)	(0.32)	(0.06)
δ1.070-1.21 (3)	164.8	4.48	0.612	0.867
	(97.07 ± 0.17)[c]	(2.55 ± 0.21)[c]	(0.38 ± 0.08) NS	(0.69)
δ>1.21 (4)	108.6	3.50	0.340	0.398
	(96.70 ± 0.17)[c]	(3.01 ± 0.16)[c]	(0.30 ± 0.01) NS	(0.46)
Human plasma				
Plasma (3)	132.3	3.32	0.614	0.498
	(96.37 ± 0.11)[b]	(3.18 ± 0.13)[c]	(0.45 ± 0.02)[d]	(0.56)
δ<1.063 (1)	65.6	2.73	0.309	0.076
	(95.57)	(3.89)	(0.45)	(0.11)
δ1.063-1.21 (3)	93.8	3.12	0.342	0.570
	(96.44 ± 0.125)[b]	(3.21 ± 0.14)[b]	(0.35 ± 0.01)[b]	(0.58)
δ>1.21 (2)	89.5	3.00	0.306	0.390
	(96.43 ± 0.01)[b]	(3.24 ± 0.01)[c]	(0.33 ± 0.01)[b]	(0.39)

[a]Percentage of total lipid by weight ± standard error.
[b]$p<0.05$.
[c]$p<0.01$.
[d]$p<0.001$.

Biomedica, Acton, MA) in 5% acetic acid. Destaining was accomplished with a solution containing 7.5% acetic acid and 5% methanol in water. The relative densities of the different bands were plotted by use of a Gilford linear scanner (Gilford Instrument Laboratories, Oberlin, OH) attached to a Beckman DU spectrophotometer, and the areas under the curves were quantitated by planimetry. With the use of known increments of particular apoproteins, linearity of chromogenicity was established over the mass ranges used in these electrophoreses. Each apoprotein band was assigned a value depending on its fractional area as determined by planimetry. This value was normalized to the amount of Intralipid associated protein per 100 mg of triglyceride. This calculation, while not defining the absolute apoprotein content because of their differing chromogenicities, does allow assessment of the relative magnitudes of similar apoproteins among the different incubations.

Methods of Assay

Triglyceride determinations on the Intralipid ethanol/ether extracts were obtained by an automatic fluorometric technique (6). Total cholesterol was assayed by the method of Abell et al. (7), and unesterified cholesterol by the method of Schoenheimer and Sperry (8). Phospholipid determinations were performed on the Intralipid extracts by the method of Ames and Dubin (9). Protein was determined by the Lowry method (10), incorporating successive ether extractions of the samples and standards. Significance of the lipid changes between plasma- and saline-incubated preparations was obtained by Student's t-test.

RESULTS

The apoprotein and lipid alteration upon plasma exposure have been defined in chylomicrons (3). For comparison of the apoprotein changes of incubated chylomicrons and Intralipid, whole chylous lymph was incubated in rat plasma in a similar manner as the 10% Intralipid incubations. The results, shown in Figure 1, demonstrate that the apoprotein changes seen in chylomicrons upon incubation in rat plasma are substantially reflected in the plasma-incubated Intralipid particle. Subsequently it was determined that the Intralipid undergoes changes in lipid composition similar to chylomicrons.

Incubations of Intralipid were made with both rat and human plasma along with the plasma lipoprotein fractions. When the composition of the emulsion was expressed on the basis of weight percent (Table I), an appreciable

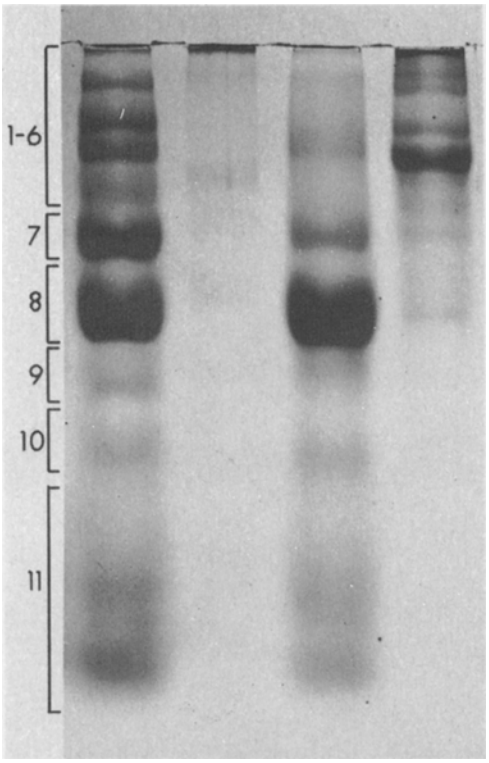

FIG. 2. SDS-polyacrylamide gel electrophoresis of apoproteins associated with Intralipid after incubation with rat plasma and plasma density fractions. From left to right: incubation with whole plasma, incubation with plasma $\delta<1.070$, incubation with plasma $1.070<\delta<1.21$, and incubation with plasma $\delta>1.21$. The Bands and their corresponding apoproteins are as in Figure 1.

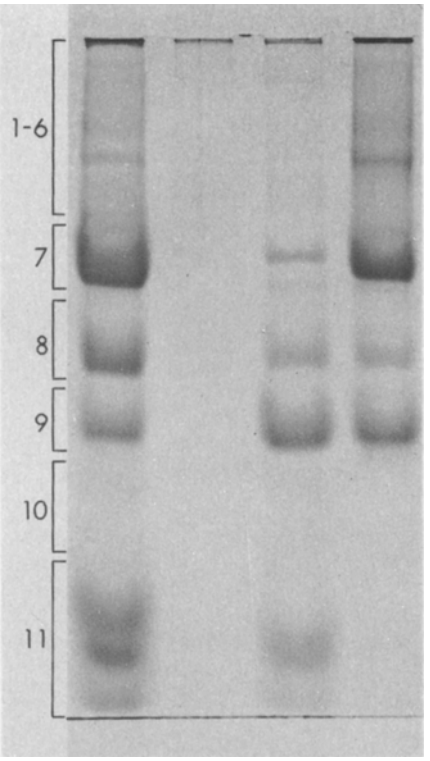

FIG. 3. SDS-polyacrylamide gel electrophoresis of apoproteins associated with Intralipid after incubation with human plasma and plasma density fractions. From left to right: incubation with whole plasma, incubation with plasma $\delta<1.063$, incubation with plasma $1.063<\delta<1.21$, and incubation with plasma $\delta>1.21$. The Bands and their corresponding apoproteins are as in Figure 1.

increase in the relative protein content of the emulsion was observed for both plasmas.

Significant increases in cholesterol and decreases in phospholipid contents were also noted in both. Cholesterol increments were uniformly somewhat higher in human plasma. The relative increment in triglyceride percentage is obviously the result of the substantial phospholipid loss. The HDL of both human (δ 1.063 <1.21) and rat (δ 1.070 <1.21) plasma reproduced the whole plasma effect on the emulsion. The fractions $\delta<1.21$ of both plasmas produced similar changes to the HDL fractions but of somewhat lesser magnitudes. Not enough incubations with the VLDL-LDL fractions were done to assess the effect in the lipid changes.

The Intralipid-associated apoproteins were analyzed by SDS-polyacrylamide gel electrophoresis and are shown in Figures 2 and 3 for incubations with rat and human plasma fractions, respectively. In Figure 2, the major Intralipid-associated proteins appear to be Band 8, which cochromatographs with arginine-rich protein (ARP), Band 11 or the Apo C peptides, and Band 7 or Apo A-IV. These apoproteins were transferred quantitatively by incubations with rat HDL (Table II), and not with incubations with density fractions $\delta<1.070$ or $\delta>1.21$ (lipoprotein-free plasma). Indeed, incubation with $\delta<1.070$ results in hardly any apoprotein transfer, while only large molecular weight proteins are transferred on incubations with rat lipoprotein free plasma. In Figure 3, showing incubations with human density fractions, the major Intralipid-associated proteins appear to be Band 7 (Apo A-IV), Band 8 (ARP), Band 9 (Apo A-I), and Band 11 (Apo C). As in the rat incubations, incubation with density fraction $\delta<1.063$ results in no transfer of apoproteins. The Apo C, Apo A-I, and ARP are transferred by HDL incubations, while incubations with lipoprotein-free plasma result in transfer of Apo

TABLE II

Intralipid Apoprotein Changes during Incubation with Whole Plasma and Plasma Density Fractions for One Hour at 37 C

Intralipid incubation	Apoprotein distribution (densitometry units/100 μg recovered triglyceride)				
	Band 7 (%)[a]	Band 8 (%)[a]	Band 9 (%)[a]	Band 11 (%)[a]	Total
Rat plasma					
In plasma	97.4 (17.1)	193.7 (34.0)	34.8 (6.1)	190.3 (33.4)	569.8
In $\delta<1.070$	---	---	---	---	59.8
In $1.070<\delta<1.21$	51.0 (7.2)	344.7 (48.7)	1.4 (0.2)	201.0 (28.4)	707.8
In $\delta>1.21$	---	7.1 (3.6)	---	13.0 (6.6)	196.2
Human plasma					
In plasma	211.2 (29.6)	13.27 (18.6)	85.6 (12.0)	250.5 (35.1)	713.6
In $\delta<1.063$	---	---	---	---	115.8
In $1.063<\delta<1.21$	8.9 (1.5)	87.0 (14.6)	262.9 (44.1)	197.9 (32.2)	596.2
In $\delta>1.21$	200.7 (49.2)	40.8 (10.0)	129.7 (31.8)	---	407.9

[a]Percentages of total apoprotein density.

A-IV and higher molecular weight proteins, as well as ARP and Apo A-I.

The apoproteins from incubations of rat lipoprotein-free plasma ($\delta>1.21$) with whole 10% Intralipid and the Intralipid top fraction ($S_f>400$) are quite different (Fig. 4). The incubation with the top fraction alone shows substantial pickup of ARP (Band 8), and modest transfers of Apo A-I and Apo A-IV, in contrast to incubation with whole Intralipid in which these apoproteins are virtually absent. Experiments in which the top ($S_f>400$) and bottom ($S_f<400$) Intralipid fractions were recombined before incubation with lipoprotein-free rat plasma ($\delta>1.21$) showed an apoprotein pattern similar to that using the whole 10% Intralipid. Incubations with the top fraction of Intralipid with the other density fractions of both rat and human plasma showed no difference in apoprotein pattern from incubations with whole 10% Intralipid.

Further investigation was made into the nature of the Intralipid bottom fraction ($S_f<400$) which appeared to inhibit uptake of ARP and Apo A-I from lipoprotein-free rat plasma onto the $S_f>400$ Intralipid fraction when incubations with unspun 10% Intralipid were run. This was done by dividing the bottom fraction into density fractions ($\delta<1.006$, $\delta>1.006$, δ 1.006-1.063, $\delta>1.063$), and recombining each fraction prior to incubation with the top fraction ($S_f>400$). The $\delta>1.063$ fraction was found to be virtually lipid-free. (Recombining in this manner had no effect on the incubations with human plasma or plasma density fractions.) A marked difference in the uptake of apoproteins resembling whole Intralipid could be seen in incubations using the $S_f>400$ fraction recombined with the $\delta<1.006$ fraction. Such changes were not so evident in recombinations using the $\delta>1.006$ fractions, and these incubations resembled more closely

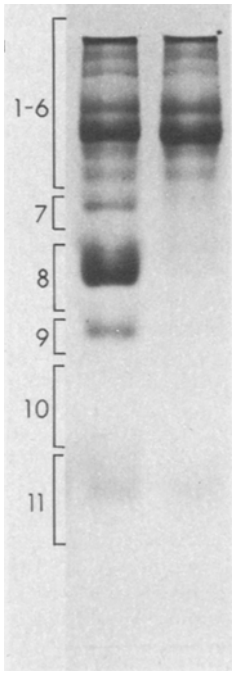

FIG. 4. SDS-polyacrylamide gel electrophoresis of apoproteins associated with Intralipid after incubation with rat plasma density fraction $\delta>1.21$. On the left are apoproteins from an incubation of the $S_f>400$ fraction of Intralipid with the lipoprotein-free plasma. On the right, whole Intralipid (unspun) was incubated with the lipoprotein-free plasma. The Bands and their corresponding apoproteins are as in Figure 1.

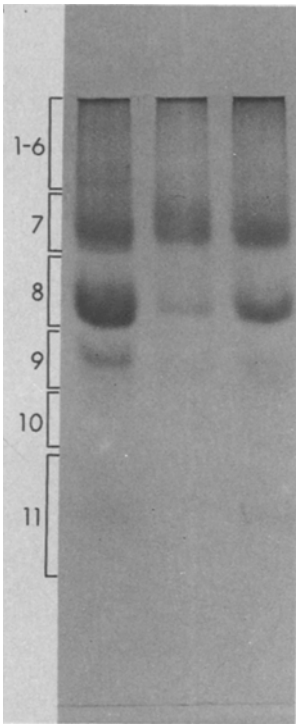

FIG. 5. SDS-polyacrylamide gel electrophoresis of apoproteins associated with Intralipid after incubation with rat plasma density fraction $\delta>1.21$. From left to right: incubation with Intralipid $S_f>400$ fraction only; incubation with Intralipid $S_f>400$ fraction plus Intralipid density fraction $\delta<1.006$; incubation with Intralipid $S_f>400$ fraction plus Intralipid density fraction $\delta>1.006$. The Bands and their corresponding apoproteins are as in Figure 1.

the incubations with the top fraction alone (Fig. 5). No effect was obtained by incubating with the top fraction recombined with the δ 1.006-1.063 or the $\delta>1.063$ fractions.

DISCUSSION

Chylomicrons and Intralipid share many functional similarities. Both consist largely of triglyceride, stabilized by a surface coat of a similar phospholipid, phosphatidylcholine. Both serve to deliver calories to body tissues in the form of triglycerides. Both have identical kinetics for their plasma elimination in dog and man (11), and both share similar kinetic behavior as substrates in vitro for post-heparin lipoprotein lipase (12). Since chylomicrons have been shown to undergo lipid and protein alterations upon in vitro and in vivo exposure to plasma (3), it would not be surprising, therefore, to see Intralipid undergoing many of the same changes. The above in vitro incubations in rat plasma have shown a similar complement of apoproteins in both Intralipid and chylomicrons. Since the apoproteins probably dictate the metabolic fate of the lipoproteins, these findings suggest that the metabolic sequences of the Intralipid and chylomicrons would be fairly similar.

The uptake of proteins onto artificial emulsions has been known for some time. Scanu and Page (13) incubated chylomicron-free human serum and an emulsion of coconut oil and found after separation that 1.5-1.8% by weight protein remained associated with the emulsion. Iacono et al. (14) infused Lipomul I.V., a cottonseed oil emulsion, into human volunteers and recovered particles with 0.8-0.9% associated protein. The incubations described in this paper have demonstrated as well a consistent and reproducible association of plasma proteins with the soybean oil emulsion, Intralipid. Moreover, the apoprotein pattern of the plasma-exposed Intralipid was similar to that seen in incubated chylomicrons and in chylomicrons after alimentary lipemia (3). Further, Intralipid incubation in HDL yields an apoprotein pattern similar to that seen in whole plasma incubations.

Elucidation of the major apoproteins of Intralipid after plasma exposure has been less clear. Scanu and Page (13) found quite similar, but not identical, "fingerprints" of proteins from chylomicrons and from incubated coconut oil emulsion. Sata et al. (15) incubated Intralipid with the $\delta>1.21$ fraction from human plasma and found Apo A-I to be a major constituent. They also described an associated apoprotein rich in proline, with molecular weight of 74,000 Daltons. In the Intralipid incubations described here, the ARP is clearly one of the major apoproteins associated after plasma exposure. Apo A-IV and the Apo C peptides are also substantially present. In human incubations, however, Apo A-I is also present to a major degree, along with Apo C, Apo-IV, and ARP. A protein of the size of the proline-rich protein (Bands 1-6) was not seen as a major constituent of Intralipid after incubations with either whole human plasma or the $\delta>1.21$ fraction.

The fact that Intralipid upon plasma exposure can acquire an apoprotein pattern similar to that of the chylomicron upon plasma exposure in vitro or in vivo suggests that the alterations in the chylomicron do not depend on any previous complement of apoproteins in the nascent state. It also suggests that the transfers are a property of the phospholipid-triglyceride emulsion, not requiring a protein mediator.

Thus, the nascent apoproteins in the chylomicron may not be necessary to effect protein and lipid transfers, nor to mediate subsequent metabolism.

Further similarities between Intralipid and chylomicrons can be seen in the changes in lipid composition after plasma incubation. Chylomicrons, harvested from the lymphatics, have been shown to lose phospholipid and gain cholesterol upon plasma exposure (3,16). Similar changes have been shown for infused Lipomul (14), and these experiments document that Intralipid loses phospholipid and gains cholesterol after in vitro plasma incubation.

Incubations of the rat $\delta > 1.21$ density fraction with Intralipid posed a unique situation, in that the transfer of ARP onto the particles of flotation greater than $S_f 400$ was absent unless particles of lesser flotation were eliminated from the incubation. These inhibitory particles were found to be primarily in the $\delta < 1.006$ (S_f 20-400) fraction. One possible explanation is that the quantity of ARP in the rat $\delta > 1.21$ fraction is quite limited, and that the smaller Intralipid particles in the $S_f < 400$ fraction preferentially compete for the limited supply. Indeed, others have found ARP in the lipoprotein-free plasma of rats (17), but evidence exists that this is, in part, an artifact of centrifugation (18). Nonetheless, the apoprotein pattern from selective incubation of Intralipid with the rat lipoprotein-free plasma resembles its chylomicron counterpart only when the smaller particles are removed from the emulsion before incubation.

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Characterization of Branched-Chain Fatty Acids from Fallow Deer Perinephric Triacylglycerols by Gas Chromatography-Mass Spectrometry

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ABSTRACT

Branched-chain fatty acids of perinephric triacylglycerols of semi-feral fallow deer (*Dama dama dama*) were analyzed by high resolution gas chromatography-mass spectrometry. Of the total fatty acids, 15.50% were Branched-chain components including 8.96% iso acids, mostly 14-methylpentadecanoic acid, 2.85% anteiso acids and 1.73% of other monomethyl-substituted acids; dimethyl-branched acids with an iso structure (1.05%) and with an anteiso structure (0.18%) were also present. Whereas the predominant iso acids and methyl-substituted iso acids had chain lengths of 13 and 15 carbon atoms, the anteiso acids and methyl-substituted anteiso acids had chain lengths of 14 and 16 carbon atoms. Methyl substitution occurred on the even numbered carbon atoms relative to the carboxyl group. The general composition is also given of the fatty acids comprising the triacylglycerols of subcutaneous (rump area) and perinephric adipose tissue.

INTRODUCTION

The branched-chain fatty acids normally occurring in tissue triacylglycerols of domesticated ruminants are mostly of the iso and anteiso series and account for 1-2% of the total fatty acids (1). These acids vary in chain length from 13 to 18 carbon atoms and are derived from the assimilation of branched-chain fatty acids of rumen bacterial lipids (2). Monomethyl branched-chain fatty acids, other than those of the iso and anteiso configuration, occur in traces in ruminant tissue triacylglycerols (3), and in enhanced proportions in those of sheep and goats given carbohydrate-rich (cereal) diets (4-6).

This paper reports the occurrence of methyl-substituted iso and anteiso acids and monomethyl-substituted acids, together with enhanced proportions of iso acids in the triacylglycerols of the fallow deer (*Dama dama dama*). Methyl-substituted iso (anteiso) acids are defined as acids with an iso (anteiso) configuration and which have an additional methyl substituent on an even numbered carbon relative to the carboxyl group; for brevity, these are referred to subsequently as methyl-substituted iso and anteiso acids (cf. ref. 22). The acids were identified using high resolution gas chromatography combined with mass spectrometry. A brief account of this work has been given in a preliminary communication (7).

MATERIALS AND METHODS

Source of Fatty Acid Methyl Esters

Samples of perinephric and subcutaneous (rump) adipose tissue were obtained from three male semi-feral fallow deer which were shot in the Midlands of England in September, 1975. Tissue triacylglycerols were extracted with acetone and their component fatty acids converted to methyl esters (8). Straight chain esters were removed from the hydrogenated total fatty acid methyl esters by urea adduct formation (9) to yield a fraction rich in branched-chain components.

Gas Liquid Chromatography (GLC)

Conventional GLC of the fatty acid methyl esters, before and after hydrogenation, was carried out on a Pye Series GCD gas chromatograph (Pye-Unicam Ltd., Cambridge, England) fitted with a flame ionization detector (FID) using 1.5 m x 4 mm i.d. glass columns packed either with 15% (w/w) ethylene glycol adipate (Supelco Inc., Bellefonte, PA) or with 10% (w/w) Apiezon L grease (Shell Chemicals Ltd., London) on acid-washed, silane-treated Celite 545 (80/100 mesh); the column operating temperatures were 175 C and 200 C, respectively, and the carrier gas was argon at 50 ml/min.

So that the bulk of the major components was excluded leaving minor components in increased concentration for analysis, five fractions of the branched-chain ester concentrate were produced by preparative chromatography in a Pye Series 104 heated dual-FID programmed chromatograph (Pye Unicam Ltd., Cambridge, England) using a 4.6 m x 9 mm o.d. glass column packed with 15% (w/w) Apiezon L grease on Celite 545 (80/100 mesh), as described above, at an operating temperature of 200 C. The carrier gas was argon at 100 ml/min.

High resolution GLC of the total branched-chain esters and the fractions obtained therefrom was carried out using a stainless-steel open

tubular column (Perkin-Elmer Ltd., Beaconsfield, Bucks, England), 100 m x 0.25 mm i.d. coated with polymerized butanediol succinate. The column was operated isothermally at 165 C with argon at 1 ml/min as carrier gas in a Pye Series 104 gas chromatograph fitted with a splitless injection system (Scientific Glass Engineering (U.K.) Ltd., London). The detector oven temperature was 250 C, and the injection port heater was maintained at 200 C.

Gas Chromatography/Mass Spectrometry (GC/MS)

The branched-chain ester concentrate and those fractions obtained by preparative GLC were subjected to analysis by GC/MS. The high resolution GC system was that described above, except that the carrier gas was helium at a flow rate of 1 ml/min and that the gas chromatograph did not have a separate detector. The gas chromatograph was interfaced through an all-glass direct inlet system maintained at 250 C to a VG Micromass 16F single focusing mass spectrometer (VG Micromass Ltd., Altrincham, Cheshire). The mass spectrometer was operated at 4 kV with an ionization energy of 70 eV and an ion source temperature of 200 C. Mass spectra were recorded at a scan speed of 1 sec/decade and a resolution of 1000.

The structures of chromatographic components were assigned from mass spectral evidence according to established methods (10). Components with an iso structure were readily distinguishable from the corresponding straight chain esters by the difference in the relative equivalent chain length (ECL) values and by the presence of a peak at M-65 and a doublet at M-55 and M-56 in their mass spectra (11); the intensity of the M-65 peak was of the order of 3% of the base peak. Compounds with an anteiso configuration were identified by the feature M-29 > M-31 and by the presence of ketene and ketene-H_2O peaks. The position of the substituent in monomethyl acids (other than iso and anteiso) and in methyl-substituted iso and anteiso acids was determined from known fragmentation patterns; particularly significant were the ions at $\frac{m}{e}$ 87 and 88 which are the base peaks for esters with 4-methyl and 2-methyl substituents, respectively, and M-76 indicating 6-methyl substitution. The presence of ketene and ketene-H_2O ions denoted substitution at other positions in the fatty acyl chain.

RESULTS

The fatty acid composition of the subcutaneous (rump area) and perinephric adipose tissue triacylglycerols is given in Table I; the analyses were carried out using conventional gas chromatography. The major fatty acids in the subcutaneous triacylglycerols were palmitic (21.0%), stearic (20.9%) and octadecenoic (27.0%); the branched-chain fatty acids represented 16.5% of the total fatty acids. The fatty acids of the perinephric triacylglycerols were more saturated than those of the subcutaneous triacylglycerols, the contribution of palmitic, stearic and octadecenoic acids being 20.6%, 37.5% and 15.3%, respectively; of the total fatty acids, 13.3% were branched-chain components. The values expressed for the branched-chain fatty acids do not include the small contribution of methyl-substituted anteiso acids which did not separate from straight chain saturated acids under the GC conditions employed. After removal of the straight-chain components by urea adduct formation, analysis of the resulting concentrate on high resolution GC showed that the proportion of branched-chain components in the perinephric triacylglycerols was 15.5%.

The methyl esters prepared from the three samples of perinephric triacylglycerols were pooled and hydrogenated. The hydrogenated total fatty acid methyl esters (1.969 g) were treated with methanol saturated with urea (2 ml/25 mg esters) yielding a nonadducting

TABLE I

Component Fatty Acids of Triacylglycerols of Perinephric (P) and Subcutaneous (S) Adipose Tissue of Fallow Deer[a]

Fatty acid	P	S
12:0	0.1	0.1
14:0	3.0	3.3
15:0	2.1	1.8
16:0	20.6	21.0
16:1	1.0	2.2
17:0	2.8	2.9
17:1	0.3	0.7
18:0	37.5	20.9
18:1	15.3	27.0
18:2	2.1	1.8
18:3	1.2	1.0
19:1	0.3	0.3
20:4	0.4	0.5
branched chain	13.3[b]	16.5

[a]Values, means of three animals, as % by weight of total fatty acids.

[b]Subsequent high resolution GC analysis of the branched-chain fatty acids (as their methyl esters) revealed that of the total fatty acids, 15.50% were branched chain components; these comprised 8.96% iso acids, 2.85% anteiso acids, 1.05% methyl-substituted iso acids, 0.18% methyl-substituted anteiso acids, 1.73% monomethyl-substituted acids (other than iso and anteiso), 0.56% polymethyl-substituted acids and 0.17% unidentified acids.

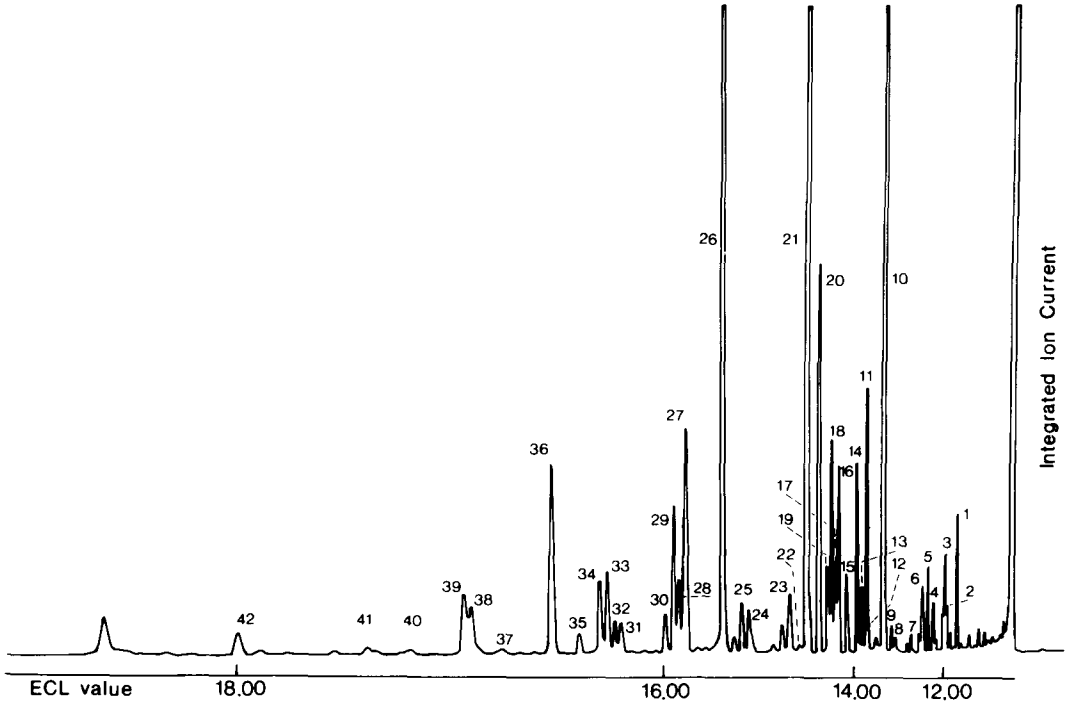

FIG. 1. Integrated ion chromatogram (magnet scan range 60 to 120 a.m.u.) of branched-chain concentrate of fatty acid methyl esters of fallow deer perinephric triacylglycerols. See text for GC-MS conditions. Peak numbers refer to those components examined by GC-MS and whose identities are given in Tables II and III. Methyl stearate (peak 42) was eluted after 56 min.

TABLE II

Methyl-Substituted Iso and Anteiso Acids of Fallow Deer Perinephric Triacylglycerols
FCL[a] Values and Identity of Components Examined by GC-MS

Chain length of iso acid	Position of additional methyl substituent				
	4	6	8	10	12
11	0.97^{2}				
13	0.92^{13}	0.80^{11}	0.80^{11}	0.85^{12}	
15	0.96^{29}	0.86^{27}	0.86^{27}	0.86^{27}	0.89^{28}
Chain length of anteiso acid					
14		1.00^{23}	1.00^{23}		
16		1.00^{37}	1.00^{37}	1.00^{37}	1.00^{37}
Other acids identified:	4,8,12-trimethyltridecanoic		FCL[a] 1.15^{15}		
	2,6,10,14-tetramethylpentadecanoic		FCL[a] 0.86^{27}		
	3,7,11,15-tetramethylhexadcanoic		FCL[a] 1.10^{38} (SRR)		
			FCL[a] 1.13^{39} (RRR)		

[a]Fractional chain length. Values taken from GC of methyl esters (see text for GC conditions). Superscripts refer to peak numbers in Figure 1.

fraction (48 mg) and an adduct fraction (1.891 g) which on further treatment with methanol/urea solution (1 ml/25 mg esters) yielded a non-adducting fraction of 163 mg. The combined nonadducting fractions (211 mg) of which the branched-chain complement (202 mg) represented 10.26% of the original total fatty acids were used in the subsequent GC-MS analysis. Figure 1 shows the integrated ion chromatogram of this fraction, the scan range of the magnet being 60 to 120 atomic mass units. The numbered peaks are those which were

examined by GC-MS, and the component identities together with their fractional chain length (FCL) values are given in Tables II and III. FCL is the fraction of a carbon number which is attributable to a methyl substituent at a specific position in the fatty acyl chain (12). The minor components in the sample were identified by examination of the fractions obtained by preparative GC.

In the urea adduct fraction, 5.24% of the total fatty acids were iso and anteiso acids. Thus, of the total fatty acids of fallow deer perinephric triacylglycerols, 15.50% were branched-chain acids (Table I); these comprised 8.96% iso acids, 2.85% anteiso acids, 1.05% methyl-substituted iso acids, 0.18% methyl-substituted anteiso acids, 1.73% monomethyl-substituted acids (other than iso and anteiso) and 0.56% polymethyl-substituted acids; 0.17% of the total acids were not identified.

Those iso acids with an odd number of carbon atoms in the chain, i.e., 12-methyltridecanoic and 14-methylpentadecanoic (Table III and peaks number 10 and 26 in Fig. 1), were the predominant branched-chain components. The methyl-substituted iso acids also had an odd number of carbon atoms in the chain, and methyl substitution occurred on the even numbered carbon atoms relative to the carboxyl group. Thus, substitution was evident at positions 4, 6, 8, 10 and 12 in iso acids with 13 and 15 carbons in the chain, whereas in the iso acids with 11 carbons in the chain, substitution was apparent only at the 4 position (Table II). With regard to the gas chromatographic behavior of these methyl-substituted iso acids, it can be seen from Figure 1 and Table II that, of the iso-C_{13} acids present, only those substituted at the 6 and 8 positions were not separated. Iso acids with a methyl substitutent in the 2 position were not detected. Acids substituted at position 4 were present in greatest abundance among the methyl-substituted iso acids and monomethyl substituted acids.

Methyl-substituted anteiso acids could not be separated under the GC conditions employed (Fig. 1 and Table II), and their identities were based solely on MS evidence. Nevertheless, by taking MS scans at different points of the chromatographic peak, it was possible to establish the presence of all the even numbered positional isomers of 14-methylhexadecanoic acid, except those substituted in positions 2 and 4.

The polymethyl-substituted acids identified in the urea nonadducting fraction were 3,7,11, 15-tetramethylhexadecanoic acid (phytanic acid) and its catabolities, 2,6,10,14-tetra-

TABLE III

Monomethyl-Branched Fatty Acids of Fallow Deer Perinephric Triacylglycerols
FCL[a] Values and Identity of Components Examined by GC-MS

Normal chain length	Position of methyl substituent						Anteiso[b]	Iso[c]
	2	4	6	8	10	12		
11								0.581
13		0.389	0.288					0.5210
15	-0.2122	0.3625	0.2924	0.2924	0.3625	0.3625		0.5426
17		0.3640						0.5641
12		0.384		0.384			0.706	0.545
14		0.3618	0.2816	0.3317	0.4119		0.7021	0.5320
16	-0.1427	0.3733	0.2931	0.2931	0.3232	0.4234	0.7036	0.5435

[a]Fractional chain length. Values taken from GC of methyl esters (see text for GC conditions).
[b]0.03, 1.74 and 1.08 wt % of total fatty acids, respectively.
[c]0.05, 1.57, 6.04, 0.65, 0.05, 0.50 and 0.10 wt % of total fatty acids, respectively. Superscripts refer to peak numbers in Figure 1.

methylpentadecanoic acid (pristanic acid) and 4,8,12-trimethyltridecanoic acid (peaks 39, 38, 27 and 15, respectively in Fig. 1). It is noteworthy that the RRR and SRR diastereoisomers of phytanate were partly resolved.

A diagnostic feature in the mass spectra of methyl-substituted anteiso acids was the presence of ketene and ketene-H_2O ions with an increment of 14 atomic mass units compared to those of the corresponding unsubstituted anteiso acids. Thus, in the mass spectrum of methyl 6,14-dimethylhexadecanoate, apart from the features denoting 6-methyl substitution and M-29 > M-31 for the anteiso configuration, ions at $\frac{m}{e}$ 237 and 219 were present.

DISCUSSION

The adipose tissue triacylglycerols of the domesticated species of ruminants so far examined contain ca. 2% branched-chain fatty acids which consist almost entirely of acids of the iso and anteiso series; normally acids with the anteiso configuration predominate (1). The content and proportions of branched-chain fatty acids in the adipose tissue triacylglycerols of feral species of ruminants, namely reindeer (*Rangifer tarandus*), red deer (*Cervus elephus*), elk (*Rangifer canadensis*) and white-tailed deer (*Odocoileus virginianus*) are similar to those found in the domesticated species (13,14). In contrast, fallow deer perinephric triacylglycerol fatty acids contain 15.5% branched-chain fatty acids including 10.0% iso and methyl-substituted iso acids and 3.0% anteiso and methyl-substituted anteiso acids. Thus, the increased proportions of iso acids in fallow deer perinephric triacylglycerols gives rise to a fatty acid composition which is atypical of ruminant depot lipids.

Monomethyl acids (other than iso and anteiso) are normally present in trace amounts in ruminant tissue lipids (3), and in considerably greater proportion in the subcutaneous triacylglycerols of lambs and goats given a carbohydrate-rich (cereal) diet (4-6). The ruminal fermentation of such diets results in enhanced availability of propionate to the host animal (15), and it was suggested that when the capacity for hepatic metabolism of propionate is exceeded, methylmalonyl-CoA, which is the carboxylation product of propionyl-CoA, accumulates and becomes available for incorporation into newly synthesized fatty acids (16). Fatty acids with methyl substituents on the even numbered carbons arise from the incorporation of methylmalonyl-CoA into the fatty acyl chain. This has been demonstrated clearly using fatty acid synthetase preparations from the liver and uropygial gland of the goose, rat liver (17,18), chicken liver, and sheep adipose tissue (19).

In fallow deer, the occurrence of monomethyl-substituted fatty acids in proportions greater than those normally found in ruminant tissue lipids (3) indicates the availability of methylmalonyl-CoA for incorporation into the fatty acyl chain. Methylmalonyl-CoA can arise from a number of metabolic pathways (20), but its origin in fallow deer is as yet unknown. It is evident that methyl-substituted iso and anteiso acids are synthesized from isobutyryl-CoA and 2-methylbutyryl-CoA, respectively (21); the substitution of methylmalonyl-CoA for malonyl-CoA yields the various positional isomers. Although the presence of methyl-substituted iso and anteiso acids in ruminant tissue triacylglycerols has not been reported hitherto, such acids do occur in other tissues such as the wax esters of *vernix caseosa*, the greasy material which covers the newborn baby (22).

It is believed that fallow deer were introduced to the British Isles from the Mediterranean region of southern Europe. Thus, it may be that the predominance of iso acids in the branched-chain fatty acid complement of fallow deer perinephric triacylglycerols is associated with an inborn error of metabolism arisen through in-breeding, although the possibility of a dietary contribution cannot be excluded. Whether this metabolic peculiarity is present in indigenous fallow deer must await further investigation.

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Comparison of Lipid Status in the Hearts of Piglets and Rats on Short Term Feeding of Marine Oils and Rapeseed Oils

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ABSTRACT

A series of 4 experiments with piglets and one experiment with rats has been conducted to establish the cardiac lipid status of weanling (3 weeks old) male animals fed fats with different contents of docosenoic fatty acids. Experimental fats were rapeseed oil (RSO) (48.0% 22:1), refined fish oil (RFO) (14.6% 22:1), partially hydrogenated fish oil (PHFO) (14.3% 22:1) and lard (0% 22:1) combined with sunflower seed oil (SFO) in different proportions in diets with 21% total fat. Lipidosis could not be detected in piglets as increased heart weights, by chemical assay for myocardial contents of triglycerides, or by accumulation of docosenoic fatty acids or nonesterified fatty acids (NEFA). In rats, diets with RSO at a level of 16% increased myocardial triglyceride and docosenoic fatty acid contents about 7 times while the effect on cardiac NEFA was inconsistent. Histological examinations of the hearts revealed stainable intracellular fat droplets in some piglets fed 16% RSO for 8 to 13 days, but not after 2, 4 and 6 and 16, 19 and 22 days of feeding. After 10 days of feeding, mild to moderate histological lipidosis was found in piglets fed diets containing 2% or more of 22:1 fatty acids, with no significant difference between RSO, RFO and PHFO in this respect. The same diets in rats gave about 5 times more histological lipidosis than in piglets. This is attributed to a difference in species response, the rat reacting in a more pronounced manner than the piglet. The cardiac lipidosis no-effect level in piglets corresponded to a daily intake of docosenoic fatty acids of 0.4 g per kg body weight. Mild lipidosis was also found in a few animals on docosenoic acid-free diets.

INTRODUCTION

It is established that feeding of diets incorporating high levels of rapeseed oil containing moderate to high levels of erucic acid causes transitory lipid accumulation in the myocardium of the male weanling rat (1-3). The condition has been described as lipidosis, and is characterized by intracellular accumulation of triglycerides and of free fatty acids, as manifested by chemical determinations and/or light microscopy (4). Partially hydrogenated fish oil, containing high levels of docosenoic fatty acids may also cause lipidosis in the rat (2,3), but it is not established to what extent such effects are caused by commercially available types of partially hydrogenated fish oils containing moderate to low levels of docosenoic fatty acids. Information on lipidosis in young mammals other than the rat is scarce, with a lack of data from work with alternative species of similar age fed similar diets. However, limited studies with young pigs indicate that this species may be less susceptible to lipidosis than the rat (5,6). The present investigation was undertaken to study the lipid status of the hearts of male weanling piglets in comparison with male weanling rats when fed diets with different contents of refined fish oil, partially hydrogenated fish oil, or rapeseed oil, to supply varying levels of docosenoic fatty acids with the objective of establishing the no-effect level.

MATERIAL AND METHODS

Diets and Experimental Design

The following fats and oils were used: rapeseed oil (RSO) (DeNoFa og Lilleborg Fabriker, Fredrikstad, Norway) refined (RFO) and partially hydrogenated (PHFO) capelin fish oil (DeNoFa og Lilleborg Fabriker, Fredrikstad, Norway) lard (A/S C.E. Basts Eftf., Copenhagen, Denmark) and sunflower seed oil (SFO) (Wilhelm Connemann, Leer (Ostfriesi), W. Germany). The fatty acid composition of the different fats and oils was determined at five different laboratories. The individual laboratory figures showed good agreement, and Table I shows the averaged compositions.

The basal ingredients were similar to those used by Gjefsen (7) to support maximum feed intake and growth in the weanling piglet. In the rat experiment, one-half of the dried skim milk was replaced by maize starch and acid casein due to the lower lactose tolerance in these animals. The basal ingredients made up 79% of the experimental diets. The remaining 21% came from added fats. Table II shows the proportions of the different fats in the different

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TABLE I

Average Fatty Acid Compositions (wt %) of Experimental Fats and Oils

Fatty acid[a]	Sunflower seed oil (SFO)	Lard	Rapeseed oil (RSO)	Partially hydrogenated capelin oil (PHFO)	Refined fish oil (RFO)
14:0	---	1.67	0.05	6.79	7.23
16:0	7.2	27.93	3.13	13.34	12.05
16:1	---	2.57	0.25	8.62	8.69
18:0	4.4	16.06	1.09	3.27	1.45
18:1	25.5	41.42	12.10	14.55	13.16
18:2	59.6	6.46	14.32	2.83	1.72
18:3	1.3	1.34	8.82	---	1.25
18:4	---	---	---	---	4.11
20:0	---	0.73	0.66	1.84	0.18
20 1	---	1.23	8.25	14.38	13.94
20:2	---	---	0.33	5.09[b]	---
20:3	---	---	---	1.38[b]	---
20:4	---	---	---	---	0.24
20:5	---	---	---	---	8.16
22:0	---	---	0.53	2.14	---
22:1	---	---	48.03	14.34	14.55
22:2	---	---	0.73	5.31[b]	---
22:3	---	---	---	1.86[b]	---
22:6	---	---	---	---	7.18

[a]Carbon atoms: number of double bonds. Fatty acids of minor importance omitted.
[b]Mixtures of different positional and geometrical isomers, mostly formed during the hydrogenation process.

TABLE II

Composition[a] (%) of Diets and Experimental Design

	Diet[c]							
Fats[b]	Lard-16	RFO-20	PHFO-16	PHFO-14	PHFO-12	PHFO-8	RSO-16	RSO-5
SFO	5.00	0.50	5.00	5.00	5.00	5.00	5.00	5.00
Lard	16.00	---	---	2.18	4.18	8.00	---	10.91
RFO	---	20.50	---	---	---	---	---	---
PHFO	---	---	16.00	13.82	11.82	8.00	---	---
RSO	---	---	---	---	---	---	16.00	5.09
Total 22:1, % of diet	0	2.98	2.29	1.96	1.69	1.15	7.86	2.45
No. of animals per diet:								
Piglet expt. I	9	---	---	---	---	---	27	---
Piglet expt. II	6	6	6	---	---	---	6	6
Piglet expt. III	7	---	7	---	7	7	---	7
Piglet expt. IV	10	---	10	10	10	---	5	---
Rat expt.	21	---	21	---	---	---	21	21

[a]The diets contained in addition to the fat/dried skim milk, piglet diets 63%, rat diets 30%, casein (acid), rat diets 13.29%; finely ground oat hulls 2% and maize starch to 100%. Added minerals and vitamins were per kg of diet: $CaHPO_4.2H_2O$, rat diets 14.60 g; $FeSO_4 \cdot 7H_2O$, 0.4 g; $MnSO_4 \cdot 4H_2O$, piglet diets 0.06 g, rat diets 0.08 g; $ZnCO_3$, 0.09 g; KI, 0.26 mg; $NaHSeO_3$, 0.22 mg; vitamin A, 10.000 I.U.; vitamin D_3, 1,000 I.U.; vitamin E, 100 I.U.; choline chloride, 0.23 g; niacin, 0.02 g, and all diets were supplemented with DL-methionine 1.8 g, zinc bacitracin 0.5 g and ethoxyquin 0.15 g per kg.
[b]SFO = sunflower seed oil, RFO = refined fish oil, PHFO = partially hydrogenated fish oil, RSO = rapeseed oil.
[c]Abbreviations: fat source-level of incorporation.

diets and also shows the number of animal fed the different diets in each experiment. Except for diet RFO-20 (source of fat-level of incorporation in diet) with 20.5% RFO which supplied a high level of polyenoic fatty acids, adequate supplies of essential polyenoic fatty acids were assured by the addition of 5% of SFO. It was intended to compare the test fats at a similar level of inclusion as well as at levels which gave equal dietary contents of 22:1 fatty acids. Due to an error in the initial fatty acid determination, the latter objective was not

entirely achieved.

In the first piglet experiment, three piglets fed the 16% RSO diet and one piglet fed the 16% lard control diet were killed after 2, 4, 6, 8, 10, 13, 16, 19 and 22 days of feeding. In piglet experiments II, III and IV, all piglets were killed after 10 days of feeding, and in the rat experiment, the rats were killed after 4 days of feeding.

Animals and Management

Male piglets of the Norwegian landrace breed, 18-21 days of age, were obtained directly from local breeders after having had access to a creep feed comprising the basal ingredient mixture with the addition of 1% of SFO. They were weaned abruptly with access only to the experimental feed. One piglet from each litter was distributed randomly to each diet according to a randomized block design (8). There were 5 piglets per cage with arrangements for recording the feed intake of individual animals. They had free access to feed for about ½ hr 3 times per day at 8.00, 12.00 and 16.00 hr, water being available *ad libitum*. Room temperature, relative air humidity, light intensity and lighting time were automatically controlled at 25 C, 60%, 20 lux and 12 hr, respectively. Three week old, male Wistar rats (Mollegaard, Denmark) were used in the rat study. Prior to weaning, they had access to a laboratory stock diet, which also was fed for three days after weaning, the experimental diets being given thereafter. The rats were kept individually in cages after weaning, and given feed and water *ad libitum*.

Recording and Sampling

The piglets were weighed individually at the start of the experiments and on the day of sacrifice. In the first piglet experiment and in the rat experiment, the animals were weighed every second or third day in the experimental period, and feed consumption was recorded daily in these experiments. In the remaining piglet experiments, feed consumption was recorded for the entire experimental periods.

Piglets and rats were anesthesized with ethyl ether 1-2 hr postfeeding and before sampling. In the first piglet experiment, blood samples were drawn from the abdominal aorta and chilled in an ice bath pending analyses of serum lipids and serum nonesterified fatty acids (NEFA). Samples for the determination of NEFA in cardiac tissue in piglet experiment I were the same as those used for the determination of total lipids, but in piglet experiment II, III and IV and in the rat experiment, they were obtained by freeze-clamping tissue from the apex (piglet experiments) or of the whole heart (rat experiment). After sampling for NEFA determination, the hearts were removed and weighed, then sectioned in transverse sections from the apex to the coronary groove in slices 5 mm thick. Every second slice was used for analyses of total lipids and/or triglycerides and immediately placed in an ice bath. The other slices were used for histological examination and were quickly placed in a buffered (9.52 g $Na_2HPO_4 \cdot 2H_2O$ + 0.818 g $K_2HPO_4 \cdot 2H_2O$ per liter) 4% formaline solution. In the rat experiment, the whole hearts from 9 animals were used for histology, the whole heart of 6 animals for NEFA determination and 3 pooled samples of 2 hearts each for triglyceride determination.

Chemical Analyses and Histological Examination

The fatty acid composition of the experimental fats and oils was determined by gas liquid chromatography (GLC) in a collaborative trial at five different laboratories. NEFA was determined by Dole's method (9) as modified by Trygstad (10) and total lipids according to Sperry and Brand (11) and Henry (12) in blood serum obtained in piglet experiment I from chilled blood (without anticoagulant) by centrifugation at 2800 g for 10 min. Total lipids in cardiac tissue from piglet experiment I was determined as described above (11,12) on a suspension obtained by homogenizing 10 g of chilled (0 C) cardiac tissue with 50 ml of water for 90 sec in an MSE homogenizer at 14,000 r.p.m., and in piglet experiments II and III as described by Folch et al. (13). Triglycerides in cardiac tissue from piglet experiment II were determined enzymatically (Boehringer, Mannheim CMBH, Biochemica Test-kit mat. no. 159 89) on lipids obtained as described above (11,12), and in piglet experiments III and IV and in the rat experiment by a combination of thin layer chromatography (TLC) and GLC on an aliquot of the Folch et al. (13) extract. Tripentadecanoin was added to the extract as an internal standard and the triglycerides were separated by TLC (14). The TLC spots containing triglycerides were hydrolyzed in NaOH and methylated as in ref. 15. The esterified fatty acids were quantitatively determined by GLC on a Carlo Erba mod. 2351 A equipped with a flame ionization detector. The glass column (6ft, 4 mm i.d.) was packed with 15% HI-EFF-4BP (Applied Science Laboratories Inc., State College, PA) on Supelcoport (100/120 mesh). Methyl esters were chromatographed isothermically at 215 C and identified by their retention times compared to known standards. Fatty acids from triglycerides

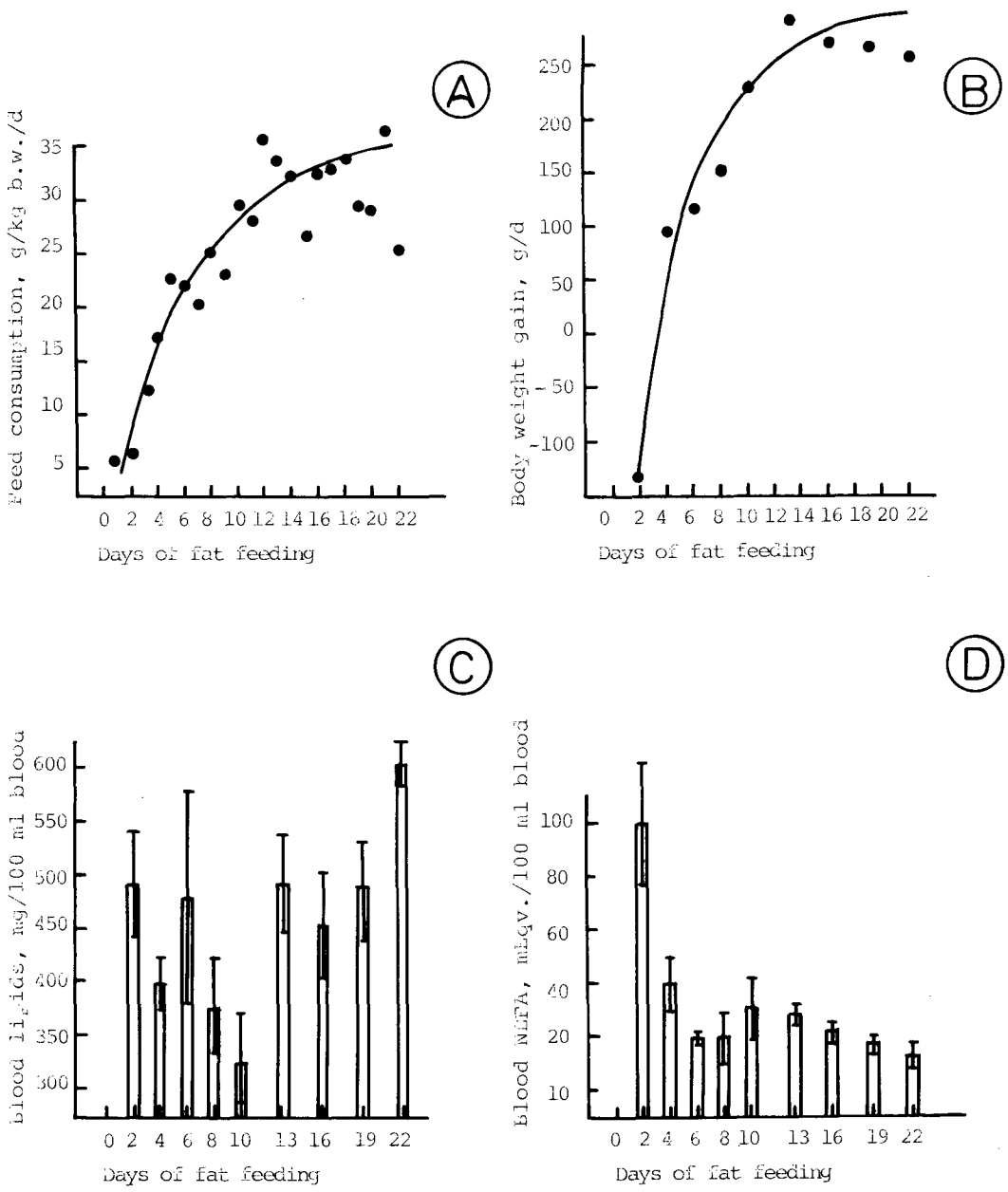

FIG. 1. Feed consumption (A), body weight gain (B), blood lipids (C) (averaged ± standard deviation) and blood NEFA (D) (average ± standard deviation) in piglets fed high fat diets for varying lengths of time in piglet experiment I.

were calculated from peak area (HP-10 integrator system). NEFA in cardiac tissue from piglet experiments I and II were determined titriometrically (9,10), and from piglet experiments III and IV and from the rat experiment by a TLC-GLC technique. The freeze-clamped samples were dropped in liquid nitrogen, ground, weighed without thawing and extracted according to Dole (9). Pentadecanoic acid was added to an aliquot of the extract as an internal standard. The fatty acids were separated by TLC as described by Litchfield (14), methylated according to Metcalfe et al. (15) and chromatographed by GLC as described for the

determination of triglycerides.

One cardiac tissue section was prepared for histological examination from each fixed cardiac tissue slice by washing the fixed slices in tap water, embedding in gelatin, freezing in liquid nitrogen and staining with Oil-Red-O stain. Sections were examined by light microscope without knowledge of dietary treatment. Intracellular lipidosis was graded as follows: 0 = negative; + = trace amounts of lipids in less than 5% of the muscle cells; 1 = lipid droplets in less than 25% of the muscle cells; 2, 3 and 4 respectively, = lipid droplets in 25-50%, 50-75%, and in more than 75% of the cells in the whole section. Since lipidosis was not equally pronounced in all parts of the heart, the section which exhibited the most pronounced degree of lipidosis determined the grading for a particular heart. A lipidosis index was calculated as the sum of the lipidosis grading (+ = 0.5, 1 = 1.5, 2 = 2.5, 3 = 3.5) for the individuals in each group divided by the number of animals in the group.

Statistical Calculations

Statistical calculations were conducted according to Snedecor (8) using nonparametric tests for the evaluation of the histological data.

RESULTS

Piglet Experiment 1

All piglets appeared in good health condition throughout the trial except for a few episodes of diarrhea of short duration which were cured by medication (Neomycini sulfas, about 0.25 g per day). Average growth and food consumption graphs are shown in Figure 1. Feed intake was low at the start and increased linearly from about day 2 to 10 and more slowly thereafter (Fig. 1A). Consequently, the piglets lost weight during the first 2 days, but gained weight during the rest of the period (Fig. 1B). The length of the feeding period had no consistent effect on total blood lipid levels (Fig. 1C), but elevated serum NEFA (Fig. 1D) in the first 2 days seemed to be associated with the reduced feed intake and likely with a negative energy balance in that period.

Heart weights and cardiac chemical lipid status are shown in Table III. Heart weights and cardiac content of total lipids and NEFA were not consistently different on the two diets and in piglets killed after different lengths of feeding. Lipidosis was detected by histological examination of hearts from 7 of the 9 piglets fed the RSO-16 diet for 6, 8 and 13 days, but in none from those fed the same diet for 2, 4, 10, 16, 19 and 22 days, and trace lipidosis was seen in the heart of one piglet fed the control lard diet for 8 days. Thus, in the piglet, the lipidosis evidently reached a maximum between 8 and 13 days of feeding and regressed thereafter. Consequently, a feeding period of 10 days was chosen in the suceeding piglet experiments.

Piglet Experiments II, III and IV

The health condition of the piglets was generally good, but a few episodes of diarrhea occured on all diets. These were, however, of short duration and were cured by medication. Occasionally, irrespective of dietary treatment, one piglet in an experiment had reduced feed intake and growth, but at post mortem examination, no macroscopical changes were seen except for the piglets in question being lean.

Feed consumption and body weight gain are shown in Table IV. Overall, the piglet consumption of the different diets, per kg of body weight, was apparently not related to the type of fat used and not significantly different for the different diets. However, in piglet experiment II, the growth rate was significantly different on the different diets with the lowest body weight gain on the RSO-16 diet. In the other piglet experiments, the growth rate was not significantly different on the different diets.

There were no significant differences in heart weight per kg of body weight between dietary treatments within experiments (Table V). In piglet experiments II and III, the content of total lipids and triglycerides in the hearts was not significantly different between the different diets within experiments (Table V). In piglet experiment IV, the piglets fed the lard diets had significantly higher content of triglycerides in their hearts than the piglets fed the other diets.

Cardiac tissue content of NEFA is shown in Table V. The titration method (9,10) yields results in equivalents of acid, the TLC-GLC method in weight units. Using freeze-clamping of the samples and grinding under nitrogen reduced average cardiac tissue NEFA levels by nearly 40% compared to chilling of the samples (Piglet experiment 1, Table III vs. Piglet experiment II, Table V). When the fatty acid composition is known, results from the TLC-GLC method can be calculated to show the corresponding equivalents of acid. Six samples which were analyzed by the titration method (9,10) and by the TLC-GLC method gave average NEFA contents of 2.76 ± 0.58 and 0.66 ± 0.15 mEq per g of wet tissue, respectively. The coefficient of correlation (r) between the two sets of data was -0.42 ($P>0.1$). Thus, data

TABLE III

Effect of Length of Lipid Feeding on Cardiac Status
(Piglet Experiment I)

	Days of lipid feeding								
	2	4	6	8	10	13	16	19	22
Heart weights, g/kg b.w.:									
Lard-16[a]	9.3	7.9	6.8	5.3	5.6	5.3	5.3	4.2	4.9
RSO-16	6.2 ± 0.7	6.7 ± 0.7	6.9 ± 0.6	5.6 ± 0.8	5.8 ± 0.2	5.7 ± 0.2	4.8 ± 0.2	4.1 ± 0.2	5.5 ± 0.3
Total lipids, % of wet tissue:									
Lard-16	2.9	3.2	3.4	3.1	2.8	3.4	3.3	3.2	3.3
RSO-16	3.3 ± 0.25	3.2 ± 0.07	3.3 ± 0.05	3.4 ± 0.32	2.8 ± 0.20	3.8 ± 0.05	3.3 ± 0.05	3.3 ± 0.17	3.5 ± 0.20
NEFA, mEq./g wet tissue[b]:									
Lard-16	5.7	4.8	5.8	7.5	5.9	5.9	5.6	5.8	4.5
RSO-16	6.6 ± 1.8	6.8 ± 2.1	5.5 ± 1.4	5.3 ± 1.0	7.2 ± 0.9	4.7 ± 0.4	5.3 ± 0.3	5.8 ± 1.7	5.0 ± 1.0
Histology:									
Incidence[c]:									
Lard-16	0	0	0	1	0	0	0	0	0
RSO-16	0	0	2	2	0	3	0	0	0
Severity[d]:									
Lard-16 Grade 0	1	1	1	0	1	1	1	1	1
+	0	0	0	1	0	0	0	0	0
RSO Grade 0	3	3	1	1	3	0	3	3	3
+	0	0	0	0	0	0	0	0	0
1	0	0	2	0	0	2	0	0	0
2	0	0	0	1	0	1	0	0	0
3	0	0	0	1	0	0	0	0	0
Lipidosis index:[e]									
Lard-16	0	0	0	0.5	0	0	0	0	0
RSO-16	0	0	1.0	2.0	0	1.8	0	0	0

[a]For explanation of diet abbreviation, see Table II. On Lard-16 1 piglet killed each time, on RSO-16 3 piglets.
[b]NEFA determined by a titration method. See text.
[c]Number of hearts with stainable lipids.
[d]Degree of stainable lipids. See text for explanation.
[e]For explanation see text.

TABLE IV

Feed Consumption, Body Weight Gain (b.w.g.) and Consumption of 22:1 in Piglet Experiments II, III, and IV, (Duration 10 Days) and in Rat Experiment (Duration 4 Days)

	Diet[a]								
	Lard-16	RFO-20	PHFO-16	PHFO-14	PHFO-12	PHFO-8	RSP-16	RSO-5	SEM[b]
Feed consumption, g/kg b.w./d.:									
Piglet expt. II	25.1	29.9	26.3	---	---	---	25.5	29.8	2.0
Piglet expt. III	20.0	---	18.9	---	16.5	16.9	---	18.5	1.7
Piglet expt. IV[c] (i)	18.4	---	21.3	20.6	18.2	---	---	---	1.6
(ii)	17.5	---	20.4	19.0	16.6	---	18.9	---	2.4
Rat expt.	117.9	111.2	108.3	---	---	---	120.6	113.7	8.6
B.w.g., g/d:									
Piglet expt. II	115	115	103	---	---	---	65	140	16
Piglet expt. III	81	---	52	---	52	58	---	73	---
Piglet expt. IV (i)	55	---	88	83	76	---	---	---	17
(ii)	24	---	85	73	78	---	60	---	27
Rat expt.	5.1	4.8	4.3	---	---	---	4.8	5.0	0.13
Consumption of C22:1, mg/kg b.w./d.:									
Piglet expt. II	---	867	605	---	---	---	1962	745	69
Piglet expt. III	---	---	433	---	284	195	---	437	31
Piglet expt. IV (i)	---	---	488	407	305	---	---	---	28
(ii)	---	---	468	376	278	---	1449	---	97
Rat expt.	---	3225	2548	---	---	---	9289	2843	91

[a]For explanation of diet abbreviations, see Table II.

[b]SEM = Standard error of mean. The lard eit not included in the calculations for consumption of 22:1.

[c]In piglet experiment IV, 10 piglets on each diet were used for the comparison of the Lard-16, PHFO-16, PHFO-14, and PHFO-12 diets and 5 piglets on each diet for the comparison of all 5 diets (i.e., including the RSO-16 diet). See Table II.

TABLE V

Relative Heart Weight and Content of Total Lipids, Triglycerides and NEFA in Cardiac Tissue in Piglet Experiments II, III and IV (Duration 10 Days) and in Rat Experiment (Duration 4 Days)

Experiment	Diet[a]								
	Lard-16	RFO-20	PHFO-16	PHFO-14	PHFO-12	PHFO-8	RSO-16	RSO-5	SEM[b]
Relative heart weight, g/kg body weight:									
Piglet expt. II	4.99	5.29	5.10	---	---	---	5.12	5.07	0.14
Piglet expt. III	5.91	---	6.26	---	5.90	5.97	---	6.12	0.25
Piglet expt.[c] IV (i)	6.32	---	6.13	6.39	6.33	---	---	---	0.24
(ii)	6.23	---	6.53	6.61	6.64	---	---	6.15	0.41
Total lipids, % of wet tissue:									
Piglet expt. II	2.53	2.58	2.63	---	---	---	2.78	2.54	0.21
Piglet expt. III	2.97	---	2.85	---	2.89	3.11	---	2.88	0.07
Triglycerides, mg/100 g wet tissue									
Piglet expt. II[d]	210	387	591	---	---	---	316	250	175
Piglet expt. III[e]	104	---	141	---	132	92	---	75	28
Piglet expt. IV[e] (i)	260	---	93	99	72	---	---	---	43
(ii)	328	---	70	87	44	---	86	---	69
Rat expt.	396	468	978	---	---	---	2713	550	182
NEFA in wet tissue:									
Piglet expt. II (m.Eq./g)[f]	3.4	3.6	4.0	---	---	---	3.7	3.4	0.4
Piglet expt. III (ug/g)[g]	141	---	104	---	87	70	---	110	22
Piglet expt. IV (ug/g)[g]	386	---	362	---	---	---	432	---	56
Rat expt. (ig/g)[g]	273	765	347	---	---	---	823	382	169

[a]For explanation of diet abbreviations, see Table II.
[b]SEM = Standard error of mean.
[c]For explanation see Table IV and text.
[d]Triglycerides determined enzymatically. See text.
[e]Triglycerides determined by a TLC-GLC method. See text.
[f]NEFA determined by a titration method. See text.
[g]NEFA determined by a TLC-GLC method. See text.

TABLE VI

Fatty Acid Composition (W%) of Cardiac Triglycerides in Piglet Experiments II, III and IV (Duration 10 Days) and in Rat Experiment (Duration 4 Days)

Fatty acid[b]	Experiment	Diet[a] Lard-16	RFO-20	PHFO-16	PHFO-14	PHFO-12	PHFO-8	RSO-16	RSO-5	SEM[c]
14:0	Piglet expt. II	2.4	2.9	2.5	---	---	---	2.2	2.4	0.13
	Piglet expt. III	2.5	---	2.4	---	2.9	2.6	---	2.4	0.33
	Piglet expt. IV[d] (i)	2.3	---	3.3	3.0	3.2	---	---	---	0.19
	(ii)	2.1	---	3.3	2.8	3.1	---	1.8	---	0.29
	Rat expt.	2.9	3.7	3.7	---	---	---	0.4	1.5	0.27
16:0	Piglet expt. II	27.4	26.0	26.8	---	---	---	25.6	26.6	0.71
	Piglet expt. III	25.8	---	27.0	---	25.3	25.8	---	24.0	1.54
	Piglet expt. IV (i)	28.0	---	24.7	25.0	27.8	---	---	---	0.53
	(ii)	28.4	---	25.1	24.5	24.6	---	17.6	---	0.89
	Rat expt.	22.2	15.7	13.6	---	---	---	5.7	17.9	0.68
16:1	Piglet expt. II	7.7	7.6	8.0	---	---	---	7.6	7.0	0.51
	Piglet expt. III	6.5	---	6.0	---	7.3	4.8	---	5.3	0.78
	Piglet expt. IV (i)	8.3	---	9.1	8.7	8.7	---	---	---	0.51
	(ii)	7.6	---	8.3	8.3	8.2	---	4.9	---	0.70
	Rat expt.	2.7	3.8	3.6	---	---	---	0.8	1.4	0.33
18:0	Piglet expt. II	9.6	10.7	8.9	---	---	---	8.8	11.2	0.88
	Piglet expt. III	12.5	---	15.7	---	10.7	17.5	---	12.6	2.36
	Piglet expt. IV (i)	8.7	---	8.4	8.3	8.8	---	---	---	0.47
	(ii)	8.8	---	8.9	8.4	9.6	---	7.4	---	0.66
	Rat expt.	8.1	5.1	6.1	---	---	---	2.9	8.3	0.43
18:1	Piglet expt. II	39.8	34.4	39.8	---	---	---	39.0	37.0	1.59
	Piglet expt. III	35.6	---	28.7	---	38.8	30.7	---	28.4	3.57
	Piglet expt. IV (i)	38.4	---	33.5	35.2	33.7	---	---	---	0.86
	(ii)	38.7	---	34.0	35.3	34.1	---	31.4	---	2.37
	Rat expt.	36.9	25.6	21.6	---	---	---	15.8	29.4	1.26
18:2	Piglet expt. II	9.3	7.2	8.7	---	---	---	9.4	9.2	0.33
	Piglet expt. III	8.6	---	7.7	---	8.0	7.0	---	7.7	0.79
	Piglet expt. IV (i)	10.4	---	11.2	11.5	12.4	---	---	---	0.45
	(ii)	10.0	---	10.7	10.8	12.9	---	14.9	---	0.82
	Rat expt.	16.5	11.2	15.6	---	---	---	13.1	16.0	1.22
20:0	Piglet expt. II	0.2	0.4	0.4	---	---	---	0.4	0.4	0.10
	Piglet expt. III	0.7	---	0.8	---	0.5	0.3	---	0.5	0.19
	Piglet expt.[d] IV (i)	0.2	---	0.3	0.3	0.4	---	---	---	0.13
	(ii)	0.1	---	0.1	0.5	0.3	---	0.1	---	0.19
	Rat expt.	0.2	nd.[e]	1.0	---	---	---	nd.	0.3	0.15
20:1	Piglet expt. II	1.1	2.9	1.6	---	---	---	1.6	1.3	0.30
	Piglet expt. III	0.7	---	1.6	---	0.9	0.9	---	0.8	0.29
	Piglet expt. IV (i)	0.8	---	3.1	2.4	2.7	---	---	---	0.30
	(ii)	0.8	---	2.9	2.5	2.5	---	3.7	---	0.39

TABLE VI (continued)

Fatty acid[b]	Experiment	Diet[a] Lard-16	RFO-20	PHFO-16	PHFO-14	PHFO-12	PHFO-8	RSO-16	RSO-5	SEM[c]
	Rat expt.	1.2	11.5	10.4	---	---	---	9.0	3.8	0.73
22:0	Piglet expt. II	0.4	nd.	nd.	---	---	---	nd.	1.6	0.18
	Piglet expt. III	0.2	---	nd.	---	nd.	nd.	---	nd.	0.08
	Piglet expt. IV (i)	0.1	0.4	---	nd.	nd.	---	---	---	0.13
	(ii)	0.2	0.7	---	nd.	nd.	---	0.1	---	0.23
	Rat expt.	nd.	nd.	0.4	---	---	---	0.9	nd.	0.18
	Piglet expt. II	1.0	2.7	1.0	---	---	---	2.7	1.7	0.51
	Piglet expt. III	1.9	---	2.2	---	0.7	2.4	---	9.1	2.38
	Piglet expt. IV (i)	0.2	---	2.1	2.1	2.0	---	---	---	0.41
	(ii)	0.2	---	1.9	2.3	1.6	---	13.0	---	1.49
	Rat expt.	6.0	18.0	13.9	---	---	---	41.7	16.6	2.88

[a]For explanation of diet abbreviations, see Table II.
[b]Carbons number: number of double bonds. Fatty acids of less importance are omitted.
[c]SEM = Standard error of mean.
[d]For explanation see Table IV and text.
[e]nd. = not detectable.

obtained by the two methods are not comparable, either in absolute figures or in relative values.

The cardiac NEFA content was 3-4 times higher in piglet experiment IV compared with experiment III for reasons which are not known. In neither experiment were there significant differences in cardiac NEFA levels between dietary treatments.

Feeding the various fat mixtures to piglets did not profoundly change the cardiac triglyceride fatty acid patterns compared with that of the lard-fed piglets (Table VI). The higher content of 20:1 and 22:1 in RSO, RFO and PHFO compared with lard increased the content of these fatty acids somewhat in the cardiac triglycerides with a slight compensatory reduction in the content of 18:1. However, the levels of 22:1 in cardiac triglycerides never exceeded those in the dietary fat and were mostly from 1/3 to 1/5 of the levels in the respective dietary fats. It is noteworthy that 20:1 and 22:1 were present at appreciable levels in cardiac triglycerides of piglets fed lard which contained no 22:1 and only a very low level of 20:1.

The different diets had no consistent effect on the composition of the cardiac NEFA, and piglets fed the lard diet which did not contain 22:1 fatty acids had about the same levels of these fatty acids in their NEFA as had those fed the diets containing 22:1 fatty acids (Table VII).

All piglet hearts appeared normal on macroscopical examination. The results of the histological examination of the hearts by light microscopy are shown in Table VIII. There were no significant differences between diets in piglet experiments II and III with regard to incidence (i.e., hearts found to contain stainable lipids) and severity (i.e., the area of the cardiac section covered with stainable lipids) of lipidosis. In piglet experiment IV, there were significant differences between diets in lipidosis incidence and severity. Of a total of 23 hearts examined from piglets fed the lard diet, traces of lipidosis (grade +) were found in one heart and more pronounced lipidosis (grade 2) in another heart which also showed pericardial inflamation (pericarditis).

No lipidosis was seen in the hearts from piglets fed PHFO at levels of 8.0, 12.0 and 14.0% of the diets (equivalent to 1.2-2.0% of 22:1 fatty acids). Diets containing from 2.3 to 7.7% of 22:1 fatty acids from RSO, RFO and PHFO when fed to piglets in experiments II and III appeared to give slightly, but not significantly, more lipidosis than the control lard diet, while 2.3% 22:1 fatty acids from PHFO did not

TABLE VII

Fatty Acid Composition of Cardiac NEFA in Piglet Experiments III and IV (Duration 10 Days) and in Rat Experiment (Duration 4 Days)

Fatty acid[b]	Experiment	Diet[a] Lard-16	RSO-20	PHFO-16	PHFO-14	PHFO-8	RSO-16	RSO-5	SEM[c]
14:0	Piglet expt. III	3.2	---	3.3	2.6	2.7	---	2.8	0.44
	Piglet expt. IV	2.1	---	2.8	---	---	2.2	---	0.18
	Rat. expt.	3.2	2.0	2.8	---	---	0.7	2.2	0.49
16:0	Piglet expt. III	21.5	---	20.0	18.5	19.0	---	17.4	1.34
	Piglet expt. IV	12.4	---	14.3	---	---	13.1	---	0.84
	Rat expt.	20.6	15.6	17.2	---	---	8.5	15.9	1.26
16:1	Piglet expt. III	2.1	---	2.5	2.7	2.5	---	2.4	0.47
	Piglet expt. IV	1.2	---	1.8	---	---	1.4	---	0.54
	Rat expt.	3.6	1.4	2.1	---	---	0.9	2.8	0.77
18:0	Piglet expt. III	28.7	---	25.5	33.3	31.8	---	32.3	3.77
	Piglet expt. IV	20.0	---	16.1	---	---	18.1	---	1.57
	Rat expt.	24.4	28.2	19.9	---	---	20.3	25.2	2.67
18:1	Piglet expt. III	20.9	---	27.5	20.6	24.3	---	20.9	1.90
	Piglet expt. IV	24.5	---	27.4	---	---	31.4	---	3.20
	Rat expt.	18.5	15.1	18.4	---	---	16.4	16.3	1.42
18:2	Piglet expt. III	8.6	---	9.1	7.8	5.8	---	7.5	1.25
	Piglet expt. IV	1.49	---	16.6	---	---	13.3	---	2.04
	Rat expt.	17.3	8.2	21.8	---	---	10.4	18.2	1.67
20:0	Piglet expt. III	0.1	---	0.2	0.1	nd.	---	0.3	0.09
	Piglet expt. IV	0.3	---	0.1	---	---	0.2	---	0.16
	Rat expt.	nd.[d]	0.1	0.1	---	---	0.2	---	0.09
20:1	Piglet expt. III	0.1	---	0.2	0.2	0.3	---	0.2	0.11
	Piglet expt. IV	0.3	---	0.3	---	---	0.8	---	0.29
	Rat expt.	0.1	4.0	2.4	---	---	2.8	1.0	0.60
20:3+	Piglet expt. III	2.8	---	3.0	3.8	4.8	---	2.5	0.44
20:4	Piglet expt. IV	3.3	---	6.4	---	---	2.9	---	2.27
	Rat expt.	8.3	9.7	6.4	---	---	3.5	7.5	2.77
22:0	Piglet expt. III	nd.	---	nd.	1.0	nd.	---	nd.	0.45
	Piglet expt. IV	nd.	---	0.4	---	---	nd.	---	0.21
	Rat expt.	nd.	nd.	nd.	---	---	nd.	nd.	---
22:1	Piglet expt. III	0.6	---	0.5	0.7	0.7	---	2.7	0.67
	Piglet expt. IV	2.2	---	2.4	---	---	3.6	---	0.93
	Rat expt.	nd.	7.4	5.6	---	---	31.7	6.7	1.97

[a]For explanation of diet abbreviations, see Table II.
[b]Carbon number: number of double bonds. Fatty acids of minor importance excluded.
[c]SEM = Standard error of mean.
[d]nd. = not detectable.

cause lipidosis in 10 piglets in experiment IV. None of the piglet hearts examined had lipidosis of a severity exceeding grade 2 (i.e., less than 50% of the section cell area containing stainable lipids as previously defined).

Rat Experiment

The health condition of the rats was excellent throughout the experiment. Feed intake was significantly different on the different diets with lowest intake on the diet containing 16% PHFO, 20% RFO and 5% RSO (Table IV). Also, growth rate was significantly different on the different diets with the lowest growth rate on the 16% PHFO diet (Table IV).

The cardiac content of triglycerides was significantly different on the different diets (Table V). Hearts of rats fed 16% RSO contained about 7 times more triglycerides than those of rats fed the control lard diet, while the hearts of the rats fed 16% PHFO, 5% RSO and 20% RFO had intermediate contents of triglycerides. Contents of NEFA in cardiac tissue were about 3 times higher in rats fed 16% RSO and 20% RFO than in those fed the control lard diet. Rats fed 16% PHFO or 5% RSO did not have contents of NEFA in cardiac tissue appreciably different from rats fed lard (Table V). None of the differences in cardiac NEFA contents were significant ($P>0.05$).

Contrary to what was found in the piglets, the feeding of diets rich in 20:1 and 22:1 fatty acids to rats caused a marked change in the fatty acid composition of their cardiac triglycerides (Table VI). RSO-fed rats had increased levels of 22:1, 20:0 and 20:1 and reduced levels of 18:0, 18:1, 16:0, 16:1 and 14:0 in their cardiac triglycerides compared with those of

TABLE VIII

Cardiac Histology in Piglet Experiment II, III and IV (Duration 10 Days) and in Rat Experiment (Duration 4 Days)

Experiment		Diet[a] Lard-16	RFO-20	PHFO-16	PHFO-14	PHFO-12	PHFO-8	RSO-16	RSO-5	Significance[b]
					Incidence[c]					
Piglet expt. II		1/6	3/6	3/6	---	---	---	4/6	3/6	n.s.
III		0/7	---	2/7	---	0/7	0/7	---	1/7	n.s.
IV		1/10	---	0/10	0/10	0/10	---	2/5	---	x
Rat expt.		1/9	9/9	9/9	---	---	---	9/9	9/9	xxx
					Severity[d]					
	Grade									
Piglet expt. II	0	5	3	3	---	---	---	2	3	
	+	1	3	1	---	---	---	1	2	n.s.
	1	0	0	0	---	---	---	1	0	
	2	0	0	2	---	---	---	2	1	
Piglet expt. III	0	7	---	5	---	7	7	---	6	
	1	0	---	2	---	0	0	---	1	n.s.
Piglet expt. IV	0	9	---	10	10	10	---	3	---	
	+	0	---	0	0	0	---	1	---	x
	1	0	---	0	0	0	---	1	---	
	2	1	---	0	0	0	---	0	---	
Rat expt.	0	8	0	0	---	---	---	0	0	
	+	1	1	0	---	---	---	0	1	
	1	0	2	0	---	---	---	0	2	xxx
	2	0	4	1	---	---	---	0	3	
	3	0	2	7	---	---	---	0	3	
	4	0	0	1	---	---	---	9	0	
					Lipidosis index[e]					
Piglet expt. II		0.1	0.3	0.9	---	---	---	1.2	0.6	
III		0	---	0.4	---	0	0	---	0.2	
IV		0.5	---	0	0	0	---	0.4	---	
Rat expt.		0.1	1.8	3.0	---	---	---	4.0	1.9	

[a]For explanation of diet abbreviations, see Table II.
[b]Significance of treatment differences, n.s. = not significant ($P>0.05$), x = $0.05>P>0.01$, xxx = $P<0.001$.
[c]Number of animals having detectable lipid droplets in their heats/total number of animals on diet.
[d]Distribution according to severity of lipidosis. See text.
[e]For explanation see text.

rats fed lard. Compared with the lard-fed rats, PHFO and RFO had less effect than RSO on cardiac triglyceride fatty acid pattern, but did cause an increase in 14:0, 16:1, 22:1 and 20:1 and a compensatory reduction in 16:0, 18:0, 18:1 and 18:2. The content of 22:1 in the cardiac triglycerides of the rats was about 30% higher than that of the respective dietary fats, and 22:1 was found in appreciable amounts in rats fed lard which did not contain 22:1.

The feeding of diets rich in 20:1 and 22:1 fatty acids significantly increased the content of these fatty acids in the cardiac NEFA to a level of about 50-90% of that of the dietary fat (Table VII).

On macroscopic examination, hearts of rats fed 16% RSO were enlarged and yellowish in appearance, while those of other treatments were normal. There were significant differences between diets with regard to incidence and severity of lipidosis (Table VIII). Severe lipidosis was found by microscopic examination in all hearts of rats fed 16% RSO. Lipidosis was also found in the hearts of rats fed 16% PHFO, 5% RSO or 20% RFO, but the severity was less than that of those fed 16% RSO.

DISCUSSION

Substantial numbers of piglets were employed in the present study, namely a total of 147 representing 63, 51 and 32 animals receiving marine oils, rapeseed oil and the control diet, respectively. Age of piglets and dietary fat levels were chosen so as to be as similar as possible to those commonly used in important rat studies, but in order to achieve maximal myocardial lipidosis, a somewhat longer period was found to be necessary in the piglet compared with the rat (i.e., 10 days vs. 4 days). The piglet proved to be a suitable animal for this type of study, having satisfactory feed consumption and growth rate and apparently maintaining good clinical conditions.

The rat experiment, although including relatively few animals, gave results in general agreement with previous observations in rats fed high levels of high erucic acid RSO (1-3,5, 16,17), thus confirming that the experimental procedure applied resembled that used by others studying the same phenomenon.

Markedly lower myocardial lipid responses of the piglet to high fat feeding, compared to the weanling rat, show a species difference. This interspecies difference was evident for all criteria tested, including myocardial triglyceride content determined chemically, absolute and relative content of fatty acids in triglycerides, and incidence and severity of stainable myocardial intracellular lipid droplets. The increase in heart weight frequently found in rats when fed high levels of high erucic acid RSO (1,2,5,16, 18) was not observed in piglets even when consuming diets with 16% RSO (7.7% of erucic acid in the diet). Feeding the rats 16% RSO increased the content of myocardial triglycerides nearly 6 times compared with that from 16% lard, while RSO and lard did not give significantly different myocardial triglyceride contents in the piglets. Although the fatty acid pattern of the myocardial triglycerides in the piglets to some extent resembled that of the dietary fat, the differences between piglets fed the different fats were minor compared with those of the rat. Docosenoic acids seemed to accumulate in rat hearts since the percentages in myocardial triglycerides were higher by 15-40% compared with those of the dietary fats. In contrast docosenoic acids did not accumulate in the myocardial triglycerides of piglets which contained only one-third to one-fifth of the level of docosenoic acids present in the dietary fat.

Fats with high contents of docosenoic fatty acids, when fed at high levels, caused an increase in incidence and severity of stainable intracellular lipid droplets in some piglet experiments over and above effects from fats without docosenoic fatty acids, but the response in the piglets was very much lower than that in the rats. Thus, when incidence and severity of lipidosis are given a combined numerical value in the *lipidosis index,* the response was 4-5 times greater in the rats compared with the piglets.

To our knowledge, the literature contains only two reports of limited studies with piglets of ages similar to those used in the present experiments and which were fed high fat diets containing high levels of docosenoic fatty acids. Feeding 10 or 20% of high erucic acid RSO to four miniature piglets for one week and to four commercial piglets for two weeks, Beare-Rogers and Nera (5) found no increase in the myocardial content of docosenoic or other fatty acids, but revealed myocardial fat droplets histologically. Myocardial fat accumulation was not demonstrated histologically by Aherne et al. (6) in four piglets fed 15% high erucic acid RSO for four weeks. Lipid and docosenoic fatty acid accumulations were not found by chemical analyses of hearts from somewhat older pigs fed high erucic acid RSO in studies by Kramer et al. (19) or in our own studies (Svaar et al., unpublished data). However, using light or electron microscopy, moderate to slight lipid accumulation was detected in some older pigs when fed high erucic acid RSO in studies

by Levillain et al. (20), and Vodovar et al. (21,22), and in our own studies (Svaar et al., unpublished data). It thus seems evident that the pig, in contrast to the rat, does not respond to diets high in docosenoic fatty acids by a transient increase in myocardial lipids detectable by chemical means, but the myocardial cells of some pigs fed high docosenoic fatty acid diets may transitorily contain lipid droplets which are detectable histologically.

The physiological reason for the difference with regard to cardiac lipid response between rats and pigs is not immediately apparent. Since the digestibility of docosenoic acids is found to be high in piglets (23) and comparable to that in the rat (24), differences in absorption are not the explanation. However, the rats consumed, per kg of body weight, about 5 times more docosenoic fatty acids on any diet than did the piglets (Table IV), and their lipidosis index was also 4-5 times higher (Table VIII). Thus, it is possible that the difference between the piglet and the rat is due to the voraciousness of the latter. On the other hand, since docosenoic acids are readily metabolized in the body (25) and serve as a source of energy, interspecies comparison of intake might better be based on metabolic body weight (i.e., body weight 0.75) than on body weight directly (26,27). Using this factor, the rats per kg body weight 0.75 consumed only one-third more docosenoic fatty acids than the piglets, a difference which can hardly explain the difference in lipidosis. It is, therefore, possible that the more moderate lipidosis in the piglets than in the rats was due to a higher metabolic capacity for handling these acids (28). This question is of utmost importance since it is related to the problem of extrapolating findings from rat studies to the human nutrition situation, and deserves, therefore, further studies.

Three types of fats were compared as sources of docosenoic fatty acids in the present study: RSO containing erucic acid (22:1ω9 *cis*) (29), RFO containing mainly cetoleic acid (22:1ω11 *cis*) and PHFO containing mainly cetoleic acid, but also some erucic acid and, in addition, other positional isomers of docosenoic acid and also respective *trans* isomers (30). When compared at a similar dietary level of fat, RSO gave consistently more lipidosis than did RFO and PHFO in both piglets and rats. At levels which supplied approximately similar dietary levels of docosenoic fatty acids, different fats were not significantly different in inducing lipidosis in either species. Our results are not entirely consistant with those previously reported where cetoleic acid (22:1ω11) in fish oil was found to cause less lipidosis than erucic acid (22:1ω9) in rapeseed oil (18,31), and brassidic acid (22:1ω9 *trans*) in hydrogenated rapeseed oil to cause less lipidosis than erucic acid (22:1ω9 *cis*) in unhydrogenated rapeseed oil (32).

Lipidosis was not generally found in piglets consuming diets with less than 2% 22:1 fatty acids. When related to feed intake and body weight, the no-effect level for cardiac lipidosis was 0.4 g 22:1 fatty acids per kilogram body weight per day, a substantial level of intake.

Some piglets and rats fed high fat diets without docosenoic fatty acids and with only trace levels of eicosenoic acids, i.e., the control lard diet, developed lipidosis which, in general, was mild, but moderate in one pig suffering from pericarditis. Thus, lipidosis was not confined to the feeding of fats high in docosenoic fatty acids. In this respect, cardiac lipidosis from high fat diets, not necessarily containing docosenoic fatty acids, has been observed in cynomolgus monkeys and man (33-35).

The low levels of docosenoic fatty acids found in the cardiac triglycerides and cardiac NEFA of the lard-fed piglets are of a mangitude similar to that previously observed in animals fed docosenoic acid-free diets (33). However, in the present study, the rats fed the lard control diet with no obvious docosenoic fatty acid component had appreciable levels of docosenoic fatty acids in their cardiac triglycerides (i.e., 6.0%), but not in their cardiac NEFA. This finding is in agreement with Utne et al. (17) who found cardiac neutral lipids of rats fed docosenoic acid-free (0.2%) lard to contain 7.7, 0.5, 0.4 and 0.3% docosenoic fatty acids after 1, 2, 6 and 12 weeks of feeding. The physiological explanation for these findings is not immediately apparent.

With regard to NEFA, two hypotheses have been advanced, first that cardiac lipidosis is due to a malfunctioning of the mitochondria caused by elevated NEFA levels in the heart of rats fed diets high in eurcic acid (36), and second that elevation of NEFA in cardiac cells is responsible for lesions of a necrotic and fibrotic nature observed in male rats after long-term feeding of rapeseed oil (37). The present study demonstrates that the determination of cardiac NEFA is rather demanding. Separation of NEFA on TLC and quantitative determination on GLC gave values for NEFA which were only one fourth of those obtained by the titration method (9,10). The lack of a correlation between data obtained by the TLC-GLC method and by the titration method would indicate that substances other than NEFA are

titrated. Immediate freezing of the tissues reduced titrable NEFA to nearly half the values found with chilling of the tissues prior to extraction. Similar effects of post mortem autolyses on the cardiac NEFA levels have also been demonstrated by Kramer and Hulan (38) in a publication appearing after this work was completed. Thus, it appears that the previously reported data on cardiac NEFA levels (3,36), on which the hypotheses on deleterious roles of elevated cardiac NEFA in lipidosis and long term lesions is based (4, 36,37), may have been erroneously high by a factor of 10-20 due to artifacts and methodology. In the present study, regardless of methodology used, no consistent differences in cardiac NEFA levels were found between piglets fed on the different dietary fats. In the rat experiment, there were no significant differences in cardiac NEFA levels between dietary treatments, but rats fed 16% RSO or 20% RFO had 2-3 times higher NEFA levels in their hearts than in those of rats fed the other experimental fats, the latter groups all being similar. Kramer and Hulan (38) observed an increase in cardiac NEFA levels in rats fed high levels of RSO rich in erucic acid for 3 days, but no comparable observations on control rats fed other fats for a similar period of time were made in that study. If the differences in the cardiac NEFA levels found in the rat experiment were real and not caused by change, cardiac NEFA levels were not related to the degree of lipidosis. The hypothesis of a role of NEFA in cardiac lipidosis seems, therefore, to be of doubtful value.

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Inhibition of Sterologenesis in Brain and Liver of Fetal and Suckling Rats from Dams Fed Di-2-Ethylhexyl Phthalate Plasticizer

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ABSTRACT

The effect of di-2-ethylhexyl phthalate (DEHP) on lipid metabolism was studied in liver and brain from fetal rats taken 3 days before parturition from dams receiving dietary DEHP during gestation. In fetuses from rats receiving 0.5% or 1.0% DEHP in a stock diet, the incorporation of ^{14}C-acetate and labeled mevalonate (^{3}H or ^{14}C) into the C_{27} sterols, C_{30} sterols, and squalene fractions of brain tissue incubated in vitro was significantly reduced between the confidence limits $P < 0.05$ to $P < 0.001$. When liver from fetuses was incubated with labeled mevalonate, incorporation of label into the C_{27} sterol and C_{30} sterol fractions was significantly reduced as well ($P < 0.02$ to $P < 0.001$), whereas incorporation of labeled mevalonate into the squalene fraction was not significantly altered. The incorporation of ^{14}C-acetate into total hepatic lipids of the fetal rats was also studied, and statistically significant reductions in incorporation were observed in the lanosterol fraction ($P < 0.001$), the combined fraction of sterol esters + squalene ($P < 0.02$), and the combined fraction of cholesterol + diglycerides ($P < 0.01$). No significant changes were observed in the incorporation of ^{14}C-acetate into phospholipids, free fatty acids, or triglycerides. In 8-day old suckling rats delivered from dams fed 0.5% DEHP for the last 16 days of gestation and maintained on the same diet during the nursing period, the incorporation of ^{14}C-mevalonate into hepatic C_{27} sterols, in vitro, was significantly depressed ($P < 0.05$) whereas in corporation into C_{30} sterols and squalene was similar to control values. In these same suckling rats, body weights were significantly lower in the control group (21.7 vs. 18.8 g, $P < 0.01$), whereas liver weight as a % of body weight was significantly higher ($P < 0.01$) in rats nursing from the DEHP-fed dams. The results indicate that the inhibitory effect of dietary DEHP on lipid metabolism in the mature rat is transmitted across the placental barrier to the developing fetus and that the abnormal pattern of lipid metabolism in rats delivered from DEHP-fed females is only partially restored to normal during the suckling periods.

INTRODUCTION

Phthalic acid esters (PAE) may now be included on the growing list of environmental pollutants. PAE have such a broad spectrum of industrial and manufacturing use (1) that annual PAE production approximates 5 x 10^8 kg (2). Unfortunately the widespread use of PAE has led to their dispersal into the environment to such an extent that PAE are found in air (2,3), water (2,3), soil (2,4), milk (5), and tissues from man (6-9) and animals, including livestock and fish marketed for human consumption (10-13) as well as marine life (2,14). Until recently (within the last 5 years), information available on the biological effects of PAE was largely limited to classical toxicology. It is now clear from our studies (15-19) and those of others (20-22) that exposure to PAE administered orally to experimental animals results in numerous alterations in tissue biochemistry and ultrastructure (20-22). Our studies have documented the fact that di-2-ethylhexyl phthalate (DEHP), the most commonly used PAE, is an inhibitor of sterologenesis in mammalian liver (16,19) and testes (19).

The present studies of sterologenesis in fetal and suckling rats from dams fed DEHP was prompted by the evidence that PAE undergo placental transfer in the rat (23) and that PAE have been associated with teratogenesis and mutagenesis in rodents (24,25) and developing chick embryos (26-28).

EXPERIMENTAL PROCEDURES

Animals and Diets

Female Sprague-Dawley rats (Upjohn strain) were bred at 250 ± 3 g (n = 23) during a 10 hr exposure to a male rat. Presence of a vaginal plug was taken as evidence of insemination. The rats were individually caged with free access to food and water and were fed either a stock diet (Purina Chow) or the stock diet supplemented with 0.5% or 1.0% di-2-ethylhexyl phthalate (DEHP) as in our previous studies (15-19). DEHP feeding was initiated either 5 days (0.5% diet) or 10 days (1.0% diet) after breeding the rats. The dams were decapitated on the 18th day of gestation and the fetuses removed for study. In one experiment, female rats fed 0.5% DEHP were permitted to deliver and to nurse the pups while continuing to receive the 0.5% DEHP diet. The pups were killed by decapitation at 8 days of

TABLE I

Incorporation of DL-Mevalonic-2-^{14}C Acid into Brain Lipids of Fetal Rats from Dams Fed 0.5% DEHP during Gestation[a]

	Incorporation (dpm/brain)[b]		
	C_{27} Sterol	C_{30} Sterol	Squalene
Control (n=7)	13570[c] ± 700	8807 ± 291	6006 ± 346
DEHP (n=7)	11509 ± 520	7693 ± 276	4782 ± 118
	P<0.05[d]	P<0.02	P<0.01

[a]Pregnant rats were fed the DEHP-containing (0.5%) diet for 13 days beginning on the 5th day of gestation. Fetal rats were delivered by Cesarean section on the 18th day of gestation.

[b]The entire brain from each fetus was incubated as described under Experimental Procedures.

[c]Values are the means ± SEM of 7 animals (n); each animal was obtained from a different dam.

[d]P values obtained using Student's independent *t*-test.

age and their tissues removed for experimentation. All animals were killed between 9 AM and 10:30 AM.

Tissue Incubations

The entire liver and brain from fetal rats were incubated intact; livers of suckling rats, being larger than fetal rat livers, were minced (15) prior to incubation in order to ensure substrate penetration of the tissue.

All incubations were performed at 37 C in 25 ml Erlenmeyer flasks in a total volume of 3.5 ml of Krebs-Ringer-bicarbonate buffer at pH 7.4 which contained 2 μCi each of sodium acetate-1-^{14}C (59 mCi/mM) and DL-mevalonic-5-^{3}H acid, dibenzylethylene diamine salt (5 Ci/mM) in combination, or 2 μCi of DL-mevalonic-2-^{14}C, dibenzylethylene diamine salt (46 mCi/mM) alone. The labeled precursors were obtained from New England Nuclear Corp., Boston, MA. The buffer was gassed with oxygen-CO_2 (95%-5%) prior to use.

The whole fetal brains used in these studies all weighed within 3% of 0.11 g. Since the consistency of the tissue did not permit us to blot the brain before weighing, we considered the tissues to be of the same weight and reported all data on a per brain basis.

Analyses

Brain lipid was saponified by treatment of the tissue with 15% alcoholic-KOH as previously described in detail (16) and the nonsaponifiable lipid fraction was extracted with n-hexane (16). Liver samples were extracted by homogenization with $CHCl_3$-methanol, 2:1, as described previously (15), and the lipid extracts washed according to Folch et al. (29). In some experiments, aliquots of the $CHCl_3$-MeOH extracts of liver were evaporated to dryness under N_2 and then the lipid residues were saponified with alcoholic-KOH (16) and treated in the same manner as brain lipids.

Fractionation of lipids in the washed lipid extracts was achieved on Silica Gel G-coated thin layer plates using n-hexane/diethylether/acetic acid (146:50:4, v/v/v) as a developing solvent. This system will separate polar lipids (phospholipids) from the various classes of neutral lipids (30) as well as fractionate tissue nonsaponifiable lipids into the C_{27} sterols, C_{30} sterols, and hydrocarbons (squalene) (16). Methods for identifying the various lipids fractionated by thin layer chromatography, and the subsequent preparation of the samples for radioactive assay by liquid-scintillation spectrometry have been described in detail previously (15,16).

RESULTS AND DISCUSSION

In view of our previous observations that DEHP feeding can significantly modify lipid metabolism in mature rats (15-18), and that DEHP is capable of placental transfer (23), we investigated lipid metabolism in tissues of developing fetuses from dams fed either 0.5% or 1.0% DEHP during gestation. The results of the studies set forth in the tables which follow attest to the fact that ingestion of DEHP by pregnant rats does result in alterations in lipid metabolism in the developing fetus.

Studies with Fetal Tissue from Dams Fed 0.5% DEHP

In these studies, pregnant rats were fed DEHP at a level of 0.5% beginning on the 5th day of gestation and the fetuses were taken on the 18th day (4 days before term) by cesarean section. Lipid metabolism was studied in

TABLE II

Incorporation of DL-Mevalonic-5-^{3}H Acid into Hepatic Lipids of Fetal Rats from Dams Fed 0.5% DEHP during Gestation[a]

	Incorporation (dpm/mg dry wt)[b]		
	C_{27} Sterol	C_{30} Sterol	Squalene
Control (n=14)	6681[c] ± 301	533 ± 46	2874 ± 186
DEHP (n=13)	5674 ± 220	331 ± 23	2550 ± 191
	P<0.02[d]	P<0.001	NS

[a]See footnote to Table I.

[b]The liver from each fetal rat was incubated as described under Experimental Procedures.

[c]Values are means ± (SEM of the number of animals (n) given in parentheses. Animals in the Control group were obtained from 8 dams and those in the DEHP group were obtained from 7 dams; not more than 2 animals were obtained from an individual dam.

[d]P values obtained using Student's independent *t*-test.

TABLE III

Incorporation of Acetate-1-^{14}C into Hepatic Lipids of Fetal Rats from Dams Fed 0.5% DEHP during Geststion[a]

	Incorporation (dpm/mg dry wt)[b]					
	Phospholipid	C_{27} Sterol + DG	C_{30} Sterol	FFA	TG	CE/SQ[c]
Control (n=14)	3738[d] ± 234	13603 ± 709	481 ± 36	68 ± 4	3580 ± 373	4429 ± 337
DEHP (n=13)	3935 ± 205	11046 ± 440	247 ± 21	59 ± 3	2795 ± 238	3289 ± 256
	NS[e]	P<0.01	P<0.001	NS	NS	P<0.02

[a,b]See footnote to Table II.

[c]DG, diglycerides; FFA, free fatty acids; TG, triglycerides; CE, cholesteryl esters; SQ, squalene.

[d]See footnote c of Table II.

[e]NS, not significantly different from Control values by Student's independent *t*-test; P values indicate statistically significant differences at the levels shown.

vitro by incubating fetal brain and liver with radioactive acetate and/or mevalonate

Incorporation of ^{14}C-mevalonate into the nonsaponifiable lipids of fetal brain is shown in Table I. Significant reductions in the incorporation of ^{14}C-mevalonate into C_{27} sterols ($P < 0.05$), C_{30} sterols ($P < 0.02$), and into the sterol precursor squalene ($P < 0.01$) was observed in brain tissue of fetuses derived from DEHP-fed pregnant femals. The extent of the inhibition of incorporation into all 3 fractions was ca. 15% (Table I).

Hepatic synthesis of nonsaponifiable lipid from labeled mevalonate was also reduced in fetuses from females fed DEHP (Table II); significant reductions in the incorporation of ^{3}H-mevalonate were observed in the C_{27} sterol fraction ($P < 0.02$) and in the C_{30} sterol fraction ($P < 0.001$). The reduced incorporation of ^{3}H-mevalonate into C_{27} and C_{30} sterols by fetal liver derived from DEHP-fed females was not paralleled by a significant reduction of incorporation into squalene suggesting, perhaps, that the inhibition of sterol synthesis in these fetuses occurs at a point beyond squalene formation in the biosynthetic pathway.

Lipid biosynthesis from ^{14}C-acetate was also studied in the fetal liver and the results are presented in Table III.

Feeding 0.5% DEHP to pregnant rats resulted in significant reductions in ^{14}C-acetate incorporation into several lipid fractions in the fetal liver. Incorporation into lanosterol was reduced 49% ($P < 0.001$), while incorporation into the combined fractions of cholesterol + diglyceride, and steryl esters + squalene was reduced 19% ($P < 0.01$) and 26% ($P < 0.02$) respectively. In contrast, ^{14}C-acetate incorporation into phospholipids free fatty acids, or triglycerides (Table III) was not significantly affected. This latter observation is interesting in that DEHP feeding inhibits phospholipid and triglyceride synthesis in the liver of mature rats (15,18). The data suggest that modification of phospholipid and triglyceride synthesis in liver may be a response related to growth and development since we have observed in some rapidly growing rats (3-4

TABLE IV

Incorporation of Acetate-1-^{14}C and DL-Mevalonic-5-^{3}H Acid into Brain Lipids of Fetal Rats from Dams Fed 1% DEHP during Gestation[a]

	Incorporation (dpm/brain)[b]					
	C_{27} Sterol		C_{30} Sterol		Squalene	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Control (n=8)	25300[c] ± 1796	19283 ± 1452	6355 ± 473	6379 ± 527	1905 ± 113	2728 ± 269
DEHP (n=6)	18377 ± 2242	11759 ± 1543	4121 ± 451	3277 ± 419	1285 ± 84	1220 ± 153
	P<0.05[d]	P<0.01	P<0.01	P<0.001	P<0.01	P<0.001

[a]Pregnant rats were fed the DEHP-containing (1.0%) diet for 8 days beginning on the 10th day of gestation. Fetal rats were delivered by Cesarean section on the 18th day of gestation.

[b]See footnote to Table I.

[c]Values are means ± SEM of the number of animals (n) given in parentheses. Animals in the Control group were obtained from 3 dams, and those in the DEHP group were obtained from 2 dams; not more than 3 animals were obtained from an individual dam.

[d]See footnote to Table I.

TABLE V

Incorporation of DL-Mevalonic-5-^{3}H Acid into Hepatic Lipids of Fetal Rats from Dams Fed 1% DEHP during Gestation[a]

	Incorporation (dpm/mg dry wt)[b]		
	C_{27} Sterol	C_{30} Sterol	Squalene
Control (n=9)	4992[c] ± 491	387 ± 53	2895 ± 695
	P<0.01[d]	P<0.01	P<0.01
DEHP, 8 days (n=6)	3009 ± 169	165 ± 6	289 ± 17
DEHP, 14 days (n=2)	2637	250	2375

[a]Pregnant rats were fed the DEHP-containing (1%) diet for 8 or 14 days beginning on the 10th or 4th day of gestation, respectively. Fetal rats were delivered by Cesarean section on the 18th day of gestation.

[b]See footnote to Table II.

[c]Values are means or means ± SEM of the number of animals shown in parentheses. Animals in each group were obtained from 2 dams treated appropriately with respect to diet and duration of DEHP feeding.

[d]See footnote to Table I.

weeks old) an actual stimulation of phospholipid and triglyceride synthesis when DEHP was fed (Bell, unpublished results).

Studies with Fetal Tissue From Dams Fed 1.0% DEHP

In these studies, pregnant rats were fed DEHP at a level of 1.0% beginning on the 10th day of gestation, and the fetuses wee taken by cesarean section on the 18th day of gestation. Fetal brain and liver was incubated with labeled lipid precursors as in the previous experiments, but only the nonsaponifiable lipids were studied.

Table IV shows that 1% DEHP feeding to pregnant rats has more pronounced effects on fetal brain lipid biosynthesis than were observed when DEHP was fed at the 0.5% level (Table I). The incorporation of both ^{14}C-acetate and ^{3}H-mevalonate into C_{27} sterols, C_{30} sterols, and squalene was significantly reduced ($P < 0.04$ - $P < 0.05$) by ca. 40% in fetal brain from DEHP-treated dams. The livers from these same fetal rats were used to study the incorporation of ^{3}H-mevalonate into hepatic nonsaponifiable lipids (Table V); included also in these experiments were livers from 2 fetal rats taken from a dam that received 1% DEHP beginning on the 4th day of gestation (Table V). In fetuses whose dams were consuming DEHP for 8 days of gestation prior to cesarean section, incorporation of ^{3}H-mevalonate into C_{27} sterols and C_{30} sterols was reduced significantly ($P < 0.01$ in each case) (Table V).

The reduction of incorporation into the sterols, relative to control values, was ca. 2 to 3-fold greater than was observed in similar fetal studies in which the dams were fed 0.5% for 13 days. In comparing the data from Tables II and V, it appears that treatment of the

TABLE VI

Incorporation of DL-Mevalonic-2-^{14}C Acid into Hepatic Lipids of 8-Day Old Suckling Rats of Dams Fed 0.5% DEHP[a]

	Pup Wt (g)	Liver Wt (% body wt)	Incorporation (dpm/g wet wt)[b]	
			$C_{27} + C_{30}$ Sterol	Squalene
Control (n=10)	21.7[c] ± 0.3	2.75 ± 0.03	390594 ± 28088	231670 ± 12602
DEHP (n=12)	18.8 ± 0.7	3.01 ± 0.06	310739 ± 20798	290598 ± 24933
	P<0.01[d]	P<0.01	P<0.05	NS

[a]Female rats were fed the DEHP-containing (0.5%) diet beginning on the 5th day of gestation and permitted to deliver and nurse the pups for 8 days while continuing to receive the DEHP-containing diet.

[b]Livers were removed from the 8-day-old suckling rats, minced, and incubated as described under Experimental Procedures.

[c]Values are means ± SEM of the number of animals (n) given in parentheses. Control animals were obtained from one dam; animals in the DEHP group were obtained in equal numbers from 2 dams.

[d]See footnote to Table I.

pregnant rats with the higher level of DEHP (1%) for a short period (8 days) has a greater effect on fetal hepatic lipid synthesis than a lower level (0.5%) for a longer period of time (13 days)

The data from the 2 fetuses whose dams were fed 1% DEHP for 14 days of gestation also had lower levels of incorporation into C_{27} and C_{30} sterols, relative to control fetuses; these same two animals also showed a lower incorporation of label into squalene, but no statistical test could be made on the data with only 2 observations.

Studies With Suckling Rats

The incorporation of ^{14}C-mevalonate into hepatic nonsaponifiable lipids of suckling rats delivered by dams fed 0.5% DEHP during gestation, as well as during the nursing period, was also studied (Table VI). It is clear from the data of Table VI that the inhibition of hepatic C_{27} sterol biosynthesis observed in the fetal rats is sustained during the postnatal period; the reduction in incorporation of ^{14}C-mevalonate into the C_{27} sterol fraction of liver in the suckling rats was significantly lower than in control livers (ca. 24% reduced, $P < 0.05$) (Table VI). Other evidence of toxicity included significantly lower body weights in pups from the DEHP-fed dams ($P < 0.01$) and significantly greater relative liver weights ($P < 0.01$).

Since neither the suckling rat tissues nor the milk from the dams was analyzed for DEHP, we are unable to say whether or not the sustained inhibition of C_{27} sterol biosynthesis reflects an accumulation of tissue DEHP (or DEHP metabolite) carried over from in utero exposure or a continuous exposure via transfer of DEHP from the dam via the milk, or both. The fact that the incorporation of ^{14}C-mevalonate into the C_{30} sterols did not differ in the two groups of suckling rats (Table VI), suggests, perhaps, that the biochemical effects resulting from in utero exposure to DEHP (Tables II and V) are reversible to some extent during the postnatal period. The fact that DEHP feeding to the dams had no significant effect on the incorporation of labeled mevalonate into squalene in the suckling rat livers (Table VI), or in the fetal rat livers (Tables II and V), is somewhat puzzling in that, in our previous studies with mature rats fed DEHP (16,18), incorporation of labeled mevalonate into hepatic squalene was significantly reduced ($P < 0.01$).

The difference in response of livers from fetal rats (Table II) or young rats (Table VI) vs. those of the mature rat (16,18), with respect to the effect of DEHP on squalene biosynthesis, may reflect adaptative changes which develop during in utero exposure to DEHP which are not operable in the mature rat.

CONCLUSIONS

The studies presented here represent a continuation of our work on the effects of phthalate esters on mammalian lipid metabolism. From these data, as well as from our previous data (15-18), it is clear that phthalate esters can significantly perturb lipid metabolism in liver, testes, heart, brain and fatty tissue; alter the pattern of plasma lipoproteins; and exert an effect on the developing fetus in utero. Although most of our studies have been conducted in rats, we have done limited studies with rabbits (17,19) and pigs (17) which indicate that the effect of phthalates on lipid metabolism is not limited to

a single species. Because of the widespread environmental contamination by phthalates and their increasing use by industry, their potential for producing serious biological effects needs to be assessed immediately.

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Linoleic Acid Absorption in the Unanesthetized Rat: Mechanism of Transport and Influence of Luminal Factors on Absorption

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ABSTRACT

Linoleic acid intestinal absorption was studied in the unanesthetized rat. At low (21-1260 μM) intraluminal concentrations, absorption took place by facilitated diffusion; while at high (1.26-2.5 mM) concentrations, simple diffusion was the predominant mechanism of transport. At low concentrations (840 μM), the equimolar additions of oleic, linolenic, and arachidonic acids or lecithin inhibited the absorption of linoleic acid. Substitution of potassium for sodium in the buffer solution, substitution of Tween 80 for sodium taurocholate, or decrease in the hydrogen ion concentration all resulted in decreased rate of linoleic acid absorption. Increase in sodium taurocholate concentration, or perfusate flow rate increased linoleic acid's absorption. These experiments demonstrate that linoleic acid is absorbed by a concentration-dependent dual mechanism of transport. The absorption rate is modified by the pH, surfactant type and concentration, the simultaneous presence of other polyunsaturated fatty acids, and the thickness of the unstirred water layer.

INTRODUCTION

Linoleic acid is the major dietary essential fatty acid in man. It is a polyunsaturated long chain fatty acid which cannot be synthesized by mammals but is synthesized readily by plants. Gastrointestinal absorption of this essential fatty acid is crucial in maintaining proper and adequate supply of this fatty acid. Dietary deficiency of linoleic acid or its intestinal malabsorption can result in reduced growth rate, wide variety of skin disorders, increased platelet aggregation, decreased prostaglandin synthesis, and perhaps increased incidences of arteriosclerotic cardiovascular disease (1).

Despite its immense biological importance and despite the fact that intestinal absorption of linoleic acid is crucial for its proper supply, information regarding its mechanism of absorption by the small intestine and the factors which may influence the absorption process is not available. We have previously studied the basic uptake mechanism of this essential fatty acid by everted gut sacs in vitro. These experiments demonstrated that, in vitro, this fatty acid is absorbed by a concentration-dependent dual mechanism of transport. Carrier-mediated passive diffusion was found to be the predominant mechanism of absorption at low concentrations while at higher concentrations, passive diffusion was found to be predominant. The absorption rate of linoleic acid in vitro was found to be influenced by the pH, surfactant type and concentration, the simultaneous presence of other polyunsaturated fatty acids, and the thickness of the unstirred water layer (2).

Since the above information was obtained in vitro with everted gut sacs, we decided to confirm and extend these observations in vivo in the unanesthetized rat. Studies using everted gut sacs have the advantages of allowing strict control over experimental variables and of providing the ability to test the absorptive process for energy dependence by the use of metabolic inhibitors (3). However, in vitro absorption studies suffer a disadvantage of a constantly deteriorating tissue which is not supplied by normal lymphatic and vascular circulations (4). Further, since they are unable to transfer lipids into the serosal compartment, everted gut sacs are especially unsuitable for the study of transmural transport of lipid compounds such as linoleic acid. Therefore, in order to gain a fuller understanding of the absorptive mechanism of linoleic acid, we have undertaken to study the process by perfusion of the fatty acid through intact small intestinal segments with well preserved vascular and lymphatic circulations in the conscious restrained rat.

MATERIALS AND METHODS

[1-^{14}C] Linoleic acid (New England Nuclear, Boston, MA) with specific activity of 50.6 mCi/mmole was used as a tracer compound. The radiochemical purity of the compound was greater than 98% by thin layer chromatography (TLC) on Silica Gel G developed in

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hexane/diethyl ether/acetic acid (70:30:1). Nonradioactive linoleic acid (Sigma Chemical Co., St. Louis, MO) was found to have less than 1% impurities by TLC. (^{3}H)Inulin (Amersham/Searle Corp., Arlington Heights, IL) with specific activity of 0.9 Ci/mmole and radiochemical purity of greater than 99% was used as a nonabsorbable marker (5). Purified grade sodium taurocholate (Calbiochem Co., San Diego, CA) was found to have less than 1% impurities by TLC (6). In some experiments, a nonionic surfactant Tween 80 (Fisher Scientific Co., Fairlawn, NJ) was used for solubilizing linoleic acid. Oleic, linolenic, and arachidonic acids with purity greater than 99% were obtained from Sigma Chemical Co.; L-α-lecithin type III-E from egg yolk was purchased from Sigma Chemical Co. Analytical Reagent grade sodium or potassium salts of monobasic or dibasic phosphate (J.T. Baker Chemical Co., Phillipsburg, NJ) were used as buffer components. A micellar solution of the surfactant in the phosphate buffer was prepared by ultrasound irradiation for 5 min at 70 watts of power with a sonicator (Artek Corp., Farmingdale, NY). The standard micellar perfusate (pH 6.5) contained the following components: linoleic acid (0.84 mm), sodium taurocholate (10 mM), sodium dihydrogen phosphate (85.67 mM), disodium hydrogen phosphate (45.56 mM), and tracer amounts of ^{14}C-linoleic acid and ^{3}H-inulin. On separate occasions, excipients such as lecithin and fatty acids other than linoleic were added to the buffer solution at equimolar concentration of 0.84 mM. The pH of the perfusion solution was varied between 5.4 and 7.4 by changing the relative concentrations of the sodium salts or potassium salts of phosphate. The osmolarity of the final solution ranged from 285 to 315 mosmoles per liter (7).

Experimental Methods

Male Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, MI) weighing 200-250 g had free access to water and Purina Rat Chow (Ralston Purina Co., St. Louis, MO), and were not fasted prior to experimentation. After the rat was anesthetized with ether, its jejunum was exposed by a midline abdominal incision. An inflow microbore tubing (S-54-HL, Scientific Products, McGraw Park, IL) was introduced into the Jejunum 5 cm distal to the ligament of Treitz and was secured in place by encircling ligatures. The jejunum, 15 cm distally to the inflow cannula, was cannulated with an L-shaped glass tubing (0.098 x 0.157 in) with a funnelled cannulating tip. The inflow tube and the outflow cannula were secured to the abdominal wall and the rat's abdomen was closed with sutures. The rat was allowed to awaken and was placed in a plexiglass restraint cage. The rat's body temperature was maintained at 37 C with a heating pad connected to a thermostatic temperature controller (Yellow Springs Instrument Co., Yellow Springs, OH) which was activated by a rectal temperature probe. A syringe pump (H 351, Sage Instruments, Cambridge, MA) was used to infuse the micellar solution at a constant flow rate of 0.53 ± 0.01 ml/min. The perfusate was collected out of the outflow cannula in six separate 10 min collections. 100 μl Aliquots were withdrawn in triplicates from each 10 min collection for determination of the remaining unabsorbed linoleic acid. Infusate samples taken prior to perfusion were used for determination of the initial specific activity of linoleic acid. At the end of perfusion, the rats were sacrificed by an overdose of ether, and the length of the perfused jejunum was measured with standardized methods of stretching and dessication (8).

Radioactivity Determinations

Aliquots of the perfusate (100 μl) were placed directly into liquid scintillation counting vials containing 10 ml of a dioxane-based scintillation cocktail (9). Radioactivity was measured to a ± 1% counting error by using a liquid scintillation counter with automatic quench calibration at ambient temperature (Beckman LS 250, Beckman Instruments, Fullerton, CA).

Calculations and Statistical Analysis

Absorption rates were calculated according to the following equation:

$$R = \frac{CPM_o - CPM_t}{CPM_o} \frac{A \cdot V}{T} \frac{10}{L}$$

where R is absorption rate (nmoles/min per 10 cm); CPM_o and CPM_t are specific activities of linoleic acid at zero time and at given time, respectively; A is amount (nmoles) of linoleic acid in 1 ml of perfusate; V is the volume (ml) of perfusate within a given interval; T is time period (min) of sample collection; and L is the length (cm) of the perfused segment. The absorption rates of linoleic acid under various experimental conditions were compared statistically to base-line data by using Student's t-test (10) and ANOVA (11). The data were plotted and analyzed using the least squares method of regression analysis (12) and a NLIN computer program.

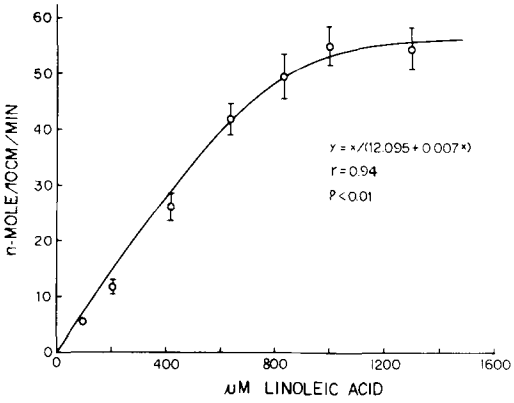

FIG. 1. Absorption rate of linoleic acid at low intraluminal concentrations. Each point represents mean ± S.E. absorption rate of at least four separate rat experiments. The rectangular hyperbolic equations were obtained by an NLIN regression analysis program.

RESULTS

Assessment of Fluid Shifts

Fluid shifts that may have taken place during the 1 hr of perfusion were evaluated by the addition of ^{3}H-inulin to the perfusion solution which contained 840 μM nonradioactive linoleic acid, 10 mM sodium taurocholate in the standard phosphate buffer (pH 6.5). Less than 1% net fluid absorption was found to take place during three 1-hr perfusion experiments. Since the amount of water absorption was minimal, the experimental results reported in this communication were not corrected for fluid shifts.

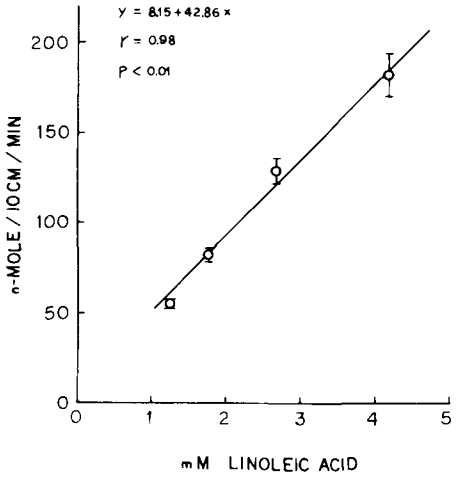

FIG. 2. Absorption rate of linoleic acid at high intraluminal concentrations. Each point represents mean ± S.E. absorption rate of at least four different rat experiments. The regression line was plotted by the least squares method.

Influence of Linoleic Acid Concentration on its Absorption Rate

The relationship between the concentration of linoleic acid and its absorption rate was studied in a micellar solution which contained varied concentrations of linoleic acid, and 10 mM sodium taurocholate in the standard phosphate buffer (pH 6.5). At first, absorption experiments were performed in the low range of intraluminal concentrations of linoleic acid (21-1260 μM). In each experiment, the variability in linoleic acid absorption rate between the different 10 min collections was negligible. At least four different animal experiments were performed at each linoleic acid concentration and the results from all the separate 10 min collections were pooled and expressed as a mean ± S.E. rate of absorption. The relationship between linoleic acid's absorption rate and its concentration at the low range of intraluminal concentrations was found to fit best to a rectangular hyperbola (Fig. 1) indicating that a saturable absorption mechanism is the predominant process of transport at this range of concentrations. On the other hand, at the higher range of intraluminal concentrations of linoleic acid (1.26-4.2 mM), the relationship between the absorption rate and the concentration fitted best to a linear plot (Fig. 2) indicating that a simple diffusion mechanism may be the dominant process of transport of linoleic acid in the higher range of intraluminal concentrations.

Influence of Other Fatty Acids and Lecithin on Linoleic Acid Absorption

In order to evaluate the possibility of interactions between linoleic acid and other fatty acids, the unsaturated long chain fatty acids oleic (18:1), linolenic (18:3), and arachidonic (20:4) were added separately at 840 μM concentration to the standard micellar solution containing equimolar concentrations of linoleic acid and 10 mM sodium taurocholate at pH 6.5. The absorption rate of linoleic acid decreased markedly as the number of double bonds of the added fatty acid increased (Table I). To assess the possible influence of the phospholipid lecithin on the absorption of linoleic acid, lecithin was added at 840 μM to the standard micellar solution containing equimolar concentration of linoleic acid solubilized in 10 mM sodium taurocholate at pH 6.5. A striking decrease in the absorption rate of linoleic acid was observed following the addition of lecithin (Table II).

TABLE I

Influence of Other Unsaturated Fatty Acids on 840 μM Linoleic Acid Absorption

Fatty acid added	No. of Animals	Absorption nmol/min/10cm[a]	P value[b]
None	5	48.5 ± 5.5	---
Oleic	4	28.4 ± 0.7	< 0.01
Linolenic	4	27.2 ± 1.3	< 0.01
Arachidonic	4	25.8 ± 0.7	< 0.01

[a]Mean ± S.E.
[b]Statistical analysis of the data was made by comparing the absorption rate of linoleic acid in the presence of other fatty acids to its absorption rate in their absence.

TABLE II

Influence of Lecithin and Potassium on 840 μM Linoleic Acid Absorption

Additive (nM)	No. of Animals	Absorption nmol/min/10cm	P Value
None	5	48.5 ± 5.5	---
Lecithin (0.84)	4	15.5 ± 0.9	< 0.01
Potassium[a] (176.8)	4	29.2 ± 1.4	< 0.01

[a]Perfusion solution was prepared by the replacement of sodium phosphate with potassium phosphate in the buffer.

TABLE III

Influence of Surfactants on 840 μM Linoleic Acid Absorption

Surfactant (nM)	No. of Animals	Absorption nmol/min/10cm	P Value
Tween 80 (5)	4	18.2 ± 1.2	< 0.01
Taurocholate (5)	4	37.4 ± 1.1	---
Taurocholate[a] (7)	4	42.8 ± 1.8	< 0.05
Taurocholate (10)	5	48.5 ± 5.5	< 0.01

[a]Absorption at 5 mM sodium taurocholate concentration served as a baseline value for comparison with absorption under the other experimental conditions.

Influence of Replacement of Sodium with Potassium on Linoleic Acid Absorption

In this series of experiments, NaH_2PO_4 and Na_2HPO_4 were replaced with KH_2PO_4 and K_2HPO_4. The final buffer solution consisted of 85.67 mM KH_2PO_4 and 45.56 mM K_2HPO_4. The total potassium ion concentration was 176.70 mM. The complete substitution of Na+ with K+ resulted in a marked decrease in the absorption rate of linoleic acid (Table II).

Influence of Surfactants on Linoleic Acid Absorption

The absorption of 840 μM linoleic acid was investigated in the presence of 5, 7, or 10 mM sodium taurocholate in the standard phosphate buffer solution. The absorption rate increased with the increase in sodium taurocholate concentration (Table III). Since sodium taurocholate is an anionic surfactant, we also studied the influence of a nonionic surfactant, Tween 80, on linoleic acid absorption. Sodium tauro-

TABLE IV

Influence of Perfusate Flow Rate on 840 μM Linoleic Acid Absorption

Flow Rate[a] (ml/min)	No. of Animals	Absorption[a] nmol/min/10cm	P Value[b]
0.14 ± 0.01	4	19.8 ± 0.7	---
0.33 ± 0.01	4	33.1 ± 1.0	< 0.01
0.53 ± 0.01	5	48.5 ± 5.5	< 0.01
4.25 ± 0.12	4	70.5 ± 10.0	< 0.01
8.65 ± 0.47	4	133 ± 12.3	< 0.01

[a]Values are mean ± S.E.
[b]Statistical differences in absorption were calculated by comparing absorption rate with the next lower flow rate absorption.

TABLE V

Influence of the Hydrogen Ion Concentration on 840 μM Linoleic Acid Absorption

pH	No. of Animals	Absorption nmol/min/10cm	P Value[a]
7.3	4	35.3 ± 3.2	---
6.5	5	48.5 ± 5.5	< 0.01
5.4	4	51.8 ± 2.4	< 0.01

[a]Statistical analysis of the data was made by comparing absorption at pH 7.4 to absorption at lower pH values.

cholate was replaced by Tween 80 in separate series of experiments at 5 mM concentration. A significant decrease in the absorption rate of linoleic acid was seen in the presence of Tween 80 (Table III).

Influence of Perfusion Rate on Linoleic Acid Absorption

To evaluate the influence of the thickness of the unstirred water layer on linoleic acid absorption, the perfusion rate was varied from 0.14 to 8.65 ml/min in order to decrease the thickness of the unstirred water layer (13). The absorption rate of 840 μM linoleic acid increased with the increase in perfusion rate (Table IV).

TABLE VI

Linoleic Acid Absorption Expressed as a Percentage of the Amount of Linoleic Infused[a]

Concentration (mM)	0.21	0.42	0.63	1.05
% Absorption	23	15	13	11
Concentration (mM)	1.26	1.68	2.52	4.20
% Absorption	9.8	8.9	8.3	8

[a]Percentage absorption represents the mean value of all the experiments performed at each concentration.

Influence of Hydrogen Ion Concentration on Linoleic Acid Absorption

The pH of the perfusion solution was varied from 5.4 to 7.4 by changing the relative amounts of the monobasic and dibasic salts of phosphate in the solution. The perfusion solution contained 840 μM linoleic acid and 10 mM sodium taurocholate. A significant increase in the absorption rate of linoleic acid was found as the hydrogen ion concentration was increased (Table V).

Percentage Absorption of Infused Linoleic Acid

The absorption of linoleic acid was calculated in terms of percentage absorption of the amount infused per hour. The mean percent value was calculated from the data used for plotting Figures 1 and 2. At low intraluminal concentrations (0.21-1.05 mM), 11-23% of the infused fatty acid was absorbed. At high intraluminal concentrations (1.26-4.20 mM), only 8-9.8% of the infused fatty acid was absorbed (Table VI).

DISCUSSION

The intestinal transport mechanism and the physico-chemical factors operant in linoleic acid absorption were studied in vivo in the

unanesthetized rat. The relationship between its concentration and its absorption rate was examined over a wide range of concentrations (21 μM-4.2 mM). At low intraluminal concentrations of linoleic acid (21-1260 μM), its absorption delineated apparent saturation kinetics (Fig. 1); therefore, in this range of concentrations facilitated transport is the predominant mechanism of absorption and may be either energy-requiring or passive. These observations, coupled with previous findings of no change in linoleic acid absorption following the additions of metabolic inhibitors and uncouplers in vitro (2), indicate that linoleic acid absorption in this range of concentrations occurs by facilitated diffusion. At higher intraluminal concentrations (1.26-4.2 mM), the relationship between the concentration and absorption rate was linear (Fig. 2), indicating that linoleic acid absorption in this range of concentrations is taking place by passive diffusion. These findings, which are consistent with the previous in vitro observations (2), lead us to conclude that passive diffusion, which is the predominant mechanism of linoleic acid absorption at high concentrations, conceals the coexisting facilitated diffusion that was dominant at lower concentrations. A similar concentration-dependent dual mechanism of transport has been reported for other nutrients such as thiamine (14,15), cyanocobalamine (16), retinol (8), and arachidonic acid (17).

At low intraluminal linoleic acid concentrations, where facilitated diffusion is the predominant mechanism of transport, 11-23% of the infused fatty acid was absorbed. On the other hand, only 8-9.8% of linoleic acid was absorbed in the millimolar range of concentration with a predominant passive diffusion mechanism of transport. Thus, it appears that the facilitated diffusion mechanism which predominates at low intraluminal concentrations is associated with a greater efficiency of absorption of linoleic acid than the passive diffusion at high intraluminal concentrations (Table VI).

In order to gain more insight into the mechanism of transport, we evaluated the influence of other polyunsaturated fatty acids on linoleic acid's absorption. The separate additions of oleic, linolenic, and arachidonic acids to the perfusion solution significantly decreased the absorption rate of linoleic acid (Table I). The addition of these long chain fatty acids causes an enlargement of the micelles which reduces their diffusion rate towards the absorptive cell membrane and results in a decrease in linoleic acid's absorption rate. The added fatty acids may also hinder linoleic acid's absorption by competing with it for binding to the fatty acid binding protein (FABP) which is thought to facilitate the intracellular transport of long chain fatty acids from the lipid cell membrane through the aqeuous cytosol to the intracellular organelles (18-20). The longer their chain length and the greater the number of unsaturated double bonds per molecule (18), the tighter is the fatty acid's binding affinity for the FABP. These properties of FABP would account for the greater inhibitory effect of arachidonic acid than that of oleic acid (18:1) on linoleic acid's absorption (Table I). These results suggest that FABP could be the common carrier involved in the absorption of fatty acids and could be responsible for the saturation kinetics of linoleic acid's absorption at low luminal concentrations (Fig. 1).

Lecithin, a common phospholipid which is secreted with bile into the proximal small intestinal lumen, was found to be a potent inhibitor of linoleic acid absorption (Table II). Lecithin may inhibit linoleic acid absorption by expanding the micellar size and thereby decreasing the rate of diffusion of the micelles towards the absorptive cell membrane (21). Furthermore, lecithin has been shown to cause changes in the physical characteristics of cell membranes (22); these changes may hinder the transfer of linoleic acid across the absorptive cell membrane.

The hydrogen ion concentration in the lumen of the small bowel decreases as a function of the distance of the intestinal segment from the pylorus. We investigated the influence of the hydrogen ion concentration on the absorption of linoleic acid by modifying the components of the buffer solution to vary the pH from 7.4 to 5.4. The absorption rate of linoleic acid increased markedly (Table V) as the hydrogen ion concentration increased. Two separate mechanisms may account for this finding. The first mechanism has to do with neutralization of the negative surface charge of the absorptive cell membrane. Since the luminal absorptive cell membrane and the micelles are negatively charged (21, 23), the amount of resistance to the micellar diffusion increases as the micelles approach the absorptive cell membrane (24). The addition of hydrogen ions to the perfusate would lower the negative surface charge of the absorptive cell membrane and thereby the resistance to the diffusion of the micellar particles. The result would be an increase in linoleic acid absorption rate (Table V). Changes in the degree of ionization of linoleic acid itself could offer another explanation for the increase in linoleic acid absorption

at higher hydrogen ion concentrations. Linoleic acid possesses a pKa of 6.5 in a sodium taurocholate micellar solution (25). Therefore, as the hydrogen ion concentration is increased, the proportion of linoleic acid in the protonated form would also increase. The shift of linoleic acid to the protonated form would cause a decrease in the negative surface charge of the linoleic acid carrying micelles and would, therefore, decrease the diffusional resistance of the micelles towards the absorptive cell membrane as well. Both of these postulated mechanisms indicate that the relative acidity of the proximal small bowel is advantageous for the absorption of linoleic acid.

We studied the influence of the bile salt concentration on the absorption rate of linoleic acid by varying the sodium taurocholate concentration from 5 to 10 mM. An increase in the sodium taurocholate concentration resulted in a parallel increase in the absorption rate of linoleic acid (Table III). As the bile salt concentration is increased, fatty acids such as linoleic would shift from the oil to the micellar phase in the lumen of the small bowel (26). Absorption of linoleic acid appears to be directly proportional to its micellar concentration which, when raised by higher sodium taurocholate concentrations, would increase the driving force of the micelle across the unstirred water layer towards the absorptive cell membrane itself (27).

When the nonionic surfactant, Tween 80, was substituted for sodium taurocholate, the absorption rate of linoleic acid decreased from 37.4 ± 1.1 to 18.2 ± 1.2 nmoles/10/cm/min. Tween 80, with a molecular weight of 1308, is a much larger molecule than sodium taurocholate and combines with linoleic acid to form a larger sized micellar particle which would be absorbed at a slower rate. It is also possible that the micellar particles formed by Tween 80 have a greater affinity for linoleic acid than those formed by sodium taurocholate. An increased affinity for linoleic acid would explain the decreased rate of linoleic acid absorption observed in the presence of Tween 80 micelles.

In order to assess the possibility of sodium dependence of the linoleic acid absorption process, we substituted potassium phosphate for sodium phosphate in the buffer base. The absorption rate of linoleic acid decreased markedly in the absence of sodium in the luminal perfusate (Table II). The transport of sugars and amino acids has been known to depend on the presence of sodium ions (28). Sodium ions could be necessary for the formation of the proper complex between linoleic acid and its carrier in much the same way as sodium is needed for the cotransport of sugars or amino acids. In the absence of sodium ions, the facilitated portion of linoleic acid's absorption is markedly inhibited (Table II), and transport could be taking place primarly via the passive diffusion route.

The unstirred water layer at the luminal cell surface is a known barrier to absorption of lipids (26,27). We investigated the influence of the thickness of the unstirred water layer on linoleic acid absorption by a stepwise increase in the flow rate of the perfusate from 0.14 to 8.65 ml/min. As the flow rate of the perfusate is increased, the thickness of the unstirred water layer is decreased. In the present series of experiments, the absorption rate of linoleic acid increased in parallel with the increase in flow rate (Table IV). This observation indicates that the unstirred water layer is indeed a significant limiting step in the absorptive pathway of linoleic acid and that factors which modify its thickness in vivo (29) may also modify the absorption rate of this essential fatty acid.

Linoleic acid is an essential dietary fatty acid in man. It is able to participate and influence the metabolism of prostaglandins. It is also thought to be important in modifying cholesterol absorption and metabolism and thereby modifying the process of atherosclerosis. The present information regarding the absorption of linoleic acid in vivo coupled with the previous observations regarding its absorption in vitro (2) provide us with an understanding of the mechanisms responsible for the absorption of this fatty acid and some of the physiological factors which modify its rate of absorption.

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Cholesterol Metabolism in the Liver and Intestine of the Chick: Effect of Dietary Cholesterol, Taurocholic Acid and Cholestyramine

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ABSTRACT

The effect of feeding cholesterol, taurocholic acid, or cholestyramine to chicks on cholesterogenesis from [1-^{14}C] acetate in liver and intestine was determined in vitro using tissue slices, and in vivo by i.v. injection of [^{14}C] acetate. The conversion of cholesterol to bile acids in liver in vivo was measured in the same treatments after i.v. injection of [^{3}H] cholesterol. Hepatic cholesterogenesis in vitro and in vivo was depressed by dietary cholesterol and taurocholate and enhanced by cholestyramine. Intestinal cholesterogenesis in vivo was depressed only by taurocholate whereas ileal cholesterogenesis in vitro was reduced by dietary cholesterol. Conversion of cholesterol to bile acids was enhanced by dietary cholesterol and cholestyramine and depressed by taurocholate. Hepatic cholesterol metabolism in the chick appears to be regulated by mechanisms similar to those reported for other species.

INTRODUCTION

Hepatic cholesterol metabolism is sensitive to dietary cholesterol which inhibits cholesterol synthesis by a negative feedback action at the level of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMG, EC 1.1.1.34) (1), and increases cholesterol conversion to bile acids (2,3). Furthermore, increasing the amount of bile acids circulating between liver and small intestine reduces the synthesis of cholesterol and its conversion to bile acids (1,4,5).

Intestinal cholesterogenesis has been reported by some to be inhibited by dietary cholesterol (6-8), whereas others were not able to observe any effect (9,10). Dietschy (11) showed that intestinal cholesterogenesis was inversely correlated with the bile acid concentration in the intestinal lumen in the rat. Subsequent experiments by Shefer et al. (12) indicated that the effect was indirect, resulting from enhanced cholesterol absorption at high bile acid concentrations.

In the chicken, dietary cholesterol has been shown to depress hepatic cholesterogenesis (13), whereas cholestyramine produces the opposite effect (14). However, in this species, the factors affecting bile acid production and the regulation of intestinal cholesterogenesis are not known. The object of the present study was to investigate cholesterol synthesis in the liver and intestine of the chick in vitro and in vivo, and, simultaneously, hepatic conversion of cholesterol to bile acids in vivo.

MATERIALS AND METHODS

Day-old New Hampshire x Leghorn male chicks were maintained in a battery brooder for 21 days and fed a commercial starter diet. Chicks were then allowed access ad libitum to the experimental diets detailed in Table I for 7 days. The diets differed from each other by the inclusion of cholesterol (Sigma Chemical Co., St. Louis, MO, recrystallized from ethanol), taurocholic acid (Sigma Chemical Co.) or cholestyramine ("Cuemid," Merck, Sharp and Dohme, West Point, PA). The control diet contained no cholesterol.

At the end of 7 days on the diet, weight gains were similar in all groups, and in vitro and in vivo incorporation tests were performed. All experiments were carried out at the same time of day. All labeled compounds used in these tests were from the Radiochemical Centre, Amersham. The in vitro tests were carried out by incubating tissue slices with sodium [1-^{14}C]-acetate (specific activity 59.5 mCi/mmol), as previously described (16). The in vivo tests were performed as follows: chicks were given an intravenous injection of 2.0 μCi sodium [1-^{14}C]-acetate (specific activity 12.0 mCi/mmol) and 6.0 μCi of (1,2-^{3}H) cholesterol (specific activity 310 mCi/mmol). The labeled compounds were dispersed in 0.9% NaCl to which 0.25% Tween 80 had been added, the volume injected into each chick being 0.5 ml. Thirty min after the injection, animals were killed by decapitation and portions of liver, jejunum and ileum were removed immediately and homogenized with 15% ethanolic KOH (w/v) in a high speed homogenizer. The jejunal segment was 5 cm long, starting from a point 10 cm distal to the common bile duct, and the ileal segment extended from 15 to 10 cm from the caecal junction. Samples were heated under reflux for 30 min, diluted with an equal volume of water, and extracted three times with

TABLE I

Composition of Experimental Diets (%)

Ingredient	Designation of dietary treatments			
	Control	Cholesterol	Taurocholic acid	Cholestryamine
Glucose	36.4	36.2	36.1	35.4
Cellulose	4.0	4.0	4.0	3.5
Cholesterol	---	0.2	---	---
Taurocholic acid	---	---	0.3	---
Cholestyramine	---	---	---	1.5
Constant ingredients[a]	59.6	59.6	59.6	59.9

[a]In % of diet: olive oil, 5; defatted soybean meal (45% protein), 50; vitamin mixture (15), 0.4; mineral mixture (15), 0.1; sodium chloride, 0.25; choline chloride (50%), 0.2; DL-methionine, 0.15; dicalcium phosphate, 2.5; calcium carbonate, 1.0.

petroleum ether. The successive extracts of unsaponifiable materials were combined and washed with 5% (w/v) aqueous KOH and then with water. The alkaline and aqueous washings were combined and added back to the corresponding saponified aqueous solutions. These were then acidified with HCl to the methyl red end point and acidic material was extracted three times with diethyl ether. Calibration of the method with taurocholic and glycocholic acids resulted in recovery of 90.3 ± 1.6 and 90.8 ± 1.4%, respectively (Mean ± SD of five replicates). The ether extracts were combined and washed thoroughly with water. Aliquots of the unsaponifiable material were taken for cholesterol determination by the colorimetric method of Searcy and Bergquist (17). Gas chromatography of the unsaponifiable fraction (15) from the different tissues revealed that over 95% of this fraction was cholesterol. Aliquots were also taken from the ether extract of acidic material for bile acid determination by the method of Singer and Fitschen (18). Radioactivity was determined in additional aliquots by liquid scintillation using separate channels for determining ^{3}H and ^{14}C simultaneously. The effects of quenching and of counts of one isotope in the channel of the other were corrected using internal standards of both isotopes.

Calculations

The extent of incorporation of labeled acetate into cholesterol and bile acids in vitro and in vivo was expressed as a percentage of the dose per g tissue. The conversion of [^{3}H]-cholesterol to bile acids was similarly expressed, but in addition, conversion rates k were also calculated, based on the linear increase in radioactivity of bile acids R_{BA} with time t, $R_{BA} = k(t-t_o)$, where t_o is the time lag before the appearance of radioactivity in the bile acids.

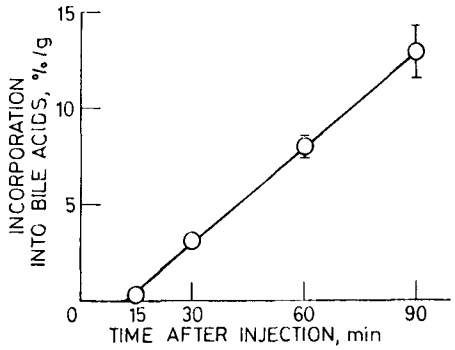

FIG. 1. Time course of incorporation of ^{14}C into hepatic cholesterol (Ch) and bile acids (BA) after i.v. injection of sodium [1-^{14}C] acetate. Each point is the mean of three chicks; SE values are shown where they do not fall within the circles. Note the different incorporation scales for cholesterol and bile acids.

Such a relation was found for the control group (see Fig. 1) and was assumed to apply also to the other groups. It can readily be seen that the constant rate of increase of radioactivity R_{BA} with time represents a special case of the general rate equation for the formation of labeled product $dR_{BA}/dt = k'S_{Ch}(t)$, where S_{Ch} represents the specific radioactivity of the cholesterol precursor and k' is the rate constant. Over the 30 min time period studied, S_{Ch} varies by less than 5%and may thus be considered constant, hence the above linear relation between R_{BA} and t.

Analysis of variance and multiple range tests were performed according to standard procedures (19).

RESULTS

The effects of the various treatments on hepatic cholesterol and bile acid concentrations in liver and intestine are summarized in Table II. Among the treatments, only dietary choles-

TABLE II

Hepatic Cholesterol and Bile Acid Concentrations and Cholesterol Concentrations in Intestinal Wall of Chicks on the Experimental Diets[a]

	Dietary treatments			
	Control	Cholesterol	Taurocholic acid	Cholestyramine
	mg/g tissue			
Liver				
Cholesterol	2.35 ± 0.03a	2.77 ± 0.03b	2.26 ± 0.04a	2.31 ± 0.04a
Bile acids	0.47 ± 0.02a	0.58 ± 0.05a	1.11 ± 0.09b	0.48 ± 0.02a
Jejunum				
Cholesterol	1.06 ± 0.06a	1.12 ± 0.01a	1.06 ± 0.04a	1.05 ± 0.02a
Ileum				
Cholesterol	1.01 ± 0.03a	1.02 ± 0.04a	0.96 ± 0.01a	1.01 ± 0.04a

[a]Means ± SE for 6 chicks. Values in rows not followed by the same letter differ significantly ($P < 0.05$).

TABLE III

Incorporation of [^{14}C]Acetate into Cholesterol in Vitro[a]

	Dietary treatments	
	Control	Cholesterol
	% of dose/g tissue	
Liver	1.73 ± 0.11a	0.95 ± 0.08b
Jejunum	0.21 ± 0.01a	0.20 ± 0.02a
Ileum	0.50 ± 0.02a	0.41 ± 0.02b

[a]Means ± SE for 5 chicks. Values in rows not followed by the same letter differ significantly ($P < 0.05$).

terol significantly affected hepatic cholesterol concentration. Liver bile acids were increased slightly by dietary cholesterol and considerably by dietary taurocholate. The dietary treatments did not alter intestinal cholesterol levels.

In vitro incorporation of [1-^{14}C]acetate into cholesterol was measured in livers and intestines of chicks receiving the control and cholesterol diets detailed in Table I, and results are presented in Table III. Dietary cholesterol depressed hepatic cholesterogenesis by nearly one-half, and also slightly reduced ileal synthesis, but had no significant effect in the jejunum. The capacity of the ileum for cholesterol synthesis in the control group was over twice that of the jejunum but less than one-third that of the liver.

The time course of the in vivo incorporation of [1-^{14}C]acetate into hepatic cholesterol and bile acids in control chicks is shown in Figure 2. After an initial lag period of ca. 12 min, ^{14}C began to appear in liver cholesterol, reaching a maximum between 30 and 60 min after injection. ^{14}C began to appear in bile acids in liver after a similar lag period, and its amount rose at

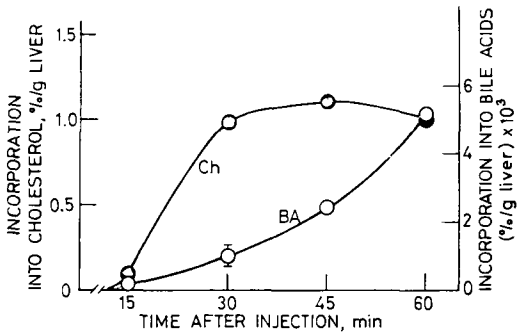

FIG. 2. Time course of incorporation of ^{3}H into hepatic bile acids after i.v. injection of [^{3}H]cholesterol. Each point is the mean of three chicks; SE values are shown where they do not fall within the circles. The regression equation is: Incorporation (% of dose/g tissue) = 0.164_t - 1.982, r = 0.98, when t is the time in minutes after the injection. The values of % incorporation per g of gall bladder bile acids were identical with the liver values.

a steadily increasing rate until 60 min. However, the radioactivity of the bile acids was comparatively low, of the order of 1% of that of cholesterol. The time course of [^{3}H]bile acid formation in the liver bile acids after injection of tritiated cholesterol is shown in Figure 1. Again, a lag period of some 12 min was observed after which the amount of ^{3}H increased linearily until 90 min. Incorporation measurements into gall bladder bile acids yielded identical results.

Results of cholesterol labeling after injection of [^{14}C]acetate are summarized in Table IV. Dietary taurocholate depressed ^{14}C incorporation into cholesterol in liver and in both intestinal segments tested. Dietary cholesterol decreased cholesterogenesis in liver, but its effects in the jejunum and ileum were not

TABLE IV

Incorporation of [^{14}C] Acetate into Cholesterol in Vivo in Chicks, 30 Min after I.V. Injection of Labeled Acetate[a]

	Dietary treatments			
	Control	Cholesterol	Taurocholic acid	Cholestyramine
	% of dose/g tissue			
Liver	0.098 ± 0.003a	0.069 ± 0.002b	0.060 ± 0.003b	0.124 ± 0.004c
Jejunum	0.030 ± 0.002a	0.031 ± 0.002ab	0.025 ± 0.002b	0.037 ± 0.002a
Ileum	0.042 ± 0.004a	0.032 ± 0.004ab	0.027 ± 0.001b	0.039 ± 0.002a

[a]Means ± SE for 6 chicks. Values in rows not followed by the same letter differ significantly ($P < 0.05$).

TABLE V

Hepatic Conversion of [^{3}H] cholesterol to Bile Acids, and Specific Activity of Cholesterol in Liver, 30 Min after I.V. Injection of Labeled Cholesterol into Chicks[a]

	Dietary treatments			
	Control	Cholesterol	Taurocholic acid	Cholestryamine
Incorporation				
% of dose/g liver	0.104 ± 0.021a	0.214 ± 0.033b	0.089 ± 0.007a	0.167 ± 0.027b
Rate constant[a], μg/min/g liver	8.0 ± 0.4a	18.8 ± 1.4b	6.8 ± 0.3c	13.0 ± 1.1d
Specific activity	±	±	±	±
Cholesterol, dpm/μg	61.1 ± 4.5a	53.9 ± 4.4a	63.3 ± 5.0a	61.2 ± 3.8a

[a]Means ± SE for 6 chicks. Values in rows not followed by the same letter differ significantly ($P < 0.05$).

[b]$k' = R_{BA}S_{Ch}^{-1}(t - t_o)^{-1}$, where R_{BA} is the radioactivity of the bile acids after 30 min; S_{Ch} is the final specific activity of cholesterol; $t = 30$ min; and $t_o = 12.5$ min.

significant. The cholestyramine treatment caused a pronounced increase in ^{14}C incorporation into liver cholesterol, but failed to affect cholesterol synthesis in the intestinal segments. Treatment effects on cholesterol specific activity (not shown) paralleled the incorporation results.

Results of bile acid labeling in the livers of chicks injected with [^{3}H] cholesterol are presented in Table V. The cholesterol and cholestyramine treatments resulted in enhanced incorporation of label, compared with the controls, while taurocholate depressed the incorporation. The same effects were found when incorporation rate constants were calculated, as described under Methods.

DISCUSSION

The incorporation of [^{14}C] acetate into cholesterol in vitro measures the capacity of the tissue under the given experimental conditions to synthesize cholesterol but does not necessarily reflect conversion in vivo. In this study, the in vitro approach yielded considerably higher incorporation values than the in vivo technique, particularly in the ileum.

Cholesterol caused a reduction of cholesterol synthesis from acetate in vitro in liver and to a lesser extent in the ileum, but it had no effect in the jejunum. These results agree with reports and magnitudes repeated for other species, in which cholesterol synthesis was more sensitive to dietary cholesterol in the liver than in the intestine (6,7,20). The guinea pig constitutes an exception to this rule (8).

The results on hepatic acetate incorporation in vivo also resemble the pattern found in other species (7,9,10), with dietary cholesterol and taurocholate showing depressing effects, while cholestyramine enhanced cholesterogenesis. Intestinal synthesis was affected significantly only by taurocholate, although cholesterol also consistently tended to depress it.

While it is reasonable to assume that the specific activity of the acetate precursor is the same in the different tissues and in the different treatments after injection, and thus the percentage incorporation of the dose per g tissue is

a suitable basis for comparing cholesterogenesis (21,22), this is not the case for bile acid synthesis from [^{3}H]cholesterol, where different cholesterol specific activities are found in different tissues and for different treatments. In that case, the general equation, according to which the rate of increase in radioactivity in the product at any one time is proportional to the specific radioactivity of the precursor at that time, should be used. But since the cholesterol specific activity was practically constant during the time interval studied, bile acid radioactivity was a simple linear function of time, so that the rate constant could readily be determined experimentally. According to both percentage incorporation and rate measurements, bile acid production was found to be stimulated by dietary cholesterol and cholestyramine, with slight differences in the quantitative effects of the treatments being observed between these two approaches.

In summary, regulation of hepatic cholesterogenesis in the chick appears to be similar to that found in other species, with dietary cholesterol depressing cholesterol synthesis and enhancing bile acid production, taurocholate depressing both cholesterol and bile acid synthesis, and cholestyramine increasing both cholesterol and bile acid synthesis. However, intestinal cholesterogenesis was influenced mainly by taurocholate, which could be due to a double feedback inhibition of cholesterol synthesis at the HMG CoA reductase level. Alternatively, the taurocholate could exert its action by increasing cholesterol absorption. However, this did not seem to be the case in rats, where feeding taurocholate did not reduce the intestinal HMG CoA reductase activity (12). It should be noted that taurocholate is not the major bile acid in the chick.

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Influence of Elevated Levels of Linoleic Acid on the Thermal Properties of Bovine Milk Fat

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ABSTRACT

The thermal properties of bovine milk fat containing 15.5% linoleic acid have been compared with those of milk fat containing a normal level (1.8%) of linoleic acid in order to examine the influence of altered triglyceride (TG) composition on their physical characteristics. The total TGs of 18:2-rich milk fat melted over the range -38 to 30 C compared with the range -33 to 34 C for control milk fat. Polymorphism exhibited by the high mol wt TGs of control milk fat was absent in the same fraction of 18:2-rich milk fat. Similarly, the complex melting thermogram of the low mol wt TGs of control milk fat and its obvious polymorphic behavior contrasted with the single broad melting peak of the low mol wt TGs of 18:2-rich milk fat. This solid miscibility in the 18:2-rich milk fat could be a consequence of the lower proportion of saturated TGs or the presence of high proportions of diene and triene TGs containing 18:2 instead of monoene and diene TGs containing 18:1.

INTRODUCTION

The positional specificity of fatty acids in the triglycerides of bovine milk fat is relatively constant (1-3), and normal seasonal fluctuations in the fatty acid composition (4), and major changes induced by special feeding procedures (5) affect the relative proportions of the constituent triglyceride species rather than their nature.

Increased levels of 18:2 in bovine milk fat were accompanied by decreased levels of 14:0 and 16:0 (3,6) thereby increasing the proportions of high molecular weight triglycerides and decreasing the proportions of low molecular weight triglycerides. Within each of these broad molecular weight groupings, there were higher proportions of diene, triene, and tetraene triglycerides and lower proportions of saturated and monoene triglycerides (3). The isolation and chemical characterization of triglyceride fractions of markedly different fatty acid and triglyceride composition from bovine milk fat have provided the opportunity to assess the contribution of these changes to the physical characteristics of milk fat.

MATERIALS AND METHODS

The milk fat used for thermal analysis was obtained from a pair of monozygous twin cows fed the same basal diet of fresh and dried grass. The diet of one cow was supplemented with formaldehyde-treated sunflower seed. The cows were in the 2nd month of lactation, and the milks analyzed were representative of the 12th day of the experimental feeding period. The fats with normal (1.8 mole %) and elevated (15.5 mole %) levels of 18:2 were each separated into triglyceride fractions of high, medium and low molecular weight by silicic acid chromatography and each fraction subjected to stereospecific analysis (3,5).

Thermal analysis of the total milk fats and their constituent fractions was carried out using the Perkin-Elmer differential scanning calorimeter (model DSC-1B), calibrated as described by Norris et al. (7). Data from the DSC was corrected for thermal lag, temperature and power calibration using a Hewlett-Packard 9830 calculator coupled to a data acquisition system. Triglycerides were melted and 5-8 mg transferred to a preweighed sample pan. The sample was shock-cooled to -100 C in liquid N_2 and, after equilibration, a heating thermogram recorded up to 50-60 C at a rate of 16 C/min. The heating thermograms for the control milk fat and its fractions corresponded closely with those obtained using a heating rate of 8 C/min (8). The tempering procedure for samples showing an exothermic transition consisted of heating the sample from -100 C at 16 C/min to the temperature of the exothermic transition, holding at this temperature for 5 min, followed by shockcooling to -100 C and equilibrating at this temperature for 5 min before recording the heating thermogram.

Liquid fat contents were estimated by constructing an integral curve from heating thermograms, assuming a constant heat of melting, after they had been corrected for temperature and power calibration and thermal-lag effects (9).

RESULTS AND DISCUSSION

Changes in Triglyceride Composition and Structure

The fatty acid and triglyceride composition

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TABLE I

Contributions of Triglyceride Fractions of High, Medium and Low Molecular Weight and Triglyceride Classes of Differing Levels of Unsaturation to Control and 18:2-Rich Milk Fats

Sample	Nature of milk fat	Proportions of fractions and classes of triglyceride in milk fat (moles %)					
		total	satd.	monoene	diene	triene	polyene
Total milk fat triglycerides	control	100	40.7	38.6	13.1	7.6	---
Total milk fat triglycerides	18:2-rich	100	25.8	26.2	26.6	11.9	9.5
High molecular weight triglycerides	control	36.1	10.6	15.9	6.4	3.2	---
High molecular weight triglycerides	18:2-rich	43.0	8.6	10.7	11.8	6.7	5.2
Medium molecular weight triglycerides	control	19.7	9.2	6.2	2.7	1.6	---
Medium molecular weight triglycerides	18:2-rich	19.5	5.5	6.2	4.3	1.8	1.7
Low molecular weight triglycerides	control	44.2	20.9	16.5	4.0	2.8	---
Low molecular weight triglycerides	18:2-rich	37.2	11.7	9.4	10.4	3.4	2.6

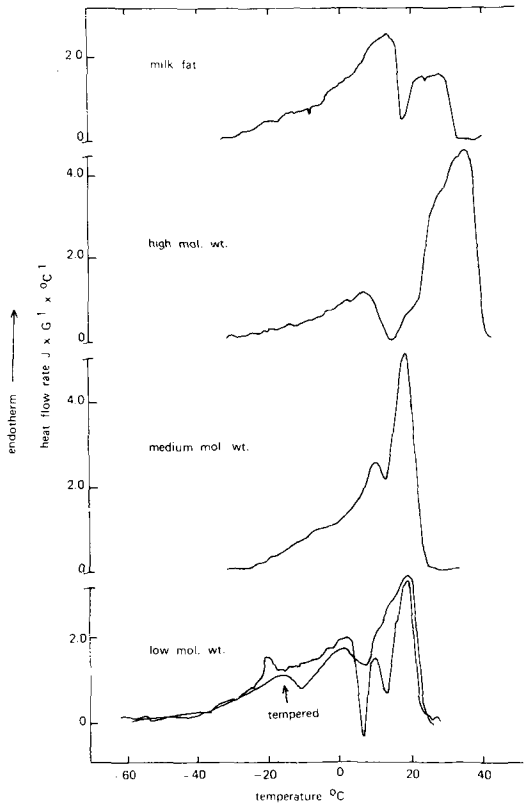

FIG. 1. Heating thermograms of the triglycerides of milk fat containing normal levels of 18:2, and its high, medium and low molecular weight fractions.

and the stereospecific analysis of the high, medium and low molecular weight triglycerides from the control and 18:2-rich milk fats which were thermally analyzed in these experiments have been described in detail (3,5). The stereospecific distribution of the major fatty acids was virtually unchanged when elevated levels of 18:2 were present (5), thus the 18:2-rich milk fat appeared to contain the same range of molecular species of triglyceride as milk fats of normal composition, although their relative proportions were considerably altered (Table I). In summary, the major changes (without regard to positional specificities) in the 18:2-rich milk fat, as compared to the control milk fat, were lower proportions of 000 (saturated fatty acids ≡ 0; monoenoic fatty acids ≡ 1; dienoic fatty acids ≡ 2) (25.8% compared to 40.7%) and 001 (26.2% compared to 38.6%) which were compensated by much increased proportions of triglycerides such as 002 (18.8% compared to 1.8%), 012 (11.0% compared to 2.8%) and 022 (9.5% compared to 0%) (3).

Melting Curves of Control and 18:2-Rich Milk Fat

The most notable features of the melting curves of the two total milk fats were an increased proportion of TGs melting below -13 C in the 18:2-rich milk fat (23% compared to 8%) and a decreased proportion of TGs melting above 17 C (13% compared to 27%). The proportion of TGs melting between -13 and 17 C remained at 64-65%. Increasing the 18:2-content of milk fat removed the broad high melting peak at 21-31 C in the melting curves as well as moving the main melting peak from 14 to 12, and lowering the temperature for the start (-33 C to -38 C) and the finish to melting (34 C to 30 C) (Figs. 1 and 2). Tempering did not remove the large gap occurring at 18 C in the control milk fat suggesting that a polymorphic transition was not occurring at that temperature unless the transition was replaced by a melting gap introduced by tempering. The liquid fat content of the 18:2-rich milk fat at all temperatures between -30 and 30 C was greater than that of the control milk fat, which was similar to results obtained by Edmondson et al. (10).

Melting Curves of Milk Triglyceride Fractions

The lower temperature for the finish of melting of the high molecular weight triglycerides from 18:2-rich milk fat (Figs. 1 and 2) was probably due to the reduction in the proportions of saturated triglycerides since, when isolated, these saturated constituents melted between 35 and 45 C (8). Tempering the control fraction removed the dip in the thermogram centered at 15 C without affecting the remainder of the thermogram, thus indicating a polymorphic transition. The absence of a division of the high molecular weight triglycerides of the 18:2-rich milk fat into two melting peaks could be due to solution effects arising from the presence of ca. 14% 002 (5). The triglycerides melting below -20 C would most likely be polyene species (Table I), e.g., 121 and 122.

The thermogram of the low molecular weight fraction of the control milk fat has a large broad peak melting between -41 and 4 C followed by an exothermic transition at 6 C, a dip at 13 C and the main melting peak at 19 C (Fig. 1). Tempering at 6 C substantially removed the exothermic transition at 6 C (Fig. 1), indicating the presence of a polymorphic transition at 6 C. In contrast, the thermogram of the low molecular weight fraction of the 18:2-rich milk fat exhibited one broad melting peak centered around 0 C (Fig. 2) corresponding closely in shape with that region in the corresponding fraction of the control milk fat melting below 6 C, which in turn is similar to the thermogram of the unsaturated triglycerides of low molecular weight in normal milk fat (8). This suggests that the saturated and unsaturated triglycerides in this fraction of 18:2-rich milk fat exhibit appreciable solid solution formation, which is in contrast to the corresponding control fraction where little solid miscibility occurs. This could be a consequence of the lower proportion of saturated triglycerides or the presence of high proportions of diene and triene triglycerides containing 18:2 instead of monoene and diene triglycerides containing 18:1 (3).

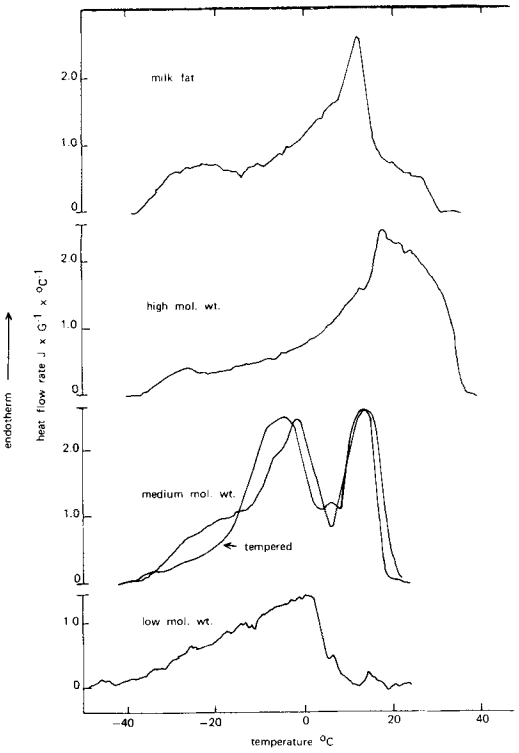

FIG. 2. Heating thermograms of the triglycerides of 18:2-rich milk fat and its high, medium and low molecular weight fractions.

The medium mol wt fraction of the control milk fat melted over the range -25 to 25 C with a single main melting peak at 19 C following a minor melting peak at 10 C (Fig. 1). On the other hand in the 18:2-rich milk fat, the medium mol wt fraction contained two major melting peaks centered at -2 and 13 C. Tempering this sample at the temperature of the dip between the melting peaks caused the first peak to be shifted from -2 C to -10 C (Fig. 2) but did

not remove the gap occurring at 5 C, suggesting that a polymorphic transition was not occurring at that temperature.

Liquid Fat Contents

At 0 C, the high, medium and low mol wt fractions of the 18:2-rich milk fat contained 21%, 56% and 79% liquid fat, respectively, as measured by integrating under the melting curves, compared to 11%, 21% and 52% for the same fractions of the control milk fat. At 20 C where the low and medium mol wt fractions of the 18:2-rich milk fat were completely in the liquid state and the high mol wt fraction was 64% liquid, the high mol wt fraction of the control milk fat was only 24% liquid and the low and medium mol wt fractions of this milk fat were 95% and 92% liquid, respectively.

The suitability of milk fats containing increased levels of 18:2 for use in the manufacture of butter with modified spreadabilities may be judged by their melting characteristics. At 2-5 C, the 18:2-rich milk fat, containing 15.5 moles % 18:2 and having 45-51% liquid phase, was considered almost spreadable, whereas the control milk fat was not (29-34% liquid phase). A problem associated with milk fats containing 15-20 moles % 18:2 is their very narrow spreadable range as a result of the fat greatly favoring the liquid state at temperatures above 20 C (>90%). This is consistent with the oiling off at 20 C observed by Wood et al. (11) in butter made from milk fat containing more than 15% 18:2.

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Effect of Lecithin on Jejunal Absorption of Micellar Lipids in Man and on Their Monomer Activity in vitro

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ABSTRACT

The effect of lecithin on jejunal absorption of fatty acids and octadecenoylglycerol was studied in healthy volunteers with a jejunal perfusion system which excluded pancreatic and biliary secretions from the test segment. Lecithin significantly reduced the absorption of oleic acid ($P < 0.05$) and octadecenoylglycerol ($P < 0.01$), while it had no effect on the absorption of ricinoleic acid. In vitro, lecithin reduced monomer activities of all three lipids; the changes were greater for oleic acid and octadecenoylglycerol than for ricinoleic acid ($P < 0.02$). From these data it is concluded that lecithin reduces monomer activity of fatty acids in mixed micellar solutions and that it can thereby reduce the absorption rates of micellar lipids. Intact lecithin is not absorbed under these conditions. Maldigestion of lecithin in pancreatic insufficiency may, therefore, aggravate the steatorrhea observed in this condition.

INTRODUCTION

Lecithin (1,2-diacyl-*sn*-glycero-3-phosphocholine) is an obligatory component of bile. It facilitates solubilization of biliary cholesterol (1) and protects the mucosa of the gallbladder (2) and small bowel (3) from the adverse effects of dihydroxy bile acids. Its role in the process of fat absorption is only incompletely defined. In the intestinal lumen, dietary and biliary lecithin is under normal circumstances hydrolyzed to lysolecithin (1-acyl-*sn*-glycero-3-phosphocholine) and fatty acids (4). Lysolecithin is effectively absorbed from the intestine (4-7). In rats with biliary fistulae, fat release from the mucosa into the lymph is significantly impaired in the absence of lecithin (8,9). On the other hand, lecithin impairs uptake of fatty acids and cholesterol by everted sacs of rat intestine in vitro (10) and inhibits the absorption of fatty acids and bile acids by the rat small intestine in vivo in the absence of pancreatic phospholipase A_2 (EC 3.1.1.4) (11). The mechanism by which lecithin exerts this effect is not clear.

This paper reports studies in the human jejunum of the effects of lecithin on absorption of fatty acids and a monoacylglycerol, 2-octadecenoylglycerol, in comparison with a nonmicellar solute, glucose. In addition, we determined monomer activities of fatty acids and 1-octadecenoylglycerol in mixed micellar solutions under the influence of lecithin in vitro. The results offer an explanation how unhydrolyzed lecithin inhibits lipid absorption.

METHODS

Materials

Chemicals used were identical to those described in a previous paper (12) with the following additions: glycodeoxycholic acid was synthesized as described previously (13); the final product was greater than 95% pure by thin layer chromatography (TLC). Deoxycholate was purchased from Schuchardt (Munich, Germany). For the in vitro experiments, 1-octadecenoylglycerol was purchased from Nu Chek Prep (Elysian, MN) and 1-octadecenoyl-[2-^{3}H]glycerol from ICN Pharmaceuticals, Clevelenad, OH. Highly purified egg lecithin (Sigma, St. Louis, MO) was further purified by column chromatography (14). The final product was greater than 98% pure by TLC. Prior to use in the partition experiments, all labeled compounds were repurified by TLC.

Perfusion Technique

The subjects were healthy male volunteers age 21 or older who gave informed written consent. The experiments were performed according to protocols approved by the Human Studies Committee of the Mayo Clinic. A 4-lumen tube was used with an occluding balloon positioned at the ligament of Treitz to exclude pancreatic and biliary secretions from the test segment of proximal jejunum (15). The intubation procedure has been described in detail elsewhere (12). Perfusates at 370 C were delivered at a constant rate of 10 ml/min through a port just distal to the balloon and were sampled 25 cm distally by siphonage. An aspiration lumen proximal to the balloon was suctioned intermittently to remove duodenal contents. Test solutions were perfused for 90 min; the first 30 min were used for equilibration. Thereafter, each study period consisted of six consecutive 10 min samples.

"Steady state conditions" were confirmed by stable concentrations of polyethylene glycol during these sequential sampling periods, and all results refer to observations during the steady state (12,16).

Experimental Design and Composition of Perfusion Solutions

Data from two groups of experiments are reported, and those of a third group of experiments reported previously (12,16) are used for comparison. Each group of experiments comprised a set of four perfusions in each of four healthy volunteers. The first group of experiments (Group I) was originally designed to study the effects of 2-octadecenoylglycerol and lecithin on fluid secretion induced by ricinoleic acid (12-hydroxy-Δ9,10-octadecenoic acid) (12). Each of the four subjects was perfused with (a) an electrolyte solution, (b) the electrolyte solution with ricinoleic acid 5 mM, (c) the electrolyte solution with ricinoleic acid 5 mM and 2-octadecenoylglycerol 2.5 mM, and (d) the electrolyte solution with 5 mM ricinoleic, 2.5 mM 2-octadecenoylglycerol and 2.5 mM lecithin. The control electrolyte solution contained (in mM); Na 120, K 10, Cl 100, HCO_3 30, glucose 11.2, xylose 11.2, and taurocholate 10 for micellar solubilization; pH was 7.5 and osmolality 280 mOsm/1. All solutions contained polyethylene glycol-4000 (PEG) 5 g/l as nonabsorbable marker. Appropriate test solutions contained [^{14}C]octadecenoylglycerol 5 μCi/1 or [^{3}H]ricinoleic acid 25 μCi/1 or both. The results of net water movement and sugar absorption have been reported previously (12,16). Here net movement of lecithin and the absorption of 2-octadecenoylglycerol and ricinoleic acid in the presence and absence of lecithin will be reported.

The second experiment (Group II) was initally designed to examine the effects of ricinoleic acid, oleic acid and two conjugated bile acids on lecithin absorption. The perfusates were: (a) electrolyte solution with 1.25 mM lecithin and 5 mM taurocholate; (b) electrolytes, lecithin, taurocholate and 5 mM ricinoleic acid; (c) electrolytes, lecithin, taurocholate and 5 mM oleic acid; and (d) electrolytes, lecithin and 5 mM glycodeoxycholate. The electrolyte solution contained the following (in mM): Na 120, K 10, Cl 100, HCO_3 30, glucose 11.2 and xylose 11.2; PEG 5 g/l with [^{14}C]PEG 5 μCi/1; pH was 7.5 and osmolality 280 mOsm/1. Solutions b and c also contained [^{3}H]ricinoleic acid or [^{3}H]oleic acid, respectively, at 20 μCi/l. Oleic acid absorption during perfusion with solution d was compared with data of fatty acid absorption during perfusion of 5 mM oleic acid in the absence of lecithin, which have been reported previously (12).

Analytical Methods

PEG was determined chemically or as [^{14}C]PEG (17). Absorption of fatty acids and octadecenoylglycerol was measured by the recovery of tritium or ^{14}C in the perfusion solutions. For isotope determinations, 1 ml of perfusate of effluent was mixed with 15 ml of a scintillation cocktail composed of toluene and emulsifier (Ready Solv VI, Beckman Instruments, Inc., Fullerton, CA) and counted in a liquid scintillation counter (Beckman, Model LS-255). Quench correction was made by external standardization. Samples containing two isotopes were counted in two channels. Counts per min were converted into disintegrations per min for each isotope with a computer program which corrected for quesching and spillover of ^{14}C into the tritium channel (18). Spillover of tritium into the ^{14}C channel was less than 1%.

Isomerization of 2-octadecenoylglycerol was measured in samples removed from the perfusion reservoir (37 C) at 0, 15, 30, 60 and 90 min and compared with that in samples of intestinal effluent taken during each 10 min "steady state" collection period (30-90 min). One ml of each sample was immediately mixed with 3 ml of heptane/ethanol/ether (1:1:1, v/v) (19) and after extraction cooled in dry ice-acetone until the organic phase could be plated on TLC plates of silica gel impregnated with boric acid. The plates were immediately developed in a solvent system containing chloroform/acetone/methanol/acetic acid (85:12.5:2.5:0.5, v/v) (20). This sytem separates 2- and 1-octadecenoylglycerol and free fatty acids. Spots containing fatty acids and octadecenoylglycerol were identified with iodine vapor, scraped off and extracted into a solution containing 10% acetic acid and the toluene-based scintillation cocktail and subsequently counted. Isomerization was calculated in percent from the ratio of radioactivities recovered from the 2- and 1-octadecenoylglycerol bands.

Effluents were analyzed for phospholipids by extracting 1 ml aliquots immediately into 5 ml of chloroform/methanol (2:1, v/v) (21). Samples were stored in tightly sealed vials until the chemical determination of lecithin as the organically bound phosphorus extracted in the chloroform phase according to the method of Kraml (22). This is a semiautomated method for the determination of phospholipids in which stannous chloride-hydrazine is used as reducing agent. Glucose was determined by the glucose-oxidase method (Boehringer).

TABLE I

Effect of Lecithin on Jejunal Absorption of Fatty Acids and Octadecenoylglycerol[a]

Test circumstances (Concentration in mM)	n	Absorption/25 cm jejunum			
		Fatty acid μmol/min	Octadecenoylglycerol μmol/min	Glucose μmol/min	Water[b] ml/min
I. Ricinoleate (RA) 5	4	14.6 ± 1.2	---	40.3 ± 4.0[c]	-1.7 ± 0.5[c]
RA 5 + Octadecenoyl-glycerol 2.5	4	14.3 ± 5.0	6.3 ± 2.4	41.1 ± 11.8[c]	-1.5 ± 0.2[c]
RA 5 + Octadecenoyl-glycerol 2.5 + Lecithin (Lec) 2.5	4	13.3 ± 3.7	2.3 ± 1.7[d]	43.7 ± 9.2[c]	-1.4 ± 0.6[c]
II. Oleate 5 + Lec 1.25	4	11.8 ± 3.2[e]	---	73.1 ± 13.1	0.5 ± 0.4
III. Oleate 5[c]	4	28.2 ± 4.1	---	77.3 ± 7.9	0.2 ± 4.1

[a]Values are mean (± SE); solutions were perfused in random sequence; data from 3 groups of experiments (I-III); perfusion rate 10 ml/min.
[b]- = Net fluid secretion.
[c]Data from previous publications (12,16).
[d]$P < 0.01$ vs. RA 5 + Octadecenoylglycerol 2.5 (paired t-test).
[e]$P < 0.05$ vs. III (unpaired t-test).

Determination of Monomer Activities

The effect of lecithin on monomer activites of fatty acids and octadecenoylglycerol was studied by the method of Sallee (23). The technique is based on the partitioning of lipids between the true aqueous solution and a solid organic phase, a polyethylene disc, and on the assumption that fatty acid interaction with micelles is similar to a phase distribution system. Polyethylene discs were incubated in mixed micellar solutions containing ^{3}H-labeled fatty acids or 1-octadecenoylglycerol. The polyethylene discs, 0.5 in. in diameter, were punched from polyethylene film, 0.006 in. thick. The discs were washed in methanol and distilled water and were dried before use to remove oil and debris from the puching operation. The average weight of the discs was 19.4 mg with a range from 18.8 to 19.6 mg. Discs falling outside this range were eliminated. All experiments were conducted in 20 mM Na phosphate buffer, pH 7.4, with 120 mM NaCl added to keep Na concentrations similar to the conditions prevailing in the intestinal lumen. All solutions contained, in addition, 10 mM taurocholate for micellar solubilization and 5 mmol/l of one of three lipids: [^{3}H] oleic acid (specific activity 0.14 mCi/mmol), [^{3}H] ricinoleic acid acid (specific activity 0.21 mCi/mmol), or [^{3}H] octadecenoylglycerol (specific activity 0.16 mCi/mmol). The concentration of added lecithin was 2.5 or 5 mM. The discs were impaled on a needle and placed into 20 cc of the test solution in a capped vial under argon atmosphere and equilibrated for 48 hr at room temperature under constant shaking at 100 oscillations per min. After completion of the incubation, the discs were rinsed with 10 mM taurocholate to remove fatty acids adhering to the surface and placed into counting vials with 10 cc of Ready Solv VI for the determination of radioactivity by liquid scintillation counting. Since disc size, specific activitires of the lipids, and lipid concentration in the incubation solution were kept constant, the change of uptake of radioactivity by the discs under the influence of lecithin is a direct experession of the changes in monomer activity. The data are reported as lipid uptake by the discs (nmol/disc). To compare the relative changes in monomer activity induced by lecithin, the data were normalized by assigning the uptake by the discs in the absence of lecithin the value of 100%. All values are reported as the mean (± SE) of 6 determinations.

Determination of absolute monomer concentrations was not possible since it requires an accurate measurement of the partition coefficient for the distribution of the fatty acids between nonmicellar dilute aqueous solutions and the polyethylene discs. In the case of oleic acid, however, an increase of fatty acid concentration from 7.5×10^{-8} M to 4.8×10^{-6} M was associated with a significant rise in the partition coefficient. This is probably due to formation of fatty acid dimers at the higher fatty acid concentrations (23).

Calculation and Statistical Analysis

Absorption of water and solutes in the 25 cm test segment was calculated from the changes in the concentrations of PEG and solutes (12,16). Absorption rates were expressed as ml/min/25 cm or as μmol/min/25 cm of jejunum and are the mean of 6 consecutive 10 min collection periods. Differences in

TABLE II

Absorption of Lecithin in the Human Jujenum[a]

Test circumstances (Concentrations in mM)	Absorption (μmol/min/25 cm)
I. Taurocholate (TC) 10	0
TC 10, Ricinoleate (RA) 5	-1.5 ± 0.6
TC 10, RA 5, Octadecenoylglycerol 2.5	0.7 ± 0.4
TC 10, RA 5, Octadecenoylglycerol 2.5, Lecithin 2.5	-1.6 ± 0.5[b]
II. TC 5, Lecithin 1.25	0.6 ± 0.4
TC 5, Oleate 5, Lecithin 1.25	0.2 ± 0.1
TC 5, RA 5, Lecithin 1.25	-1.4 ± 0.2[c]
Glycodeoxycholate 5, Lecithin 1.25	-0.2 ± 0.6

[a]- = Net secretion; results from 2 groups of experiments (I and II). Four volunteers were studied in each group; 4 solutions were perfused in random sequence in each group; perfusion rate 10 ml/min.
[b]Different from 0 ($P < 0.01$).
[c]Different from 0 ($P < 0.05$).

TABLE III

Effect of Lecithin on Partitioning of Lipids into Polyethylene Discs

	Uptake of Lipids into Polyethylene Discs (nmol/disc)[a]			
	Lecithin concentration (mM)			
Lipid tested	0 (Control)	1.25	2.5	5
Oleate 5 mM	124.8 ± 6.7	106.9 ± 9.8	77.5 ± 4.4[b]	38.0 ± 1.5[b]
Ricinoleate 5 mM	4.0 ± 0.3	3.4 ± 0.2	2.9 ± 0.2[c]	2.5 ± 0.2[c]
Octadecenoylglycerol 5 mM	3.9 ± 0.4	---	2.1 ± 0.3[d]	1.5 ± 0.2[c]

[a]Values are mean (±SE) of 6 determinations. All solutions contained 10 mM taurocholate, 20 mM Na-phosphate buffer (pH 7.4) and 120 mM NaCl. Polyethylene discs (0.5 in. in diameter, 0.006 in. thick) were incubated in 20 cc of test solution for 48 hr at room temperature.
[b]$P < 0.001$ vs. Control
[c]$P < 0.005$ vs. Control } Unpaired t-test
[d]$P < 0.01$ vs. Control

net movement of water or solututes and in the partitioning into polyethylene discs were evaluated statistically by paired or unpaired t-tests.

RESULTS

Effect of Lecithin on Absorption of Micellar and Nonmicellar Solutes (Table I)

Ricinoleic acid induced net fluid secretion (12). The addition of lecithin significantly reduced absorption of 2.5 mM octadecenoylglycerol ($P < 0.01$), while it had no effect on water transport or the absorption of ricinoleic acid or glucose. Oleic acid absorption in the presence of lecithin was significantly less than during perfusion with oleic acid alone ($P < 0.05$), while net water movement and absorption of glucose were comparable between the two groups of subjects.

Absorption of Lecithin (Table II)

Lecithin recovery was determined under eight different experimental conditions. In no instance was significant net absorption of lecithin observed. Small but significant net secretion of phospholipid occurred in the presence of ricinoleic acid ($P < 0.05$).

Isomerization of Octadecenoylglycerol

Isomerization of 2- to 1-octadecenoylglycerol was assessed in 7 of 8 studies. Isomerization proceeded at the same rate in vitro as during perfusion through the jejunal segment. At the beginning of the perfusion, 83.6 ± 2.4% were present as 2-octadecenoylglycerol and after 90 min, 74.3 ± 2.1%.

Effect of Lecithin on Monomer Activities of Fatty Acids and Octadecenoylglycerol

Lecithin (2.5 and 5 mM) reduced monomer activity of all three lipids significantly ($P < 0.01$) (Table III).The more polar ricinoleic acid was significantly less affected by the addition of 5 mM lecithin than oleate ($P < 0.001$) and octadecenoylglycerol ($P < 0.02$) (Fig. 1).

DISCUSSION

Lecithin significantly reduced the absorption

of oleic acid and octadecenoylglycerol from mixed micellar solutions in the human jejunum. This observation is in agreement with observations in the rat small intestine where 3 mM lecithin reduced the absorption of 2 mM linoleic acid (11) and 2 mM diether phosphatidylcholine reduced the absorption of 6 mM oleic acid (10) in vivo.

Fatty acids affect water and solute transport, whereby a close linear relationship exists in the jejunum between changes in water transport and solute transport, including the absorption of fatty acids (16,24). Water transport and glucose absorption were not affected by the addition of lecithin in the current studies. Therefore, the observed changes in lipid transport can be definitely attributed to the addition of lecithin to the perfusion solutions.

The mechanism by which lecithin inhibits the absorption of fatty acids is not well established. Since lecithin by itself was not absorbed under any experimental condition, the observed effect of lecithin has to be due to intraluminal events. Lecithin expands the size of mixed micelles (25), and it has been postulated that expansion of micellar size will reduce monomer activity of the lipids present in mixed micellar solutions (26). Our studies confirm this prediction. The more polar ricinoleic acid was less affected than oleic acid and octadecenoylglycerol, which supports the concept that fatty acid interaction with mixed micelles is similar to a phase distribution system (22). The theoretical limitations of this analogy have been discussed elsewhere (27). Since the rate-limiting step for absorption of most long chain fatty acids is the monomer concentration at the brush border membrane (28), reduction in monomer activity will result in a reduction of fatty acid absorption. If this explanation is correct, the dissociation of the effects of lecithin on the absorption of oleic acid and octadecenolyglycerol on one hand, and on the absorption of ricinoleic acid on the other hand, will require an explanation. The changes in monomer activity for ricinoleic acid were less than those of the two other lipids (Fig. 1). The differences at the lower concentrations of lecithin, however, probably were not big enough to explain the different effects on fatty acid absorption. This would suggest that the rate-limiting step for the absorption of the more polar ricinoleic acid is not its monomer concentration at the mucosal surface, but rather the uptake by the mucosal cell membrane or steps in the further disposal of the fatty acid by the enterocyte. Uptake by the enterocyte, for example, can be rate-limiting for other more water-soluble fatty acids such as

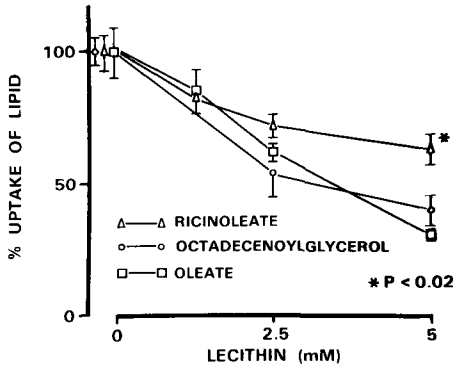

FIG. 1. Effect of lecithin on uptake of lipids (5 mM) into polyethylene discs. Values are mean (± SE) of six determinations. Polyethylene discs (0.5 in. in diameter, 0.006 in. thick) were incubated in 20 cc of test solution for 48 hr at room temperature. All solutions contained 10 mM taurocholate, 20 mM Na-phosphate buffer (pH 7.4), 120 mM NaCl, and ^{3}H-labeled lipids. For purposes of comparison, lipid uptake by the discs in the absence of lecithin was assigned the value 100%. * $P < 0.02$ ricinoleate vs. oleate or octadecenoylglycerol (unpaired t-test).

octanoic acid (29). In addition, activation of ricinoleic acid by Acyl-CoA synthetase (EC 6.2.1.3) in the rat intestinal mucosa was less efficient than the activation of oleic acid (30). Although the changes in monomer activity would adequately explain the effects of lecithin on the absorption of oleic acid and octadecenoylglycerol, the present experiments do not exclude the possibility that reduced diffusion of the enlarged mixed micelles across the unstirred water layer (31) contributes to the reduction of lipid absorption as well.

Recovery of phospholipids relative to PEG was at least 100% of the amount infused, indicating a lack of absorption of intact lecithin. A significant but small net secretion of phospholipids was observed in the presence of ricinoleic acid. This probably reflects the shedding of membrane phospholipids and is in agreement with observations in the rabbit intestine (32). The small rate of phospholipid secretion noted in only one of the three lecithin-free perfusion solutions also provides evidence against any major exchange between intraluminal and membrane phospholipids. Thus, the present data support and extend the findings of others (5,6,8) that lecithin is not absorbed in the absence of pancreatic phospholipase. Our observations are in conflict with those of Wingate et al. (3) who used an identical perfusion system to study the effect of lecithin on water secretion induced by glycodeoxycholate and found 72-90% absorption of lecithin. Since

the experiments in Group I suggested that lecithin was not absorbed in the presence of 10 mM taurocholate, we proceeded to mimic the conditions used by Wingate et al. (3) in the experiment of Group II and to compare the effect of taurocholate and glycodeoxycholate on lecithin absorption. The disagreement in the observations can be explained by differences in methodology, since in the present study phospholipids were extracted immediately into chloroform-methanol, whereas Wingate et al. extracted phospholipids only after storage of their samples for several weeks or months. This could have resulted in hydrolysis of the lecithin.

The rate of isomerization of 2-octadecenoylglycerol in vitro follows first order kinetics and varies with temperature and pH (33). The current experiments demonstrate that during the in vivo perfusion of a segment of intestine, isomerization of 2-octadecenoylglycerol occurred at the same rate as in vitro.

Our observations may have clinical relevance. Normally, phospholipids are hydrolyzed to lysolecithin and are readily absorbed. The absorbed lysolecithin may improve the release of absorbed lipids from the enterocyte (8,9). In cases of pancreatic insufficiency, however, lecithin digestion is impaired due to the lack of phospholipase. The nonabsorbable intact lecithin will then further impair absorption of other lipids such as fatty acids, monoacylglycerols and probably fat-soluble vitamins. This hypothesis is further supported by the observation that the diether analogue of lecithin, which cannot be hydrolyzed, inhibits absorption of lipid in feeding experiments in the rat (34). Thus, in pancreatic insufficiency, steatorrhea is the result of impaired hydrolysis of triglycerides, as well as impaired absorption of free fatty acids due to the presence of nonabsorbable phospholipids in the intestinal lumen.

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Thiobarbituric Acid Test for Detecting Lipid Peroxides

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ABSTRACT

The thiobarbituric acid (TBA) test has been used in the field of medical science in recent years to detect lipid peroxides. In this case, it is necessary for hydroperoxides to be decomposed to secondary products during the reaction. When purified methyl linoleate and methyl linolenate monohydroperoxides were used as the sample for the TBA test, they did not decompose entirely to secondary products, but did so completely when an iron catalyst (ferrous sulfate) was added. However, the iron catalyst also accelerated the autoxidation of coexisting unsaturated fatty acids. Therefore, the addition of antioxidants was required. Fifteen min of heating was sufficient to complete the reaction. With additions of catalyst and antioxidant to the TBA test, it may be possible to make useful distinctions between hydroperoxides and secondary products of lipid oxidation.

INTRODUCTION

The thiobarbituric acid (TBA) test is one of the common methods used for detecting oxidation of lipids. This test is simple to conduct and is also highly sensitive, although its specificity is rather uncertain. Therefore, it has been used in the field of food science for the past two decades, and during this period, some improved methods have been proposed and several reports on the TBA method have been made (1,2).

In the field of medical science, the relationship between lipid peroxides and diseases has been discussed (3,4), and the application of the TBA test to blood and tissues has been investigated (5-7).

The TBA test is, in general, the reaction between TBA and some aldehydes contained in the so called secondary products formed from lipid peroxides. There are two absorption maxima associated with TBA test, one at ca. 450 nm and the other at 532 nm. Patton et al. (8-10) showed that the latter is apparently specific for malonaldehyde and products which will derive malonaldehyde during the test. Sinnhuber and Yu (11) demonstrated the mechanism of pigment formation between malonaldehyde and TBA reagent. 1,1,3,3-Tetraethoxypropane which liberates malonaldehyde and ethyl alcohol under mild acid conditions was used as the standard substance (12). The mechanism of malonaldehyde liberation from linolenic acid has been suggested (13-15). However, secondary products from linoleic acid also form a red pigment with the TBA test. It has been reported that 2,4-alkadienal forms the red pigment, but the red pigment formed by 2,4-alkadienal was suggested to be caused by further oxidation of alkadienal to malonaldehyde (16-21).

In the field of food science, the TBA test has been used to detect so called secondary products, that is, the value has been used as a guide in evaluating deterioration of oils. However, in the field of medical science, it has been used to test for lipid peroxides. Therefore, there are some differences in procedures of the two fields. The heating time required by the former (22) is shorter than that required by the latter (5-7), because the former application determined the existence of only secondary products which coexist with lipid peroxides. On the other hand, the latter application aims to ascertain the presence of only lipid peroxides, and the heating time required is longer. However, the prolonged heating alone is not enough to decompose hydroperoxides to their secondary products.

To examine the suitability of the TBA method for the detection of lipid peroxides, the authors used purified methyl linoleate monohydroperoxides and methyl linolenate monohydroperoxides for the substrate of the TBA test. The results showed that the decomposition of hydroperoxides by the conventional methods (5-7,22) was not complete.

To determine the presence of lipid peroxides, it is necessary for hydroperoxides to decompose to their secondary products during the heating procedure. Some catalyst must be added. The first evidence that prooxidant trace metals (copper) induce increased absorption in the test was provided by Patton and Kurtz (23). Iron is also an effective catalyst for the decomposition of hydroperoxides. Therefore, an iron catalyst could be used for this reaction. But the iron catalyst would also accelerate autoxidation of coexisting unsaturated fatty acids. Therefore, the addition of an antioxidant is also required.

In this paper, a modified TBA test for detecting lipid peroxides is proposed.

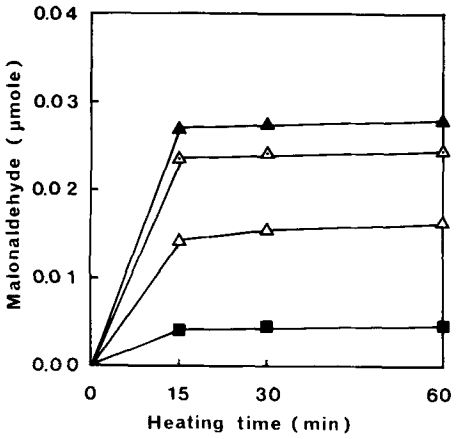

FIG. 1. Effects of iron catalyst on the TBA test of MLHPO. Each tube contained 10 μmoles of MLHPO and 1-100 μmoles of ferrous sulfate. Ferrous sulfate, ▲ 100 μmoles, △ 10 μmoles, △ 1 μmole, ■ none.

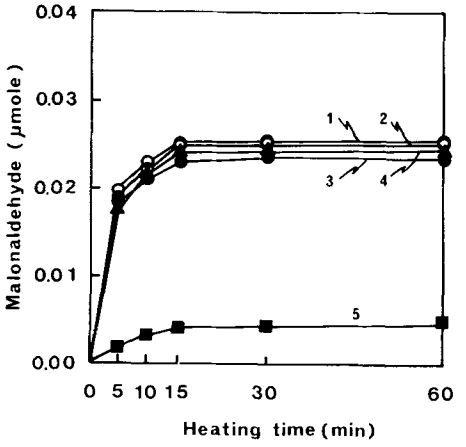

FIG. 2. Effects of BHT addition to the TBA test mixture (MLHPO) containing the iron catalyst. Each tube contained 10 μmoles MLHPO, 10 μmoles ferrous sulfate, and 0.1-1 μmole BHT. BHT amount: (1). 0.1 μmole, (2). 0.5 μmole, (3). 1 μmole, (4). without BHT, (5). without both BHT and ferrous sulfate.

MATERIALS AND METHODS

Methyl linoleate (Tokyo Kasei Co.), methyl linolenate (Tokyo Kasei Co.), methyl arachidonate (Sigma Chem. Co. St. Louis, MO), glycerol trilinoleate (Nakarai Chem. Co., Kyoto), and glycerol trilinolenate (Nakarai Chem. Co.) were obtained from commercial sources. Fatty acid esters were dissolved in hexane, and were purified through a Florisil column (24). Their purities were estimated to be higher than 99% by gas chromatography.

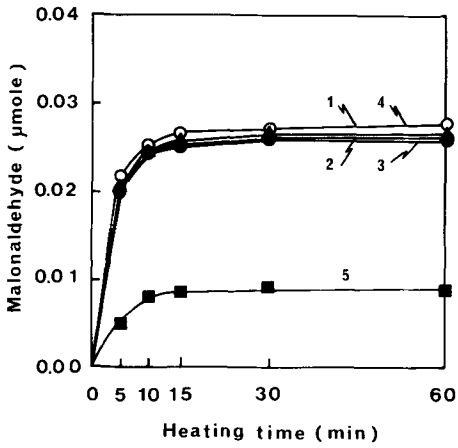

FIG. 3. Effects of BHT addition to the TBA test mixture (MLNHPO) contianing the iron catalyst. Each tube contained 5 μmoles MLNHPO, 10 μmoles ferrous sulfate, and 0.1-1 μmole BHT. Symbols and numbers are the same as in Figure 2.

1,1,3,3-tetraethoxypropane was obtained from Tokyo Kasei Co.

Methyl linoleate monohydroperoxides (MLHPO) and methyl linolenate monohydroperoxides (MLNHPO) were fractionated by silica gel column chromatography from fatty acid esters autoxidized at 37 C (25). Their purities were calculated to be over 95% from the measurement of peroxide value (26).

As a catalyst for decomposing hydroperoxides, ferrous sulfate ($FeSO_4.7H_2O$) was used. Butylated hydroxytoluene (BHT) was used as an antioxidant.

The Ottolenghi method (22) was chosen for the TBA test. Two ml of 0.36% TBA solution and 1 ml of 35% trichloroacetic acid were added to the sample (0.1-0.2 ml hexane solution) in a 20 ml test tube. A glass bead was put on the test tube as a cap. The mixture was heated for 15 min in a boiling water bath. After cooling, 1 ml of glacial acetic acid and 2 ml of chloroform were added. The mixture was then shaken and centrifuged. The optical density of the supernatant was determined at 532 nm using a 1 cm cuvette. The volume of aqueous layer is estimated to be ca. 4 ml. When ferrous sulfate solution ($FeSO_4.7H_2O$ 2.78 g/100 ml water) and 0.1 ml of BHT solution (BHT 220 mg/100 ml ethyl alcohol) were added, the volume of the aqueous layer is estimated to be ca. 4.2 ml. Ferrous sulfate solution was prepared daily. In these experiments, running blanks are necessary. When the color intensity was too high, the optical density was measured after the solution was diluted with 50% acetic acid. The values were converted to the amount

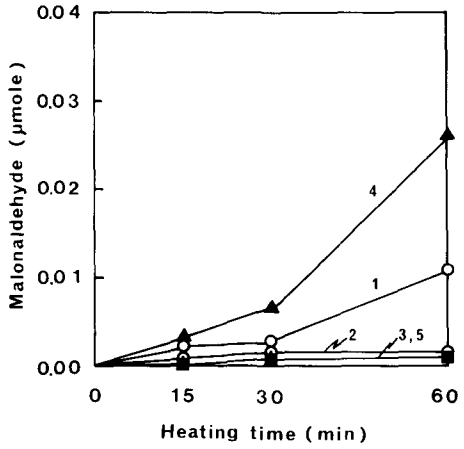

FIG. 4. Effects of ferrous sulfate and BHT additions to the autoxidation of methyl linoleate. Each tube contained 100 μmoles of methyl linoleate, 10 μmoles ferrous sulfate, and 0.1-1 μmole BHT. Symbols and numbers are the same as in Figure 2.

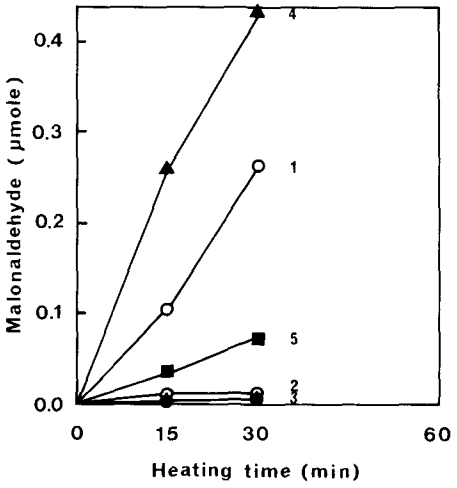

FIG. 5. Effects of the iron catalyst and BHT additions on the autoxidation of methyl linolenate. Each tube contained 85 μmoles of methyl linolenate, 10 μmoles of ferrous sulfate, and 0.1-1 μmole BHT. Symbols and numbers are the same as in Figure 2.

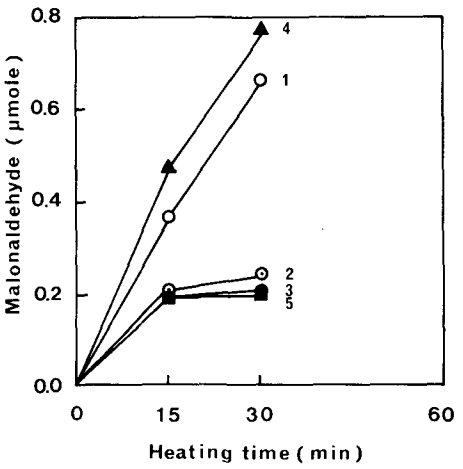

FIG. 6. Effects of the iron catalyst and BHT additions on the autoxidation of methyl arachidonate. Each tube contained 15 μmoles of methyl arachidonate, 10 μmoles of ferrous sulfate, and 0.1-1 μmole BHT. Symbols and numbers are the same as in Figure 2.

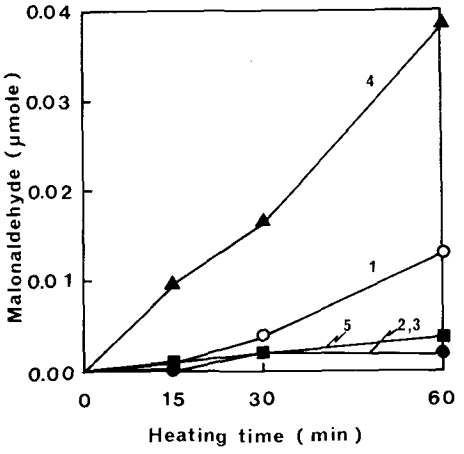

FIG. 7. Effects of the iron catalyst and BHT additions on the autoxidation of trilinoleate. Each tube contained 14 μmoles of trilinoleate, 10 μmoles of ferrous sulfate, and 0.1-1 μmole BHT. Symbols and numbers are the same as in Figure 2.

of malonaldehyde (O.D. 1 at 532 nm corresponded to 0.02 μmole malonaldehyde when the aqueous layer is 4.2 ml) as shown in the figures. As a standard, 1,1,3,3-tetraethoxypropane (11) was used.

Most of the experiments in this paper were tests involving heating times (Figs. 1-8). The experiments were designed to evaluate four or five different conditions. In each condition, the color intensities were measured at two to five different heating times by taking two tubes each time. Blank tubes were also tested at each heating time. For instance, in the case of Fig. 1, four different conditions were tested three times. Therefore, 30 tubes were incubated at the beginning including blank tests.

RESULTS

Hydroperoxides cannot be decomposed

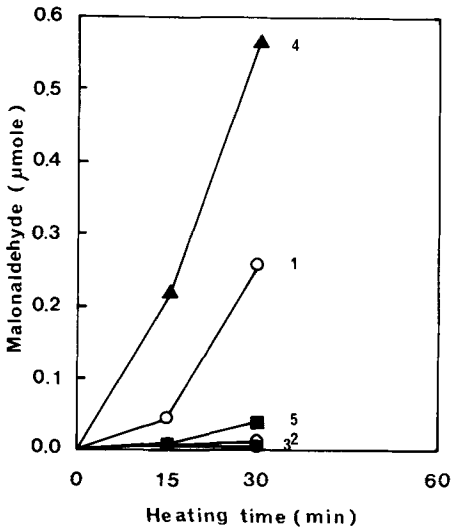

FIG. 8. Effects of the iron catalyst and BHT additions on the autoxidation of trilinolenate. Each tube contained 19 μmoles of trilinolenate, 10 μmoles of ferrous sulfate and 0.1-1 μmole BHT. Symbols and numbers are the same as in Figure 2.

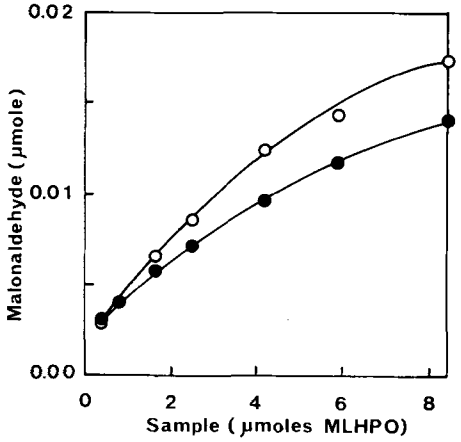

FIG. 9. Calibration curve of TBA test on MLHPO with and without the presence of methyl linoleate. Each tube contained 10 μmoles ferrous sulfate and 1 μmole BHT. ○ 0.42-8.4 μmoles MLHPO, ● 0.42-8.4 μmoles MLHPO and 100 μmoles methyl linoleate. Heating time was 15 min.

completely to secondary products during the TBA reaction. To accomplish this in a short time, the addition of an iron catalyst is necessary. Figure 1 shows the effect of different concentrations of the iron catalyst. When the iron catalyst was added to MLHPO, the rate of formation and the amount of red pigment were measured. Without any iron catalyst, the

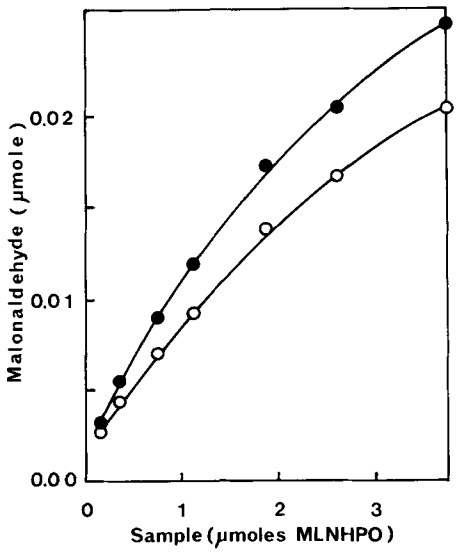

FIG. 10. Calibration curve of TBA test on MLNHPO with and without the presence of methyl linolenate. Each tube contained 10 μmoles ferrous sulfate and 1 μmole BHT. ○ 0.19-3.8 μmoles MLNHPO, ● 0.19-3.8 μmoles MLNHPO and 10 μmoles methyl linolenate. Heating time was 15 min.

resulting color intensity was very low, but a value five times higher was obtained by adding ferrous sulfate 10 μmole/tube. The greater the amount of iron catalyst added, the higher was the value obtained; but the iron catalyst also accelerates the autoxidation of coexisting unoxidized unsaturated fatty acids. Fifteen min of heating was enough to complete the reaction.

To depress the autoxidation of unsaturated fatty acids coexisting, antioxidants must be added. However, radical reaction also is involved in the decomposition of hydroperoxides, and antioxidants may cause the color intensity to decrease. Figures 2 and 3 show the effect of BHT in the reaction mixture. By the concentration used here, no effect from the antioxidant was observed. When the data of Figures 2 and 3 are compared, the color yield per mole of MLHPO is about one-half that of MLNHPO.

When both hydroperoxides and unsaturated fatty acids are present, the complete decomposition of hydroperoxides is achieved by the addition of an iron catalyst, although there is a posibility of accelerating the autoxidation of unsaturated fatty acids. Figures 4-8 show the effect of antioxidant added to the reaction conditions containing an iron catalyst. For methyl linoleate (Fig. 4) and methyl linolenate (Fig. 5), the addition of 1 μmole BHT/tube

prevented most of the autoxidation from occurring. However, at the lower level of BHT, the formation of hydroperoxides progressed under long heating time. As arachidonic acid (Fig. 6) is very easily oxidized, no complete inhibitory effect of the antioxidant was observed. Glycerol trilinoleate (Fig. 7) and glycerol trilinolenate (Fig. 8) acted in the same way as their corresponding fatty methyl esters.

From the above results, it seems that the addition of 10 μmoles of ferrous sulfate and 1 μmole of BHT per tube was the most appropriate for the TBA test of hydroperoxides.

Figures 9 and 10 show the relationship between amount of hydroperoxides and the optical density in the TBA test. When methyl linoleate coexisted with hydroperoxides, a value slightly lower than that from hydroperoxide alone was obtained (Fig. 9), but in the case of MLNHPO (Fig. 10), it was shown that the value included that of coexisting autoxidized linolenate. The strange results of Figure 9 in which coexisting linoleate made the value lower could not be explained, but this tendency was reproducible in several repetitions of the experiment.

The TBA test containing an iron catalyst and an antioxidant was applied to the autoxidation process of soybean oil. The values were compared with those of PV and ordinary TBA test as shown in Figure 11. Without the iron catalyst, secondary products were detected, and with the catalyst, it was possible to detect both the secondary products and peroxides. The TBA test with the iron catalyst showed a curve similar to that of the peroxide value.

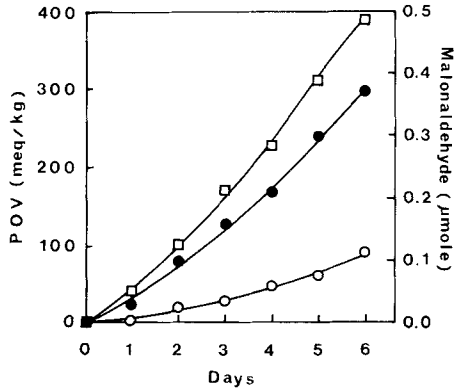

FIG. 11. Application of the TBA test to the autoxidation of soybean oil. Soybean oil, 50 g, was incubated in a Erlenmeyer flask of 1,000 ml. In the case of PV determination, 1-2 g of oil was used in each experiment. In the case of TBA test, 0.1 ml sample oil was taken with a pipet. • with 10 μmoles ferrous sulfate and 1 μmole BHT, ○ without both ferrous sulfate and BHT, □ peroxide value (PV) (26).

DISCUSSION

When the TBA test was applied to purified MLHPO and MLNHPO, only a partial decomposition of the hydroperoxides was achieved by heating in an acid medium, and the reaction was not completed until an iron catalyst was added. It also must be recognized that the TBA test does not determine only the secondary products when hydroperoxides coexist. A part of hydroperoxides may decompose to their secondary products even in the absence of an iron catalyst. The absorbance at 532 nm in the test reflected these facts (Figs. 1-3). It is also noticeable that less than 1% of MLHPO or MLNHPO contributed to the formation of malonaldehyde (Figs. 2 and 3).

On the other hand, the iron catalyst must catalyze the autoxidation of unsaturated fatty acids in the presence of unoxidized unsaturated fatty acids (Figs. 4-8). One μmole of BHT was added to 100 μmoles methyl linoleate, to 85 μmoles of methyl linolenate and to 15 μmoles of methyl arachidonate. Autoxidation was limited in the case of linoleate and linolenate, but with arachidonate addition of 1 μmole BHT could not completely prevent autoxidation. However, since the content of arachidonic acid is not usually as high as those of linoleic and linolenic acids in living materials, 10 μmoles of the iron catalyst and 1 μmole BHT per tube were chosen as the appropriate amounts for the reaction. This amount of antioxidant did not influence the pigment formation from hydroperoxides (Figs. 2 and 3). Fifteen min heating was enough to complete the reaction (Figs. 1-3). The longer heating time promotes only the autoxidation of unoxidized fatty acids.

Generally, two absorption maxima are characteristic of the TBA test as it is applied to autoxidized oils. The yellow pigment around 450 nm can be eliminated by adding 0.1% sodium sulfite (anhydrous) to the TBA reagent solution (20,27). However, the addition of sulfite interfered with the effect of an iron catalyst in the TBA test procedure proposed here.

Although Nishigaki et al. (6) have already discussed the addition of iron salt to the TBA test, the materials they used were not purified. Therefore, their conclusions are quite different from ours.

By using both the TBA test proposed here and the ordinary TBA test, it may be possible to make useful discussions between hydroperoxides and secondary products of lipid oxidation. Further, this method may be useful for

detecting peroxides in blood serum. Direct application of this method to complicated systems such as meat or tissues which contain heme (iron) compounds and large amounts of protein may necessitate further studies (28).

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The Use of Essential Fatty Acid Deficient Rats to Study Pathophysiological Roles of Prostaglandins. Comparison of Prostaglandin Production with Some Parameters of Deficiency

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ABSTRACT

In a retrospective study on essential fatty acid deficient (EFAD) rats used to study pathophysiological roles of prostaglandins (PGs), slight increases in the linoleic acid content of the diet were found to gradually restore the depressed growth rate and to increase the reduced endogenous PG production. These apparently poorly deficient animals had a serum triene tetraene ($\omega 9$:$\omega 6$) ratio much higher than the value of 0.4 used as a criterion for EFA deficiency by nutritionists. Changes in body weight, serum $\omega 9$:$\omega 6$ and platelet PG production were not correlated with each other. Feeding rats on a diet containing <0.1 mg/g/linoleic acid led to decreasing platelet PG production as the degree of EFA deficiency increased. At this high level of deficiency, a serum $\omega 9$:$\omega 6$ ratio of 6 or over was achieved. This high ratio may be taken as an *indicator* of the degree of EFA deficiency required for studies on PG deprivation, but PG production by the tissue investigated or by platelets should preferentially be measured.

INTRODUCTION

Since the first description by Burr and Burr (1) of the development of essential fatty acid (EFA) deficiency symptons in rats fed a fat-free diet, the use of this deficiency disease has been restricted mostly to nutritional studies (2). However, several recent studies have indicated the usefulness of the EFA deficiency state in the study of possible pathophysiological roles of prostaglandins (PGs), because EFAs are the endogenous precursors of PGs and the lack of the latter may account for some changes seen during EFA deficiency (3). Thus, alterations in platelet function induced by EFA deficiency in rats (4-6) and in newborn babies (7) may be attributable to lack of PGs and/or thromboxanes; dermal changes produced by the deficiency syndrome in rats (8-9) and mice (10) may also be related to changes in PG production; and EFA deficiency in rats has been used to study the roles of PGs in models of acute (11-14) and chronic inflammation (15-18). It should be noted, however, that many consequences of dietary EFA deficiency cannot be prevented by supplementation with PGs, even over a long period of treatment (19). In most of these studies, the criterion accepted for EFA deficiency was that of Holman (20), namely a triene/tetraene ($\omega 9$:$\omega 6$) ratio greater than 0.4. This ratio is based on the fact that, under EFAD conditions, the normally abundant tetraenoic fatty acid, arachidonic acid ($20{:}4\omega 6$), is replaced in tissue lipids by 5,8,11-eicosatrienoic acid ($20{:}3\omega 9$). A value of 0.4 or lower is considered by most nutritionists to be normal, indicating that the minimum requirement for EFAs is being met (2). However, the applicability of this value as an indicator of the minimum nutritional EFA requirement has recently been questioned (21). The present report shows that a value for the serum $\omega 9$:$\omega 6$ ratio of 0.4 is also inadequate to ensure a sufficient degree of EFA deficiency for studies on PG deprivation. Since PGs are very potent compounds, it was considered necessary to reassess the methods for determination of EFA deficiency to obtain rats which had sufficiently reduced endogenous PG-producing capacity to be useful in studies on pathophysiological roles of PGs. We present here a retrospective analysis of data on EFAD rats obtained over a 3 yr period, demonstrating the need to incorporate measurement of PG production into determination of the extent of EFA deficiency during such studies on endogenous PGs.

METHODS AND MATERIALS

Animals and Diet

All experiments were carried out with male albino rats of the Wistar strain (TNO Central Breeding Institute, Zeist, The Netherlands). Control animals were fed a normal laboratory animal diet, containing 3.5% of its calories as linoleic acid (Hope Farms, Woerden, The Netherlands), after weaning and were caged on sawdust bedding or on "Sol-Speedi-Dry" (Metallochemie, Ramondt, The Netherlands), an absorbant grit. In order to induce EFA deficiency, pregnant female rats were fed the EFAD diet (Table I) from 5 days before delivery and throughout the weaning period (5). After weaning, the male pups were also placed on the EFAD diet, kept in wire cages or on "Sol-Speedi Dry" bedding and were used for

TABLE I

EFAD Diet for Rats[a]

	%
Casein	20.0
Glucose (cerelose)	65.0
α-Cellulose	4.0
[b]Hydrogenated coconut oil	4.0
Dried yeast	1.0
$CaCO_3$	1.0
$Ca(PO_4)_2$	1.6
KCl	1.0
NaCl(iodinated)	0.5
MgO	0.3
[c]Vitamins and trace elements (added in glucose)	1.4
dl-Methionine	0.2

[a]Prepared by Hope Farms, Woerden, The Netherlands.

[b]Coconut oil Constituents (5): stearic acid (18:0) 9.5, palmitic acid (16:0) 8.5, myristic acid (14:0) 20.0. lauric acid (12:0) 49.0, capric acid (10:0) 6.3, caprylic acid (8:0) 6.2.

[c]Vitamins and trace elements (per kg diet)- Vitamin A 18,000 I.E, Vitamin D_3 2,000 I.E, Vitamin K_3 30 mg, Vitamin E 125 mg, Thiamin HCl 20 mg, Riboflavin 12 mg, Pyridoxine HCl 15 mg, Niacin 40 mg, dl Ca-pantothenate 35 mg, Vitamin B_{12} 50 μg, Biotin 300 μg, Folic acid 8 mg, Inositol 220 mg, 50% Choline chloride 4,000 mg, Fe $(CO_3)_2$ (57% Fe) 200 mg, Cu $(CO_3)_2$ (55% Cu) 30 mg, MnO (62% Mn) 100 mg, ZnO (78% Zn) 60 mg.

experiments at 14-16 weeks old. Data shown in this paper were obtained from rats fed an EFAD diet which were used, one week after determination of EFAD parameters, for experiments on platelet aggregation and release reaction (5,6,22,23), carrageenan paw oedema (11,13,14), adjuvant arthritis (16) and experimental granuloma models (17,18,24). Rats which yielded appreciable amounts of PGE-like material either during platelet aggregation in vitro or in any tissue fluid (e.g., inflammatory exudate) were not utilized for any published experimental investigations on pathophysiological roles of PGs. Some of the more recent data were obtained from rats used in studies which are, as yet, unpublished.

Available Parameters of EFA Deficiency

Apart from the commonly used ω9:ω6 fatty acid ratio, several other parameters may be used as indicators of EFA deficiency and these have been reviewed (1-3,10,25,26). They include diminished growth rate, scaly, dry skin, loss of hair, degeneration of gonads, increased epidermal DNA synthesis, swelling of mitochondria, increased transepidermal water loss leading to increased water intake, and decreased formation of PGs by various tissues. Since the ω9:ω6 ratio is the most objective of the above parameters, we chose this as one parameter of EFA deficiency, together with production of PGE-like material by aggregating platelets. This latter parameter was chosen since platelet aggregation, coupled with bioassay, is a quick and convenient routine technique, widely used in laboratories carrying out PG research. Change in body weight was also chosen as a simple parameter suited for routine measurement.

Serum Fatty Acid Determination

For serum fatty acid and platelet PG determinations, two EFAD and two normal rats were randomly chosen from the complete batch of EFAD and litter-mate control rats (20-22 rats of each type) utilized for each experimental investigation. Heparinized blood was obtained, either by cardiac puncture or by cutting off the end of the tail, and left to stand for 1 hr before collection of serum. The serum was saponified with 1 N methanolic potassium hydroxide at 100 C, and unsaponifiable material removed by extraction with pentane/ether (1:1). This extract was then washed with 0.1 N sodium hydroxide to recover any dissolved fatty acids. The combined soaps were acidified to pH 2 with phosphoric acid and the fatty acids extracted with pentane/ether (1:1). The antioxidant butylated hydroxytoluene (BHT) was added, the mixture was evaporated to dryness and fatty acids methylated with diazomethane before being subjected to gas liquid chromatography (GLC). For determination of the linoleic acid content of the rat diet, fatty acids were extracted from the food, in a Soxhlet apparatus, with either n-hexane or chloroform/methanol (2:1) and then methylated using the boron trifluoride method before being subjected to GLC. Separation and determination of fatty acids was carried out on a Packard Becker Model 419 gas liquid chromatograph, using a SCOT/OV-275 column (80 m length, 0.5 mm int. diameter, N_{eff} = 82,000, N_{th} = 109,000 (20:3ω9) at a split ratio of 1:25 and the following temperatures: Injector/Column/Detector, 220:200:220 C.

Platelet PG Production

Platelet aggregation in the presence of phospholipase A_2 (EC 3.1.1.4) (40 μg) was carried out as described previously (23), and aliquots of the supernatant were bioassayed for PGE-like material against authentic PGE_2 on the isolated rat stomach strip as described by Ferreira and de Souza Costa (27).

Expression of EFAD Parameters

Differences in body weight and PG produc-

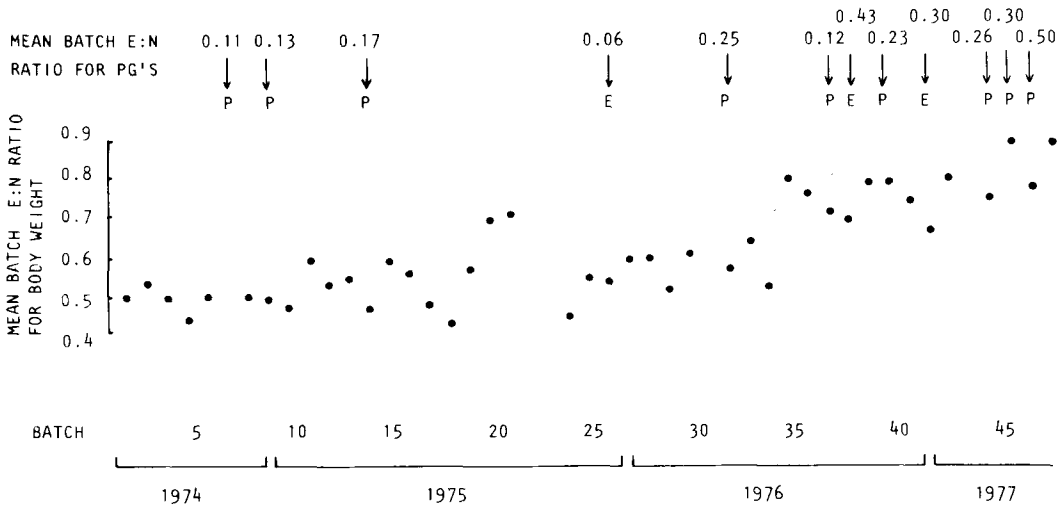

FIG. 1. Body weights and values for PGE-like material obtained from batches of 13-14 week old EFAD (E) and normal (N) rats over a 2½ yr period. Lower section: mean batch E:N ratios for body weight (each point represents the value calculated for 18-22 EFAD and 18-22 normal rats per batch). Upper section: mean batch E:N ratios for PGE-like materials formed by aggregating platelets (P), as described elsewhere (6,23), or for PGE-like material detected in exudates from kaolin pouch granulomata or carrageenin sponge granulomata (E), as described elsewhere (17). The arrows indicate the groups of animals from which the mean batch E:N ratios for PGs were obtained.

tion were quantitated by calculating mean EFAD/Normal (E:N) ratios for batches of 18-22 EFAD and 18-22 control rats delivered at the same time to the Dept. of Pharmacology. Because serum $\omega 9{:}\omega 6$ ratios in normal rats were always <0.01, this EFA deficiency parameter was expressed as the mean serum $\omega 9{:}\omega 6$ ratio in EFAD rats only.

Statistical Analysis

Correlations between changes in various parameters of EFA deficiency were assessed by determination of the Spearman rank correlation coefficient, r_s (28).

RESULTS

Retrospective Analysis of Body Weight and PG Production in EFAD Rats

Figure 1 summarizes published and unpublished data on body weight and PG production by platelets and in inflammatory exudates obtained from batches of EFAD and normal rats over a period of ca. 2½ yr. It can be clearly seen that during 1976 and early 1977 both the depressed body weights and the depressed endogenous PG production in EFAD rats tended to increase towards the values for control rats, i.e., E:N ratios tended towards unity. These later rats thus became useless for studies on PG deprivation. The reason for this change in E:N ratios became apparent following analysis (in January 1977) of the linoleic acid content of the EFAD diet (Table II). New supplies of coconut oil used to prepare the diet (Table I) unexpectedly contained higher amounts of linoleic acid, and these had gradually been incorporated into the diet.

Despite the tendency for the E:N ratios for both body weight and PG production to increase with time, when values for PG production were compared with those for body weight in the same batches of rats, there was no significant correlation between the two ($r_s=0.511$, $p>0.05$, when using E:N ratios for PG production by platelets and in exudates; $r_s=0.619$, $p>0.05$, when using E:N ratios for PG production by platelets only). However, since these data were obtained from a variety of different studies, more controlled investigations were necessary.

Relationships between Various Parameters of EFA Deficiency and EFAD Diet

In order to provide an indication of the degree of EFA deficiency required for studies on PG deprivation, a detailed analysis of body weight, platelet PG production and the serum $\omega 9{:}\omega 6$ ratio was carried out on both the poorly EFA deficient rats (batches 44 and 45, early 1977) and rats which were fed a diet containing <0.1 mg/g linoleic acid (as suggested by B. Folkersma, Unilever Research Labs., Vlaardingen). In these studies, the methods

TABLE II

Data Obtained from Eight Different Batches of Rats Delivered to the Pharmacology Department, Rotterdam at 13-15 Weeks Old

Batch No.	Delivery date	Mean batch E:N ratio[a]		Serum ω9:ω6[b]	EFAD diet analysis	
		Body weight	Platelet PGs		Date	Linoleic acid (mg/g food)
					Jan. 1977	1.0[c]
44	3/15/77	0.77	0.18	3.5	Mar. 1977	0.17[c]
45	4/5/77	0.90	0.18	3.7		
1	8/9/77	0.82	0.13	6.1		
2	8/30/77	0.76	0.12	8.2		
3	9/20/77	0.75	0.11	7.2	Sept. 1977	0.08[d]
4	10/11/77	0.77	0.10	5.0		
5	11/1/77	0.86	0.10	6.4		
6	4/25/78	0.62	0.08	6.0		

[a]Determined as $x_1 + x_2 \ldots\ldots x_n / y_1 + y_2 \ldots\ldots y_n$ where x is the individual value for one EFAD rat, y is the individual value for one normal rat, n is 20-22 for body wt. (depending on the batch), n is 2 for platelet PGs.

[b]Values from 2 EFAD rats per batch only. Values for normal rats were always <0.01.

[c]Extraction of linoleic acid carried out with n-hexane.

[d]Extraction of linoleic acid carried out with chloroform/methanol (2:1).

TABLE III

Relationships between Body Weight, Platelet PG Production and Serum ω9:ω6 in EFAD Rats[a]

Relationship	r_s	P
E:N ratio for body weight vs. E:N ratio for platelet PGs	0.506	>0.5
E:N ratio for body weight vs. Serum ω9:ω6	-0.327	>>0.5
E:N ratio for platelet PGs vs. Serum ω9:ω6	-0.345	>>0.5

[a]Values for the Spearman rank correlation coefficient were calculated using the data shown in Table II.

described above were used and the data are shown in Table II. It can be seen that, while the serum ω9:ω6 ratios in EFAD rats from batches 44 and 45 were very much higher than 0.4, the nutritionists' criterion for EFA deficiency (2), PG production by platelets was still appreciable. After changing the EFAD diet to the one containing <0.1 mg/g linoleic acid, the serum ω9:ω6 ratio in EFAD rats increased further and the E:N ratio for platelet PG production decreased to much lower values. Although the E:N ratios for body weight also showed a tendency to decrease with increasing EFA deficiency, the change was very slight (Table II).

Possible correlations between the changes in the three parameters were investigated and the results are presented in Table III. There was no significant correlation between any of the parameters.

DISCUSSION

During studies designed to investigate the effects of PG deprivation on various pathophysiological functions, we observed that rats which had been fed an EFAD diet began to produce appreciable amounts of endogenous PGs. The data presented here show that this was a gradual effect brought about by an unforeseen increase in the linoleic acid content of the EFAD diet. The retrospective analysis of the accumulated data revealed that the depressed growth rate of EFAD rats was also gradually reversed, apparently in parallel with the increasing PG production. However, since the two parameters were not significantly correlated, it is clear that growth rate and endogenous PG production are differentially sensitive to changes in EFA intake. This was further confirmed in the more detailed converse study, in which rats were made progressively more EFA deficient, where changes in body weight and in PG production were also not significantly correlated. The data in Table II suggest that, while PG production progressively falls with increasing EFA deficiency, body weight decreases erratically then falls more rapidly when EFA deficiency becomes more extensive (cf. batch 6, Table II). It is thus obvious that body weight cannot be used as an indicator of the extent of EFA deficiency in studies on PG deprivation.

Analysis of the serum ω9:ω6 ratio in the

rats which were poorly EFA deficient according to body weight and PG production revealed an unexpectedly high value for this fatty acid ratio. This paradox was probably attributable to preferential acumulation of the PG precursor, arachidonic acid (20:4ω6), by some tissues as compared with others. In fact, in rats or mice fed an EFAD diet, it has been observed that while the brain or erythrocyte ω9:ω6 ratio may rise well above 0.4, the arachidonic acid content of these tissues remains relatively high (Deby and Bacq, personal communication, refs. 29,30). Apparently, 20:3ω9 levels in erythrocytes increase under EFAD conditions, rather than 20:4ω6 levels decreasing. After feeding rats the diet containing <0.1 mg/g linoleic acid, the desired decrease in endogenous PG production was associated with a further increase in the serum ω9:ω6 ratio. However, the fact that the changes in the two parameters were not significantly correlated indicated that, as with PG production and body weight, serum fatty acid levels also show a differential sensitivity to changes in EFA intake. In fact, body weight and serum ω9:ω6 values were also not significantly correlated.

These findings have several implications for the use of EFAD animals to study pathophysiological roles of PGs. First, while being a simple and easily performed test for EFA deficiency, measurement of body weight is not a valid check on deficiency for studies on PG deprivation. Second, although the *rate* of change in the serum ω9:ω6 ratio is not correlated with the rate of change in endogenous PG production, a value for this ratio of 6 or above does appear to be a valid *indicator* of the degree of EFA deficiency required to study effects of PG deprivation. However, this value cannot be used as a criterion of deficiency per se in the same way as a value of 0.4 is used by nutritionists as a criterion for the minimum EFA requirements (2). To obtain the highest possible ω9:ω6 ratio, it is obvious that the lower the linoleic acid levels in the EFAD diet the better, although it is possible that a complete absence of linoleic acid may result in undesirable degeneration of membranes. Our data indicate that a level of <0.1 mg/g in the diet is suitable for studies on PG deprivation. It is also clear from our results that fairly regular analysis of the fatty acid content of the diet should be carried out to avoid unexpected (and costly) increases in linoleic acid levels. A further implication of our findings is that measurement of endogenous PG production is essential when using EFAD animals for studies on pathophysiological roles of these local hormones. Whenever possible, the PG assay should be carried out on the tissue under investigation. This is of particular importance when studying the role of PGs in the brain and other tissues, in which arachidonic acid is preferentially accumulated. In several recent studies, EFAD rats have, in fact, been used to study the role of PGs in the brain (30-33). In some cases though, it is not possible to directly measure PG production by the tissue under investigation. Under these circumstances, production of PGs by platelets, as determined in the present studies, would appear to be a good check on the degree of EFA deficiency. The data summarized in Figure 1 show that PG production in inflammatory exudates, at least, is similar to that by aggregating platelets, when expressed as an E:N ratio.

In conclusion, we suggest that, while assay of PG production by the studied tissue is prefered, when using EFAD animals to study effects of PG deprivation, determination of the serum ω9:ω6 ratio and platelet PG production is a useful secondary means for estimating the extent of the deficiency. When using the mean E:N ratio for platelet PG production, the values we obtained in rats may be comparable with values in other related animal species, such as mice (10) and rabbits (34). Certainly, the data presented here offer guidelines for further studies involving the use of EFAD animals to study pathophysiological roles of PGs.

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COMMUNICATIONS

Stearoyl-Coenzyme A Desaturase Activity in Novikoff Hepatoma

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ABSTRACT

The activities of microsomal stearoyl-CoA desaturation, NADH-cytochrome b_5 reductase, NADH-cytochrome c reductase, and the content of cytochrome b_5 were similar in livers of normal and host rats. On the other hand, stearoyl-CoA desaturation activity was absent in Novikoff hepatoma. The activities of NADH-cytochrome b_5 and NADH-cytochrome c reductases in the hepatoma microsomes were 4.8% and 2.2%, respectively, of those in normal liver. Furthermore, in hepatoma microsomes, cytochrome b_5 was absent. An active stearoyl-CoA desaturation was reconstituted only on addition of both cytochrome b_5 and the terminal desaturase enzyme to the hepatoma microsomes. These results indicated that a complete absence of cytochrome b_5 and terminal desaturase is responsible for the lack of stearoyl-CoA desaturation in Novikoff hepatoma microsomes.

INTRODUCTION

It is well recognized that physiological control of lipogenesis is defective in primary and transplanted hepatomas (1-3). The stearoyl-CoA desaturase activity in Morris hepatomas 5123C and 7777 and in hepatomas SS1K and SS1H maintained in mice was 12-40% of that of their host livers (4-6), whereas the activity in Morris hepatoma 7800 was two-fold higher than in normal liver (6). The conversion of stearoyl-CoA to oleoyl-CoA is catalyzed by the microsomal stearoyl-CoA desaturase system, which consists of NADH-cytochrome b_5 reductase, cytochrome b_5 and the terminal desaturase protein. A recent report by Mercuri and DeTomas (7) indicated that the fast growing hepatoma SS1K, which has 20% of the desaturase activity of normal mouse liver, contained one-fifth of the NADH-cytochrome b_5 reductase and similar activity of the NADH-cytocrhome c reductase as compared to normal liver, although no experimental data were presented. Since the turnover number of the NADH-cytochrome b_5 reductase is 1,000 times higher than that of desaturase (8,9), these authors concluded that the low activity of NADH-cytochrome b_5 reductase was not limiting the overall desaturation and that the reduced desaturation capacity of the hepatoma microsomes was due to the low level of the terminal desaturase protein. This conclusion is equivocal since no direct measurements of the contents of cytochrome b_5 and terminal desaturase were made. Hence, we have carried out a systematic study of the stearoyl-CoA desaturase system and its electron transport components in the rapidly growing Novikoff hepatoma.

MATERIALS AND METHODS

Novikoff hepatoma was implanted in male Holtzmann rats, weighing 150-200 g (10). Microsomes from livers and hepatoma cells were prepared as described previously (10,11).

The activities of microsomal stearoyl-CoA desaturase and of NADH-ferricyanide, NADH-cytochrome c and NADPH-cytochrome c reductases were measured as described by Wilson et al. (11). The microsomal content of cytochrome b_5 was measured from the NADH-reduced minus oxidized spectra between 429-409 nm using a full scale calibration of 0.02 and 0.05 absorbances (11). Reconstitution of desaturase activity using microsomes, purified cytochrome b_5 (detergent extracted from rabbit liver) and rat liver terminal desaturase was accomplished by the method of Joshi et al. (12). Terminal desaturase was purified from rat liver microsomes by the method of Strittmatter et al. (9).

RESULTS AND DISCUSSION

The activity of NADH-ferricyanide reductase reflects the activity of the flavoprotein, NADH-cytochrome b_5 reductase, while the NADH-cytochrome c reductase activity is a measure of the flavoprotein and the amount of cytochrome b_5. The results presented in Table

TABLE I

Activities of the Components of Stearoyl-CoA Desaturation in Microsomes from Novikoff Hepatoma and Normal and Host Rat Livers

Source of microsomes	NADH-Ferricyanide reductase nmol/min/mg protein	NADH-cytochrome c reductase[a] nmol/min/mg protein	NADPH-cytochrome c reductase nmol/min/mg protein	cytochrome b_5 pmol/mg protein	stearoyl-CoA desaturation pmol/min/mg protein
Normal liver[b]	3920 ± 110	410 ± 30	50 ± 4	350 ± 20	590 ± 14
Host liver[b]	3600 ± 71	350 ± 6	50 ± 3	260 ± 30	420 ± 55
Novikoff hepatoma[c]	187 ± 35	9 ± 1	1.2 ± 0.1	0[d]	0

[a]Addition of antimycin A (0.45 μM) did not affect the enzyme activity indicating absence of mitochondrial contamination.
[b]Values are mean ± SEM from 3-4 rats.
[c]Values are mean ± SEM from 3 different microsomal preparations.
[d]Not measurable; value is < 10 pmol/mg protein.

I show that the stearoyl-CoA desaturase activity of host liver microsomes was similar to the activity in normal rat liver. The similar desaturase activity of the microsomes from the two sources was further correlated with identical activities of NADH-ferricyanide and NADH-cytochrome c reductases and the content of cytochrome b_5 in the livers of normal and host rats. These results indicated that in the livers of rats carrying rapidly growing Novikoff hepatoma cells, there was not a drastic reduction of stearoyl-CoA desaturase components and, therefore, host liver should be capable of making oleic acid as effectively as liver of normal rat. The normal ability of host liver microsomes to catalyze stearoyl-CoA desaturation was also shown in animals bearing SS1K hepatomas (4) and Morris hepatomas 5123C and 7800 (5,6).

In contrast to the host liver, the stearoyl-CoA desaturase activity was completely absent in Novikoff hepatoma microsomes (Table I). The hepatoma microsomes contained only 4.8% of the NADH-ferricyanide reductase and 2.2% of the NADH-cytochrome c reductase activities that are present in normal liver. The latter value indicated that the hepatoma microsomes contain very low levels of cytocrhome b_5. Indeed, the direct determination of cytochrome b_5 showed that these microsomes had less than 10 pmol of cytochrome b_5 per mg protein. The NADPH-cytochrome c reductase, which is presumably involved in the desaturation system when NADH is replaced by NADPH (13), is also reduced by an equivalent amount as NADH-cytochrome C reductase in the hepatoma microsomes. These results suggest that the absence of stearoyl-CoA desaturation in the hepatoma microsomes may be due to the total absence of cytochrome b_5 and/or low levels of NADH-cytochrome b_5 reductase. However, it is also possible that the terminal desaturase protein is totally absent.

The absence of specific components of the desaturation system in Novikoff hepatoma microsomes was deduced from the ability of exogenous components to generate an active desaturation in the hepatoma microsomes. The stearoyl-CoA desaturase activity of the normal and host liver microsomes decreased by ca. 65% when assayed in the presence of Triton X-100 (Table II; cf Table I). Supplementation of normal and host liver microsomes with cytochrome b_5 did not change the desaturation activity, indicating that cytochrome b_5 is not limiting in these microsomes. However, the addition of the terminal desaturase to normal and host liver microsomes resulted in 20- and 40-fold increase in desaturase activity, respectively, suggesting that the amounts of flavoprotein and cytochrome b_5 present in these microsomes are in excess over the terminal desaturase. When hepatoma microsomes were supplemented with the terminal desaturase alone, no enzyme activity was observed, which confirmed the absence of cytochrome b_5 (Table II), nor did incubation of the hepatoma microsomes with cytochrome b_5 alone generate an active stearoyl-CoA desaturation indicating the absence of terminal desaturase in these microsomes. On the other hand, when hepatoma microsomes were supplemented with both cytochrome b_5 and terminal desaturase, an active desaturation of stearoyl-CoA was observed. This reconstituted activity of the hepatoma microsomes was four times higher than the activity of normal liver microsomes (Table II). These results clearly indicate that the low NADH-cytochrome b_5 reductase activity of the hepatoma microsomes is not limiting the stearoyl-CoA desaturation in these microsomes. It is the absence of cytochrome

TABLE II

Reconstitution of Desaturase Activity by Added Cytochrome b_5 and Terminal Desaturase

Component added		Stearoyl-CoA desaturation[a] pmol/min		
Cytochrome b_5 pmol	terminal desaturase (μg)	Normal liver	Host liver	Novikoff hepatoma
0	0	90	40	0[b] (0[c])
250	0	85	51	0[b] (0[c])
0	20	1720	1780	0[b] (0[c])
250	20	2175	1875	300[b] (390[c])

[a]400 μg of microsomal protein was preincubated with or without cytochrome b_5 and terminal desaturase in 50 μl containing 1% Triton X-100 for 10 min (12). Stearoyl-CoA desaturation was assayed as described in text.
[b]NADH-ferricyanide reductase activity of the microsomal preparation was 4.29% of normal liver.
[c]NADH-ferricyanide reductase activity of the microsomal preparation was 7.1% of normal liver.

b_5 and terminal desaturase protein which is responsible for the loss of stearoyl-CoA desaturation in Novikoff hepatoma. It is clear from the data presented in Table II that reconstitution of desaturation activity with the terminal protein is much greater in normal or host liver microsomes with endogenous cytochrome b_5 than in hepatoma microsomes supplemented with a higher concentration of exogenous cytochrome b_5. This may result either from the lower efficiency of exogenous cytochrome b_5 to form a catalytically active complex than with endogenous cytochrome b_5 or from the differences in the lipid composition of these microsomes. The Novikoff hepatoma lipids contain about twice the amount of oleic acid and one-fourth the amount of archidonic acid as compared to host liver (14). Since the host liver is efficient in stearoyl-CoA desaturation, it is likely that it may be one of the sources of oleic acid to the growing tumor.

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The Effect of Exercise on Plasma High Density Lipoproteins

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ABSTRACT

The influence of vigorous activity in man on plasma lipids and lipoproteins is reviewed, with particular emphasis on high density lipoproteins. Both cross sectional and longitudinal (or training) studies have been reported, many of them of less than ideal design. Nonetheless, a consistent pattern emerges in which increased exercise levels lead to lower plasma concentrations of triglycerides and very low density lipoproteins, and of low density lipoproteins. High density lipoprotein levels increase. Sometimes, but not uniformly, plasma total cholesterol level falls as the result of these changes. The increase in plasma high density lipoprotein appears to be the result largely of an increase in the less dense HDL_2 subfraction. Plasma apolipoprotein A-I levels (but not apo-A-II levels) seem to increase concomitantly. The precise biochemical mechanism responsible for these changes has not been elucidated; but the recent finding of increased lipoprotein lipase activity in adipose tissue and muscle of endurance runners suggests that increased lipolytic rate of triglyceride-rich lipoproteins may be an initial step in a sequence of events leading to higher plasma levels of HDL_2.

INTRODUCTION

The topic of exercise in relation to plasma high density lipoprotein (HDL) concentration now lies at the intersection of two areas of increasing scientific study and of considerable public health concern. Interestingly, both exercise and HDL concentration had received Cinderella treatment for many years, while the preponderance of investigative attention was directed towards their more glamorous sisters, diet and low density lipoprotein (LDL), respectively. Remarkable changes of scientific emphasis have occurred recently, particularly in the United States, dating from about 1975. In this year, the classic paper of Miller and Miller (1) appeared, pulling together numerous existing lines of evidence suggesting that low levels of plasma HDL are strongly associated with increased risk of coronary heart disease (CHD) in man. Intense activity in this area has continued in the realms of epidemiology, biochemistry and physiology, with the great majority of subsequent reports supporting and extending the original hypothesis (2). The concept that relatively high plasma HDL levels are associated with less CHD, and that a high HDL level might actually be "protective," has become familiar to a large section of the general public (3). Almost coincidentally with this upsurge of interest in HDL as a "negative" risk factor for CHD, increased exercise in leisure time has become progressively more popular in the United States (4,5). The present paper attempts to review the current state of knowledge of the influence of the more vigorous, aerobic types of exercise upon plasma lipid and lipoprotein levels, with particular attention to studies of high density lipoproteins in man. Cross sectional studies will be reviewed first, followed by longitudinal (or training) studies and finally by consideration of some biochemical mechanisms that may be involved.

CROSS SECTIONAL STUDIES

Plasma Triglycerides

It is now clear from increasing numbers of cross sectional studies that very active individuals generally exhibit lower plasma concentrations of triglycerides, and thus of very low density lipoproteins (VLDL) than sedentary individuals of the same sex and age range. Bjorntorp et al. (6) reported significantly lower triglyceride concentrations for 15 physically well trained men aged 52-56, compared with 45 sedentary control men. Hurter et al. (7) similarly found lower plasma triglyceride levels in younger male long distance runners compared to sedentary men. Using standardized assays (8), Wood et al. (5) examined 41 male and 43 female long distance runners, and relatively sedentary control groups, randomly selected from three northern California towns, while Martin et al. (9) reported on 20 young elite male runners and sedentary controls. As shown in Table I, the more active groups were found to have significantly lower triglyceride levels, for both sexes. Lehtonen and Viikari (10) measured lower triglycerides in 23 men aged 35-68 who were running or skiing 83 km per week in comparison with 15 healthy but inactive men aged 33-58. The same authors (11) also found lower plasma triglyceride levels in 12 Finnish lumberjacks compared with levels in 15 electricians; this study suggests that vigorous physical activity at work, as well as vigorous leisure time activity, results in relatively low plasma triglyceride concentrations.

These and other studies are consistent in their findings of low mean triglyceride levels in physically very active groups. It is noteworthy that the spread of values (as indicated by standard deviations) is also consistently lower in the active groups. Measurements of VLDL cholesterol in male and female long distance runners have confirmed that the triglyceride-rich VLDL is indeed at a very low level in these very active individuals (5).

Plasma Total Cholesterol

Numerous cross sectional studies have addressed the question whether or not the vigorously active lifestyle is associated with relatively low levels of plasma total cholesterol. Several reports indicate that significantly lower total cholesterol levels (compared to sedentary controls) are associated with vigorous activity, for instance in cross-country runners and skiers (6); in middleaged male (12) and female (5) long distance runners; and in young elite (national class) long distance runners (9). On the other hand, many studies have failed to show significant differences between active groups and sedentary controls, for instance in male marathon runners aged 24-43 (7), in English male civil servants aged 40-64 who reported vigorous leisure time activity (13), in Norwegian male cross-country skiers aged 16-74 (14), in Finnish male runners and skiers aged 35-68 (10), and in Finnish lumberjacks (11). A study by Montoye et al. (15) on 1060 males and 119 females from the Tecumseh Community Health Study reported no relationship between maximal oxygen uptake (a measure of physical fitness) and plasma total cholesterol concentration, when the effects of age, weight and adiposity were removed. Several other apparently contradictory cross sectional studies have been reviewed by Naito (16). In summary, there is at present no clear consensus on the question of physical activity level in relation to plasma total cholesterol concentration.

There seem to be several reasons for this lack of consensus. In a number of studies, the difference in level of physical fitness between the active and the sedentary groups may have been too small for an effect to be apparent; and in some instances, the numbers in the groups were quite small. In addition, less than adequate total cholesterol assays in some studies may have contributed to variance to such an extent that true differences between groups were obliterated. Again, lack of appropriate analytical control on certain concomitants of high levels of physical activity (leanness, abstinence from cigarette smoking, possible dietary differences) may have led to reporting of significant differences in total cholesterol level that were not due to differences in exercise level *per se*, or alternatively may have obscured true differences when they did exist.

The traditional interest in total cholesterol level derives, of course, from the positive correlations usually found in westernized populations between plasma total cholesterol concentration and risk of future CHD. This relationship results, in turn, from a strong positive correlation in many populations between plasma total cholesterol and the concentration of the atherogenic low density lipoprotein (LDL) fraction. As the proportion of the total cholesterol carried in LDL becomes less, and the proportion carried in HDL becomes greater, the predictive power of a total cholesterol measurement presumably becomes less. In populations, HDL cholesterol concentration generally correlates poorly or not at all with total cholesterol (17); however, in very active populations HDL cholesterol (which constitutes a larger than usual proportion of the whole) may correlate positively with plasma total cholesterol level (14). In view of these considerations, and of the fact that a total cholesterol measurement contains within it the elements of a positive (LDL) and a negative (HDL) risk factor, the predictive importance of total cholesterol is probably considerably diminished, particularly in physically active populations. The focus of attention, then, has moved on from total cholesterol to the lipoprotein fractions, notably HDL.

Plasma High Density Lipoprotein

One of the first observations of the association of the vigorously active lifestyle with plasma HDL concentration was that of Carlson and Mossfeldt in 1964 (18); male Swedish skiers showed higher mean HDL cholesterol levels than have been reported for the general male population (19). The twin observations that male Eskimos in northwest Greenland have high plasma HDL levels, and low CHD mortality rates, have been ascribed a common cause – the high physical activity level in this population (20).

Our group has conducted several cross sectional studies in middleaged male and female runers (5,12) and in young, elite long distance runners (9), each in comparison with a randomly selected control group of appropriate age and sex. Plasma lipid and lipoprotein concentrations recorded are summarized in Table I. Comparisons are made between groups of dedicated long distance runners and age- and sex-matched control groups, without any

attempt to adjust for factors such as adiposity and smoking habits. From these crude comparisons, it was clear that the active groups, in each case, exhibited significantly and importantly lower plasma total and LDL cholesterol concentrations, and high HDL cholesterol concentrations.

In an attempt to further characterize the HDL elevation that appeared to be frequent among very active runners, we and our coworkers examined a subset of the individuals described in Table I, using the analytical ultracentrifuge for quantitation of HDL subfractions (HDL_2 and HDL_3) and radial immunodiffusion for assay of apolipoproteins A-I and A-II (21). Seven male runners (aged 42-58) and 6 premenopausal female runners not on hormones (aged 34-46) were compared to randomly selected control groups of similar age believed to be representative of the U.S. adult population (Table II). Our conclusions from this small study are as follows: the elevated plasma HDL cholesterol concentration characteristic of runners (male or female) represents an increased total mass of the HDL fraction. This increase is predominantly in the less dense, larger particle size HDL_2 subfraction, and is accompanied by an elevation of plasma apolipoprotein A-I but not of apolipoprotein A-II. Male runners thus exhibit an HDL pattern similar to that generally found in women.

Mean plasma HDL cholesterol in a group of 220 male Norwegian skiers was significantly higher than that of several other groups of relatively sedentary Norwegian men (14). Twenty-three male Finnish runners and skiers (mean age 44) had an HDL cholesterol that was significantly higher than that of an inactive control group of similar age, according to Lehtonen and Viikari (10). These same authors (11) also reported recently that Finnish lumberjacks have significantly higher HDL cholesterol levels than relatively sedentary electricians of similar age (75 vs. 55 mg/100 ml). This is of interest, since Finnish lumberjacks have often been cited as a group that is not protected against CHD (they have a high rate) even though they have a very high level of occupational activity, because they eat a particularly atherogenic diet. This study indicates that they do have a high plasma HDL cholesterol level, as one might anticipate; but surprisingly, their reported mean total cholesterol (224 mg/100 ml) and calculated LDL cholesterol (138 mg/100 ml) were not particularly high by U.S. standards. Probably cigarette smoking is an important risk factor in this population. In spite of their high occupational activity level, they apparently exhibit a high frequency of

TABLE I

Plasma Lipid and Lipoprotein Cholesterol Concentrations[a] and Ratios in Runners and Controls (5,9)

	Runners (Age 35-59)		Random controls (Age 30-59)		Elite male runners (age 21-34) (n=20)	Random male controls (age 26-30) (n=72)
	Males (n=41)	Females (n=43)	Males (n=743 or 145[b])	Females (n=934 or 101[c])		
Triglycerides (mg/100 ml)	70 ± 24[d]	56 ± 19[d]	146 ± 105	123 ± 89	74 ± 25[d]	92 ± 37
Total cholesterol (mg/100 ml)	200 ± 22[d]	193 ± 33[d]	212 ± 38	209 ± 38	175 ± 26[d]	189 ± 36
Cholesterol in (mg/100 ml):						
LDL	125 ± 21[d]	113 ± 33[d]	139 ± 32	124 ± 34	108 ± 25[d]	124 ± 36
HDL	64 ± 13[d]	75 ± 14[d]	43 ± 10	56 ± 14	56 ± 12[d]	49 ± 11
VLDL	11	7	28	28	11	15
Ratio: HDL/LDL cholesterol	0.51	0.66	0.31	0.45	0.56	0.39
Ratio: Total cholesterol/ HDL cholesterol	3.1	2.6	4.9	3.7	3.1	3.9

[a]Mean ± SD.
[b]n=145 for LDL-, HDL-, and VLDL-cholesterol concentrations.
[c]n=101 for LDL-, HDL-, and VLDL-cholesterol concentrations.
[d]Mean for runners is significantly different ($p \leq 0.05$) from appropriate control group.

TABLE II

Concentration[a] of HDL Subfractions (HDL_2 and HDL_3), HDL Cholesterol and Apolipoproteins A-I and A-II in Long Distance Runners and Randomly Selected Controls Aged 30-59 (21)

	Runners		Random controls	
	Males (n=7)	Females (n=6)	Males (n=38)	Females (n=20)
HDL_2	119 ± 47[b]	218 ± 79[b]	53 ± 44	122 ± 85
HDL_3	259 ± 22[c]	220 ± 38[c]	227 ± 45	220 ± 28
Apo-A-I	163 ± 14[b]	176 ± 26[b]	120 ± 20	130 ± 22
Apo-A-II	30 ± 1[c]	30 ± 2[c]	33 ± 5	34 ± 5
HDL Cholesterol	70 ± 10	85 ± 27	---	---

[a]mg/100 ml; mean ± SD.
[b]Mean for runners is significantly different ($p<0.05$) from control.
[c]No significant difference between means for runners and controls.

smoking.

Finally, a recent study by Hartung et al. (22), reported in abstract, indicates that a group of 59 male marathon runners and a second group of 85 male joggers showed significantly higher plasma HDL cholesterol levels than a sedentary control group (65 vs. 58 vs. 44 mg/100 ml, respectively). Thus, the joggers exhibited increased HDL cholesterol levels although their mean jogging distance was no more than 11 miles/week. The study also failed to show any significant relationship of dietary intake to HDL level for men in these different activity level classes.

LONGITUDINAL TRAINING STUDIES

Longitudinal studies should be more revealing than cross sectional comparisons in at least one respect: self-selection of groups, which is the rule in cross sectional studies, is reduced, and each participant acts as his own control. However, few if any "ideal" longitudinal training studies have been reported. New and different problems beset the longitudinal approach. It is more difficult to obtain adequate numbers of participants in training studies; sometimes the length of the trial has been too short to represent a real-life conditioning program; if the training program is prolonged, dropouts may become a problem, and the self-selection process is reintroduced; and in some cases the lipid or lipoprotein assays were probably poorly quality controlled, so that methodological "drift" may have become a serious problem for studies that lasted for periods of months. This methodological problem compounds the high biological variance of some lipids (particularly triglycerides) so that large numbers of participants may be required in some circumstances to obtain statistically significant differences following training.

Plasma Triglycerides

In the early 1960s, it was noted that vigorous exercise following a fatty meal reduces the degree of alimentary lipemia (23,24). Since that time a number of studies have been reported in which the effect of acute or chronic exercise upon plasma triglyceride levels was studied longitudinally. These are briefly discussed under the headings of acute studies, and long term studies on normal subjects, on hyperlipoproteinemic subjects and on CHD patients, respectively.

The *acute* effects of vigorous exercise on plasma triglycerides have been studied by several investigators. Carlson and Mossfeldt (18) found a significant mean decrease in triglycerides from 108 to 69 mg/100 ml in male participants in an 85 km ski race. Carlson and Froberg (25) reported on a group of twelve men who walked 50 km per day for 10 days with very little caloric intake. Plasma triglyceride fell by more than 50% after three days, and then rose slightly over the next seven exercising days. Participants lost a considerable amount of weight during the walk. Oscai et al. (26) exercised seven hypertriglyceridemic, middleaged men during four successive days by having them run 3-4 miles in 40 min each day. Mean plasma triglyceride at baseline was 235 mg/100 ml, while levels after successive days of exercise were 173, 136, 119 and 104 mg/100 ml. Most of these differences were significant.

Hurter et al. (7) measured triglycerides in 14 trained athletes before and after a marathon race and found a small but insignificant increase. A variable degree of hemoconcentration probably occurs in such very acute exercise studies, tending to increase concentrations of all lipoproteins temporarily. This effect is seldom the object of correction, or even discussion, in these reports.

Long term training studies in apparently *normal subjects,* with initial triglyceride levels generally within the normal range, have yielded a mixed bag of significant decreases in plasma triglyceride concentration (e.g., 27) and insignificant decreases and increases (28-31). Some studies have investigated the effect of a combined exercise and caloric restriction program upon plasma triglycerides. Lewis et al. (32) followed plasma triglyceride levels in 22 obese women aged 30-52 who lost an average of 17% of their body fat during a 17-week program including both jogging and voluntary caloric restriction, and found no significant difference at the end of the program. Widhalm et al. (33) found no significant change in triglyceride level in 7 girls and 7 boys (aged 11-13) who were overweight, following a 3-week program of weight loss through a low calorie diet and exercise.

Several investigators have examined triglyceride changes following exercise in subjects who were initially *hyperlipoproteinemic.* Holloszy et al. (34) studied 15 professional men, aged 35-55, whose initially high mean triglyceride (208 mg/100 ml) fell to a mean of 125 mg/100 ml during the course of a 6-month program of distance running and calisthenics. Oscai et al. (26) found a progressive decrease in triglycerides to normal levels among 7 hypertriglyceridemic males during 4 days of exercise. Gyntelberg et al. (35) found a significant decrease in mean plasma triglyceride in 5 Type IV subjects who exercised by walking for 30 min on a treadmill for 4 days. This decrease occurred whether or not the increased caloric expenditure was compensated for by increased food intake. Lampman et al. (36) examined the effect of 10 weeks of high intensity physical training on 23 middleaged men with Type IV hyperlipoproteinemia and reported a significant decrease in both plasma triglycerides and insulin. Melish et al. (37) reported (abstract) on 29 men with Type II hyperlipoproteinemia (elevated LDL); 18 exercised for 6 months while 11 acted as sedentary controls. Plasma triglyceride fell from 155 to 110 mg/100 ml in the exercising group, while there was no change in the controls.

Lastly, some observations have been made in subjects with *proven CHD.* For instance, Erkelens et al. (38) reported (abstract) that 18 survivors of myocardial infarction who exercised moderately for 3 months showed an unchanged triglyceride concentration.

From this selection of longitudinal studies, it appears that relatively high levels of plasma triglyceride can be considerably lowered by a program of vigorous physical activity. Low initial levels of triglyceride are less predictably affected by exercise, perhaps because at such low levels a larger proportion of the total plasma triglyceride is carried in the LDL and HDL fractions, and less in the VLDL, which is the lipoprotein fraction most decreased by exercise. Triglyceride reduction by exercise in postmyocardial infarction subjects and other people with CHD has apparently been rather unsuccessful, probably due to the relatively low level of fitness that the subjects were able to attain.

Plasma Total Cholesterol

As might be predicted from the results of cross sectional studies discussed earlier, longitudinal studies have provided no clear consensus on the question of exercise in relation to plasma total cholesterol concentration. A review of some early studies has been published (39).

In several studies on normal subjects (27-29) a significant decrease in total cholesterol has accompanied an exercise program. Campbell (40) noted that vigorous, dynamic sports, in particular cross-country running and tennis, resulted in significant decreases in total cholesterol in young male students, whereas relatively static sports, such as wrestling and weight training did not. Melish et al. (37) found a significant reduction in total cholesterol following a 6-month exercise program in a group of Type II hyperlipoproteinemic men (elevated total cholesterol and LDL cholesterol). Considerable reductions in plasma total cholesterol have been reported in circumstances where caloric restriction and significant weight loss accompanied the exercise (25,33). A report in abstract by Weltman et al. (41) suggests that an exercise program alone is equally as effective as exercise plus diet (presumably a weight-loss diet) in bringing about reduction of plasma total cholesterol concentration.

On the other hand, a number of authors have reported no significant change in total cholesterol concentration during a variety of short term and longer term exercise programs (7,18,30,31,34,42,43). Several of these studies showed considerable reductions in mean cholesterol with exercise, although statistical significance was not reached. In three studies (26,35,36), Type IV men (with elevated triglycerides and VLDL) showed a reduction of triglyceride level with exercise, but cholesterol concentration change was not significant. In one study (32), where considerable weight loss accompanied completion of an exercise program in obese women, significant reduction in total cholesterol was not seen.

Almost certainly these apparently conflicting results reflect two situations: (a) in many studies inadequately quality-controlled cholesterol assay methods were employed; and (b) plasma total cholesterol concentration changes are the sum of changes in the cholesterol content of the major lipoprotein classes, HDL, LDL and VLDL. As we shall review in the next section, HDL tends to increase with exercise, while LDL and VLDL tend to decrease; thus, it is not surprising that the behavior of plasma *total* cholesterol is somewhat unpredictable during exercise programs of varying length and intensity.

Plasma HDL and LDL

Training studies in which plasma triglycerides and total cholesterol were the only measures used as indicators of plasma lipoprotein changes are of limited value. A number of longitudinal studies have included measures of the major specific lipoprotein classes, usually by assay of the cholesterol (esterified plus unesterified) content of the separated HDL, LDL and VLDL fractions. These reports will now be considered.

The early report by Carlson and Mossfeldt in 1964 (18) on men before and after participation in an 85 km ski race showed a slight but nonsignificant decrease in LDL cholesterol. During the course of the 500 km 10-day walk reported by Carlson and Froberg (25), the participants showed a large and continuous decrease in LDL cholesterol (129 mg/100 ml at baseline reduced to 61 mg /100 ml at the tenth day) and an increase in HDL cholesterol that was significant at the sixth day of the walk (62 mg/100 ml increased to 70 mg/100 ml). Participants experienced considerable weight loss during the walk. The 39 male subjects (age 18-59) in Altekreuse and Wilmore's training study (29) showed a significant increase in the mean *proportion* of alpha lipoprotein (HDL) in total lipoprotein after 10 weeks. The proportions of LDL and VLDL in total lipoprotein each decreased significantly. Although absolute concentrations of lipoprotein fractions were not determined in this study, it was one of the first to show a significant redistribution of plasma lipoproteins in an apparently desirable direction in a group of initially rather sedentary men following only ten weeks on a vigorous, but not unduly demanding exercise program (an average of only 5 miles per week was run at an average speed of 7.5 miles per hour). The mean weight loss reported was only 2 kg.

Lopez et al. (27) studied 13 young male medical students who underwent an exercise program (jogging, cycling, calisthenics) of four daily 30-min sessions per week for 7 weeks. The mean concentration of alpha lipoprotein (HDL) increased significantly. Beta lipoprotein (LDL), and also LDL cholesterol both decreased significantly, in agreement with the proportional changes found by Altekreuse and Wilmore. Lewis et al. (32) found a decrease in mean LDL cholesterol and an increase in HDL cholesterol (neither of which reached significance) in their study of 22 obese women who lost an average of 4.2 kg body weight during a 17-week program of jogging and self-determined caloric restriction. However, a significant increase in the ratio HDL cholesterol/LDL cholesterol was observed following the program (0.38 increased to 0.43).

Leon et al. (43) observed a significant increase in HDL cholesterol in 6 obese young college men who exercised for 1.5 hr/day for 16 weeks (3% loss of body fat).

The remaining reports of which we are aware, describing recent longitudinal work in the exercise-plasma lipoprotein area, were all published in 1978, and most are at present in abstract form. Ratliff et al. (31) studied 14 previously untrained middleaged firefighters who exercised three times per week for 20 weeks, while 14 others acted as sedentary controls. The only significant change in lipoprotein cholesterol levels was an increase in HDL cholesterol (42 to 50 mg/100 ml) in exercisers. The percent of body mass as fat decreased significantly in the exercise group compared to the controls. Weltman et al. (41) looked at the effects of either 10 weeks of exercise or 10 weeks of exercise plus a weight-loss diet in groups of middleaged sedentary men and women, vs. an untreated control group. There was a significant decrease in total cholesterol and LDL cholesterol, and a decrease in the LDL cholesterol/HDL cholesterol ratio in the exercising groups whether or not the exercise was accompanied by a weight-loss diet. Gilliam and Burke (42) studied 14 girls (aged 8-10) during a 9-day training period. A significant increase in HDL cholesterol was found, but data on LDL-cholesterol were not given. Melish et al. (37) worked with men (aged 35-58) with Type II hyperlipoproteinemia, who were already being treated (by diet only) for their elevated plasma LDL cholesterol. Eighteen were assigned to an exercise group for 6 months and 11 to a sedentary control group. The exercisers showed significant decreases in total cholesterol, and LDL cholesterol but no change in HDL cholesterol. Total body weight did not change in the exercisers, but estimated body fat mass fell from 22.5 to 20.0%. Widhalm et al. (33)

observed 7 boys and 7 girls aged 11-13 during a 3-week weight reduction program at a diet camp. An average weight loss of 4.7 ± 1.1 kg occurred as a result of a 1000 kilocalorie per day diet and a daily sports program. There was a significant decrease in LDL cholesterol accompanied by a nonsignificant *decrease* in HDL cholesterol. It is impossible in this study to separate the effects of exercise from those of caloric restriction. Erkelens et al. (44) reported on 18 survivors of myocardial infarction who underwent a 3-month exercise training program. Compared to the untrained state, mean HDL cholesterol increased significantly after only one week on the exercise program (35 to 40 mg/100 ml) and remained significantly elevated after 3 months of training. There was no significant change in plasma total cholesterol or triglycerides; LDL cholesterol level was not reported.

In summary, reported training studies cover a range of different groups – men and women, old and young, normal and hyperlipoproteinemic. Many of the studies fell considerably short of the "ideal" longitudinal design. Notwithstanding these shortcomings, a common pattern of change in plasma lipoprotein concentrations runs through these reports that is consistent with the findings of the cross sectional studies and strongly suggests that the apparently desirable changes can indeed be induced in initially sedentary people by exercise training programs. The major changes seen are a reduction of plasma triglyceride and VLDL cholesterol levels (especially where VLDL levels are initially elevated); a reduction of LDL cholesterol level and an increase of HDL cholesterol level; and not infrequently a resultant reduction of total cholesterol level. So far as we are aware, no *longitudinal* exercise studies have yet reported data on HDL subfractions or on apolipoprotein concentrations. A critical question is whether or not concomitant physiological changes, apart from increasing physical fitness *per se*, are partially or even totally responsible for the observed changes in lipoprotein pattern. Loss of fat tissue mass is clearly a prime candidate, and this and other possible factors are dicussed in the next section.

ASSOCIATIONS AND MECHANISMS

In considering the plasma lipoprotein pattern (and notably the high HDL concentration) apparently characteristic of the relatively fit, physically trained individual, we must take note of other common characteristics of such individuals that might at least in part explain differences from sedentary controls. Some of these factors will now be examined.

Sex, Age and Inheritance

These nonenvironmental influences affect plasma HDL levels, and should be borne in mind when comparing active with sedentary groups. Women generally show lower levels of plasma triglyceride (45) and VLDL (46) than men of similar age. Women also generally show higher levels of plasma HDL cholesterol and total HDL mass (46) than men of similar age. The increment in total HDL in women is predominantly the result of an increased concentration of the less dense HDL_2 subfraction, rather than of the HDL_3 subfraction (Table II). The sex differential in HDL cholesterol concentration is maintained when groups of male and female runners, each training at a similar level, are examined (Table I). However, male runners show lower levels of triglycerides and higher HDL cholesterol levels than do relatively sedentary women of similar age (Table I); and this seems to be reflected in a higher plasma concentration of apolipoprotein A-I in the male runners (Table II). Traditional sex differences in plasma lipoprotein concentrations can thus be overcome by physical activity.

As seen in large scale, cross sectional studies (47), there are age trends for all plasma lipids and lipoproteins for both sexes. These trends are, of course, not necessarily the same as changes with time that might be observed if a single cohort was followed for many years. In particular, there appears to be a slow but steady rise in plasma HDL cholesterol for women from the ages of 20 to 65. For men between the ages of 20 and 50, there appears to be almost no change in population HDL cholesterol means, as viewed cross sectionally. Women runners maintained their HDL cholesterol at higher than sedentary control levels across the age span 30-59. The considerable increase in triglycerides with age in the general population (45) is not seen in groups of male and female runners (5).

Families in which high levels of HDL cholesterol (above 75 mg/100 ml) seem to be inherited have been studied by Glueck et al. (48,49). These hyperalphalipoproteinemic individuals seem to live longer and to suffer less CHD than does the general U.S. population. While the frequency of such individuals in populations is probably low, their occurrence should be remembered when data from small-group exercise training studies are examined. For instance, a single individual with an initial, sedentary HDL cholesterol concentration of, say, 125 mg/100 ml could considerably influence the statistical outsome of a small "n"

study.

In summary, training studies should be subject to sex-specific analysis, and the influence of age (though generally fairly small) should be eliminated or adjusted for. The possible occurrence of individuals with familial hyperalphalipoproteinemia should be kept in mind.

Socio-Economic Status

There is some indication, somewhat contrary to traditional views, that plasma concentrations of cholesterol and triglycerides in populations are inversely related to socio-economic status (that is, groups with more education and higher incomes tend to have lower plasma lipid levels). It is quite probable that a similar but positive correlation between plasma HDL level and socio-economic status may emerge from ongoing analyses. Certainly, there are many indications (but few published reports) that individuals of higher socioeconomic status are particularly well represented among long distance runners. Cross sectional comparisons of HDL levels of active and sedentary groups, in which socio-economic status is held constant, do not seem to have been carried out, but would be of interest.

Smoking

A negative association of cigarette smoking and plasma HDL cholesterol has been reported for men and women of the Framingham study (50). When heavy drinkers were eliminated, a significant negative relationship, independent of alcohol consumption and obesity, was found for both sexes between number of cigarettes smoked per day and HDL cholesterol concentration. The authors of this study consider several other possible associated characteristics of smokers (adiposity, alcohol intake, dietary pattern) that might be responsible for the observed association, but they do not consider the physical activity habits of smokers (perhaps because such data were not available to them). At one extreme of the physical activity spectrum (for instance in marathon runners), cigarette smoking is at a very low level, or is nonexistent (5). It appears very probable to us that differences in physical activity level may, in fact, largely account for the HDL cholesterol differences between smokers and nonsmokers in the Framingham study. It seems unnecessary to propose a direct depressing effect of some component of cigarette smoke upon plasma HDL, although such may, of course, prove to be the case. In our study of runners (5), smoking and nonsmoking sedentary controls showed similar HDL cholesterol levels.

Oral Contraceptives and Estrogen

Estrogen administration in women is associated with increased HDL cholesterol levels while progestin use is associated with decreased levels. The effect on HDL of a combination oral contraceptive depends on its formulation (51). The use and nature of such compounds should be taken into account in exercise studies including women, if plasma lipoproteins are to be investigated. In our study of long distance runners (5), the great majority of the women took no hormone preparations, indicating that the high mean HDL cholesterol (Table I) was not the result of unusually prevalent estrogen-taking among women runners. It has also been shown that oral contraceptive use is associated with higher plasma triglyceride levels in women (52). It is of interest that the small proportion of women runners in our study (5) who were taking oral contraceptives showed the typical, low plasma triglyceride concentrations of women runners not on hormones, suggesting that vigorous activity may effectively counteract the hypertriglyceridemic effects of oral contraceptive use.

Adiposity

Very active individuals tend to be lean (i.e., they have a relatively low percentage of total body weight in the form of fat). Since adiposity is correlated with plasma levels of several lipids and lipoproteins, the relative contributions of leanness on the one hand, and physical activity level on the other, are difficult to dissect out. By matching runners to controls on the basis of relative weight in our studies (5), we estimated that about 50% of the difference in triglyceride level between runners and sedentary controls could be accounted for by the greater relative weight of the controls. Body fat percentage (from hydrostatic weighing) would be a preferable measure of adiposity; but a very large sedentary control group on which this expensive determination had been performed would be required to adequately match a group of lean runners.

The situation with regard to the interrelations of HDL, physical activity and adiposity is more obscure, and requires further investigation. Several studies have shown that relative weight is inversely related to plasma HDL cholesterol (12,53), and indeed very active individuals tend to show low relative weights (5). On the other hand, Hulley et al. (54), studying men in the Multiple Risk Factor Intervention Trial (MRFIT) in San Francisco, found a nonsignificant negative association between HDL cholesterol and body mass index when baseline data were subject to

multivariate analysis. *Changes* in body mass index over a one-year period of observation were negatively associated with changes in HDL cholesterol, but again the association was not significant in the multivariate analysis.

In several training studies, an increase in plasma HDL cholesterol has been accompanied by at least some reduction in percent body fat (31,32). But in others, HDL cholesterol has increased without any significant loss of weight (27). Lehtonen and Viikari (10) considered young male runners' low adiposity not to be responsible for their high HDL cholesterol, since a sedentary control group of young men were lower than the runners in this respect, but nevertheless had HDL cholesterol levels as low as those of much more obese, older sedentary males. A study by our group showed a small but significant decrease of both plasma triglyceride and HDL cholesterol immediately after a mean diet-induced weight loss of 8.6 ± 3.9 kg in 15 considerably obese females (55). A follow-up 6 months later in those women who had maintained the weight loss showed no significant difference between baseline and post-weight-loss plasma HDL cholesterol. This study suggests that moderate weight loss by caloric restriction alone may have little effect on plasma HDL cholesterol. In the study of Gyntelberg et al. (35) on Type IV subjects, an exercise program resulted in reduction of plasma triglycerides and weight loss, but *no* change of HDL cholesterol level.

The possibility exists that the low adiposity of very active individuals in part explains their characteristically high HDL cholesterol levels. However, better designed studies are required to settle this important point in a convincing manner.

Diet and Alcohol

Active individuals may tend to select food differently from sedentary individuals, and in a way that could result in a high HDL cholesterol level. Certainly very active individuals will, in the steady state, comsume more calories than otherwise similar, sedentary individuals. However, our preliminary studies by dietary questionnaire suggested that the composition of the diet (intake of saturated and polyunsaturated fat, P/S ratio, cholesterol intake) was similar between runners and sedentary controls (5). We have recently confirmed this impression by administration of 3-day food records to male and female runners and controls. Lehtonen and Viikari (10) also found similar dietary intakes in their runners and skiers as compared with sedentary controls. Hartung et al. (22) found diet unrelated to HDL cholesterol in men of different activity levels.

Increasing alcohol intake is known to be associated with increasing plasma HDL cholesterol level (56,57). In our studies, a larger proportion of runners than controls reported drinking, but among the drinkers the amount of alcohol consumed appeared to be similar between the two groups. This has also been confirmed recently by administration of 3-day diet records. When the drinking runners were compared to nondrinking runners, or the drinking controls to nondrinking controls, no significant difference in plasma HDL cholesterol concentration emerged. Certainly, abstinent long distance runners maintain much higher HDL cholesterol levels than have been reported for moderate drinkers in population studies of (presumably) rather sedentary individuals.

Amount of Exercise in Relation to HDL Level

There are some indications that relatively low intensity training (jogging three times per week) for reasonably short periods (20 weeks) can produce significant increases in HDL cholesterol (31). In the study of Erkelens et al. (38), an increase in HDL cholesterol was seen after only one week of training in myocardial infaction survivors. Leon et al. (43) found a significant increase in HDL cholesterol in obese young men following a 16-week program of walking. Hartung et al. (22) conclude "Even men who jog 11 miles per week have significantly higher HDL levels than sedentary men."

However, there does sometimes seem to be a positive correlation between amount of exercise and level of plasma HDL. Lehtonen and Viikari (10) found a correlation between HDL cholesterol level and the reported weekly mileage of runners and skiers; while Enger et al. (14) report for their skiers: "The fastest skiers, presumably the best trained, have a trend towards higher levels than the slowest."

Biochemical Mechanisms

The precise mechanisms whereby increased physical activity level leads to a higher plasma concentration of HDL, and to other changes of lipoproteins, is currently not known. Several pieces of the metabolic jigsaw puzzle are thought to be important, and will be briefly enumerated.

Plasma VLDL concentration is inversely correlated with HDL cholesterol concentration. Very active groups show remarkably low plasma triglyceride levels. Long distance runners have been shown recently to have higher levels of muscle and adipose tissue lipoprotein

lipase activity than sedentary controls, and whole-body lipoprotein lipase activity was estimated as 1.5-2.3 times higher in the runners. Serum HDL cholesterol level and lipoprotein lipase activity of adipose tissue correlated highly (58). It seems quite probable that increased physical activity leads to increased muscle and adipose tissue lipoprotein lipase activity which, in turn, leads to lowered plasma triglyceride concentration and ultimately to increased HDL concentration.

On the other hand, decreased *production* of plasma VLDL has been demonstrated in the chronically exercising rat (59).

The enzyme LCAT (lecithin/cholesterol acyltransferase), which may be involved in the transfer of unesterified cholesterol from cells to "nascent" HDL, has been reported to have increased activity in exercising individuals (27). The predominant apolipoprotein of HDL, apo-A-I, which is increased in runners compared to controls (Table II), is an essential activator of the LCAT reaction. HDL cholesterol (unesterified) has been shown recently to be the preferred source of cholesterol (as compared with LDL cholesterol) for incorporation into human biliary cholesterol (60) and bile acids (61). Interesting interrelationships among subclasses of HDL and LDL are apparent when the lipoprotein spectrum of male or female runners is compared with that of appropriate sedentary controls (62).

No doubt in the near future these fragments will be put together to reveal the entire sequence of events among the lipoproteins that ensues when the sedentary individual begins to exercise.

CONCLUSIONS

Consideration of published reports indicates that regular endurance sports such as skiing and running generally result in a characteristic pattern of plasma lipids and lipoproteins. Strenuous occupational activity seems to have the same effect. Specifically, very active individuals are usually distinguished from relatively inactive individuals of the same sex and similar age by their low plasma VLDL concentrations, relatively low LDL concentrations and high HDL concentrations. As a consequence of this, plasma triglyceride concentration is low; total cholesterol concentration is often, but not invariably, lower than is the case in the sedentary condition.

A number of aspects of this subject remain unclear and indicate the need for further study. The extent to which certain physiological concomitants of the active lifestyle, rather than exercise *per se*, may account for the lipoprotein differences remains to be determined by experiments designed to distinguish these factors. Leanness is the chief potential candidate here. The mechanism by which increased exercise (or its accompanying changes) leads to the characteristic lipoprotein pattern is not known, although there are already some interesting glimpses of the overall sequence of events. Finally, the important general question remains unanswered: does change from the lipoprotein pattern of the average north American to that typical of the chronic exerciser (however achieved) result in worthwhile reduction of atherosclerotic disease?

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A Review of the Unique Features of HDL Apoproteins

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ABSTRACT

The human plasma high density lipoproteins (HDL) are a heterogeneous ensemble of five proteins associated with both neutral and polar lipids. The sequences of all five proteins are known. ApoA-I and apoA-II are the major protein components; apoC-I, apoC-II and apoC-III are the minor protein components. All these apoproteins spontaneously recombine with phospholipids to give stable lipid-protein complexes and freely exchange between the two major HDL subclasses, HDL_2 and HDL_3. In addition, ApoC-I, apoC-II, and apoC-III exchange between HDL and very low density lipoproteins. Furthermore, certain HDL apoproteins are activators for plasma enzymes that are important in lipid metabolism. ApoA-I and apoC-I activate lecithin/cholesterol acyltransferase; apoC-II is an activator of lipoprotein lipase. The regions of apoC-I and apoC-II that are involved in the activation of these enzymes have been localized with synthetic peptides. Studies of synthetic and native fragments of apoA-II, apoC-I, apoC-II, and apoC-III as well as model lipid-binding peptides have identified specific regions with structural features common to lipid-binding proteins. These special properties, which include helical potential, sequences with a critical amphipathic length, and high hydrophobicity of the nonpolar side of the amphipathic helix, are the determinants of HDL structure and metabolism.

INTRODUCTION

The human plasma lipoproteins are operationally defined according to their densities as the high, low and very low density lipoproteins (HDL, LDL, and VLDL), respectively. The lipoproteins and their component lipids and proteins are the primary vehicles for the transport of plasma lipids and are also important as activators of some of the lipid catabolizing enzymes (1). Because of the inverse correlation of coronary artery disease and the plasma HDL levels, increasing interest has been focused upon the HDL (2). HDL contain a variety of lipid and protein components whose macro- and microscopic structures may be correlated with their stability and with the mobilization of lipids and apoproteins among other lipoproteins and tissue. The major components of HDL are apoprotein, cholesterol and phospholipids (Fig. 1). The proteins are apoA-I, apoA-II and minor amounts of apoC; the latter are also found in the VLDL (1).

Herein, we shall summarize five properties of the HDL apoproteins which distinguish them from other proteins. HDL apoproteins are water-soluble even in the absence of other surfactants. ApoA and apoC of HDL spontaneously associate with phospholipids and self-associate in the absence of lipids (3). HDL apoproteins exchange or transfer in vitro and in vivo (4). Some of the HDL apoproteins activate the enzymes of intravascular lipid metabolism (5,6). In HDL and in lipid-protein recombinants, a large fraction of the HDL apoproteins probably has a helical conformation; this observation has promoted the design of the amphipathic helical model of apoproteins in lipoproteins (7).

All of the interactions of the HDL apoproteins have not been studied adequately. Herein we focus on the properties of native apoproteins and their native and synthetic fragments that illustrate unique properties of HDL apoproteins that can be associated with specific segments of their primary structure.

DYNAMICS OF LIPID-PROTEIN INTERACTIONS

ApoA-I, the major apoprotein of HDL, associates with DMPC (dimyristoylphosphatidylcholine) to give soluble complexes which are sufficiently stable to isolate by gel filtration. DMPC is a synthetic lecithin which undergoes a transition of its acyl chains from a gel (solid) to a liquid crystalline (fluid) phase at 23.5 C (8). ApoA-I and DMPC applied separately elute from the column at different positions (Fig. 2A). In Figure 2B, apoA-I and DMPC coelute after being mixed for a few minutes at the transition temperature of DMPC, 23.5 C. At temperatures above and below the transition temperature of DMPC (Fig. 2C and D), complete association of DMPC and apoA-I is not achieved even after a 48 hr incubation, indicating enormous temperature effect on the rate of association of DMPC with apoA-I. The rate of apoA-I-DMPC association has also been studied by measuring the rate of disappearance of liposomal turbidity. The results are summarized in Fig. 3A and B. By this index for interaction, the maximal rate of association of

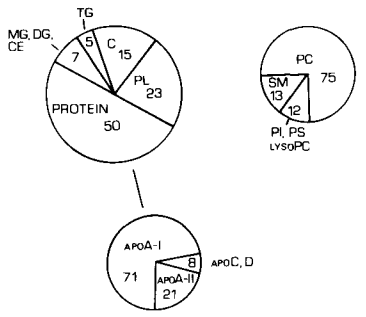

FIG. 1. Composition of HDL. The large circle contains the entire composition of HDL; the lower and right hand circles contain the detailed compositions of the protein and phospholipid components (Permission granted by Baylor College of Medicine).

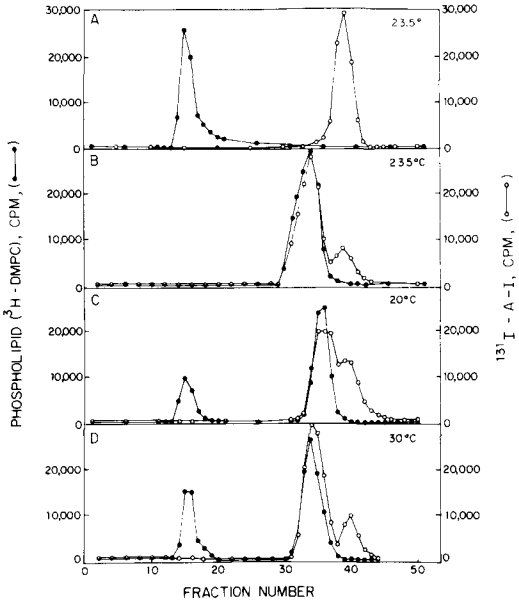

FIG. 2. Gel filtration of (A) DMPC liposomes (3 ml of 0.5 mg/ml) or apoA-I (3 ml of 0.1 mg/ml). (B) DMPC-apoA-I complexes formed after 5 min incubation at 23.5 C of DMPC (1.5 mg) and apoA-I (0.3 mg) in a final volume of 3 ml. (C) DMPC-apoA-I complexes formed after a 48 hr incubation of DMPC (1.5 mg) and apoA-I (0.3 mg) at 20 C in a final volume of 3 ml. (D) DMPC-apoA-I complexes formed after a 48 hr incubation of DMPC (1.5 mg) and apoA-I (0.3 mg) at 30 C in a final volume of 3 ml. Conditions: 1.6 x 90 cm column of Sepharose 4B; flow rate 8 ml/hr; 4.4 ml/fraction. Each sample was run separately in a water-jacketed column at 23.5 C or at the temperature of complex formation. DMPC and apoA-I concentrations were measured by scintillation counting of [^{3}H] DMPC and γ-counting of [^{125}I] apoA-I, respectively. Recovery of liposomes alone was 50%. Recovery of DMPC with apoA-I complexes was >90%. Protein recovery was quantitative, ± 5%. (Permission granted (9)).

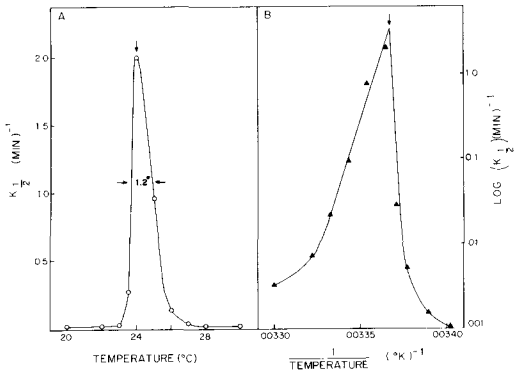

FIG. 3. Temperature dependence of the rate of apoA-I-DMPC association. Linear plot of $k\frac{1}{2}$ vs temperature, where $k\frac{1}{2} = 1/t\frac{1}{2}$ and $t\frac{1}{2}$ is the half-time for the initial reaction rate. (Permission granted by Baylor College of Medicine).

apoA-I with DMPC occurs at the transition temperature of the lipid. We suggest that this enhanced rate of association occurs at lattice defects located at the border of coexisting frozen and fluid DMPC (9).

Hypothetically, addition of an impurity to a crystalline lattice such as a phospholipid matrix would enhance the rate at which apoA-I binds to DMPC. To test this we selected a physiologically important "impurity," cholesterol, and found that the combined effects of cholesterol and temperature alter the rate of association of apoA-I with DMPC over three orders of magnitude. The maximum rate is always observed at 12.5 mole % cholesterol at any temperature and always at T_c, independent of cholesterol content (10). Figure 4 contains a model which is consistent with our data. ApoA-I, as a monomer, inserts into a defect in a lipid matrix created at the border of cholesterol-rich and pure DMPC phases. The maximal rate observed was 2 min $^{-1}$ if the process is considered to have first order kinetics. We have observed similar behavior with apoC-II, apoC-III and apoA-II. Thus, the rapid and spontaneous solubilization of phospholipids is a singular chemical property common to the HDL apoproteins.

Differential scanning calorimetry gives a clue to the structure of lipids in lipoproteins. As a result of incorporation of HDL apoprotein, the phospholipid matrix may be crystallized as reported for apoHDL and apoA-I by Tall et al. (11). Similar results are shown in Fig. 5 for the complex of apoC-III and DMPC. In the absence of protein, the DMPC exhibits the usual melting pattern with its main endotherm at ~ 24 C. With increasing amounts of apoC-III, the endo-

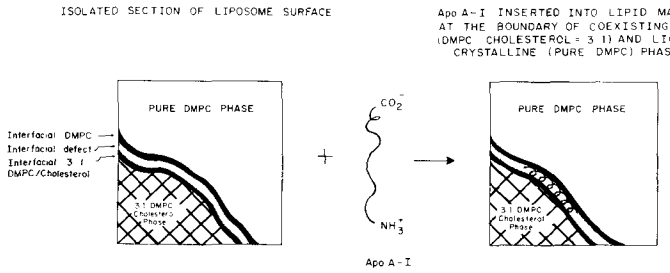

FIG. 4. A hypothetical model for the mechanism by which apoA-I associates with a DMPC matrix. On the left is shown a section of the surface of a liposome in which a DMPC and a 1:3 cholesterol/DMPC phase coexist. Each phase is bounded by interfacial lipid; the interfacial lipid of each phase is separated from that of the other phase by a hole or channel defect. ApoA-I may insert into this defect to give the initial lipid-protein intermediate on the right. The apoA-I in the lipid matrix is drawn as a helical structure since it probably exists as such as HDL and in apoA-I:PC complexes. (Permission granted (10)).

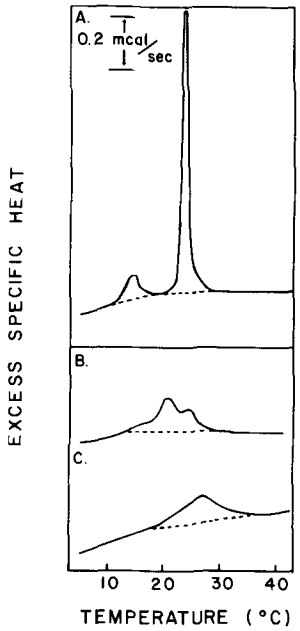

FIG. 5. Traces from the differential scanning calorimetry of (A), DMPC liposomes; (B), DMPC single-bilayer vesicles; (C), DMPC: apoC-III complex with a lipid to protein ratio of 66:1. Samples (~(8 mg) were measured in a sealed 75 μl stainless steel pans and run against an equal volume (50 μl) of water. The liposomes were prepared by dispersing a known weight of DMPC in buffer at >24 C for 10'. DMPC vesicles were prepared by ultrasonic irradiation. Complexes were prepared by mixing DMPC liposomes with apoC-III; after chromatography on Sepharose CL-4B, the fractions containing the complexes were pooled and concentrated. (Permission granted by Baylor College of Medicine).

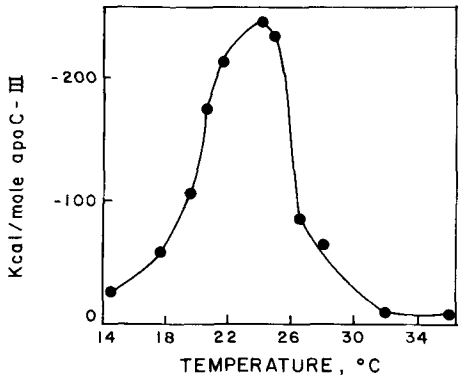

FIG. 6. Temperature dependence of the enthalpy of interaction of apo-C-III (200 μg) with vesicles of DMPC (2 mg). Mixing was achieved in an LKB Batch Microcalorimeter, calibrated with an electrical standard. (Permission granted by Baylor College of Medicine).

therm at 24 C disappears; concomitantly, a new broad endotherm assigned to the complex appears at ~ 27 C. Since crystallization is an exothermic process, this result suggests that between the endothermic maximum of the vesicles and that of the complex the association of apoC-III with DMPC should be exothermic. The temperature dependence of this process (Fig. 6) confirms this prediction. The enthalpy of association of apoC-III is maximal at T_c, i.e. -240 kcal/mole protein or about 3.6 kcal/mole DMPC. The result requires that the heat of association of apolipoproteins with phospholipids contributes to the free energy of lipid-protein association.

STRUCTURE-FUNCTION RELATIONSHIPS OF THE APOLIPOPROTEINS

Support for the hypothesis that the functions of the apolipoproteins can be localized to specific regions of their primary structure has been provided by studies of lipid-binding fragments of apoC-III and of the activation of lipoprotein lipase by fragments of apoC-II; each has certain functions which are

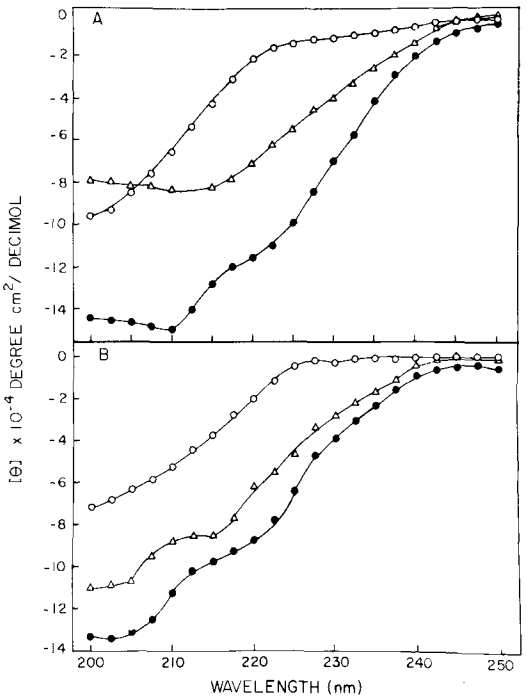

FIG. 7. Circular dichroic spectra of the fragment of apoC-III containing residues 40-79. (A), apoC-III$_B$ with added DMPC; (B), apoC-III$_B$ with added 1-palmitoyl-2-oleoyl phosphatidylcholine; o, protein alone; Δ, 13 moles lipid per mole of peptide; o, 39 moles lipid per mole of peptide. Spectra were measured through a 0.5 mm cell path in a Cary 61 Spectropolarimeter. (Permission granted (12)).

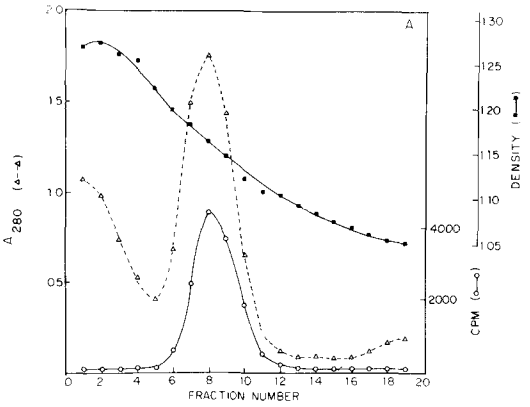

FIG. 8. Ultracentrifugal distribution of an apoC-III$_B$-DMPC complex in a density gradient of cesium chloride. The protein was monitored by measuring the absorbance at 280 nm. ^{3}H-Labeled DMPC was quantitated by liquid scintillation counting. The density was determined by refractometry. (Permission granted (12)).

associated with specific regions of the apoprotein sequence.

Cleavage of apoC-III provides two peptides, each containing half the native molecule, ApoC-III$_A$ (residues 1-40) and apoC-III$_B$ (residues 41-79). Lipid-binding by physical methods shows that apoC-III$_A$ does not bind to DMPC or to 1-palmitoyl-2-oleoylphosphatidylcholine (12). These lipids and apoC-III$_A$ do not form a complex sufficiently stable for isolation by density gradient ultracentrifugation. In addition, there are no changes in the ultraviolet, circular dichroic or fluorescence spectra of apoC-III when this peptide is combined with lipid. By contrast, apoC-III$_B$ (Fig. 7) undergoes major spectral changes as assessed by circular dichroism. These changes suggest that the protein becomes more helical with addition of increasing amounts of lipid. Figure 8 shows that apoC-III$_B$ and DMPC form a stable complex which can be isolated in a salt gradient. The enthalpy of association of apoC-III$_A$ with lipids is nil (-35 kcal/mole) while that of apoC-III$_B$ (-170 kcal/mole) is similar to that of intact apoC-III (-240 kcal/mole). Thus, the lipid-binding region of apoC-III is found in the carboxyl terminal half. We have correlated the sequence of apoC-III with helical probability (13), β-structure probability (13) and hydrophobicity (14). The mean residue hydrophobicity of various segments, shown at the top of Fig. 9, is notable in three respects. ApoC-III$_A$ region is predicted to have the greatest helical content; it does not, however, bind lipid. ApoC-III$_B$ has a higher hydrophobicity and a lower predicted helical content; upon binding lipid, it becomes highly helical. Shorter analogs of this protein containing 20 amino acid residues have been synthesized. Lipid-binding studies show that some associate with lipids and concomitantly form a helical structure; removal of 4 residues from the amino terminal end abolished this behavior.

Thus, we have modified our design of a lipid-binding protein to accommodate the following three features. The peptide need not have helical structure in solution but must have the potential to form a helix. The peptide must have a certain length, the critical amphipathic length of ca. 20 residues. Finally, as shown in Fig. 10, the peptide must have a high hydrophobicity; when arranged in a helical structure, the hydrophobic residues should appear on one face of the helix which, presumably, would penetrate the lipid matrix. In the helical segment of apoC-III (40-72), there is a distribution of the charged residues on one face of the helix with the hydrophobic residues appearing on the opposite face. Similar though less definitive studies of apoA-I, apoA-II, apoC-I, and

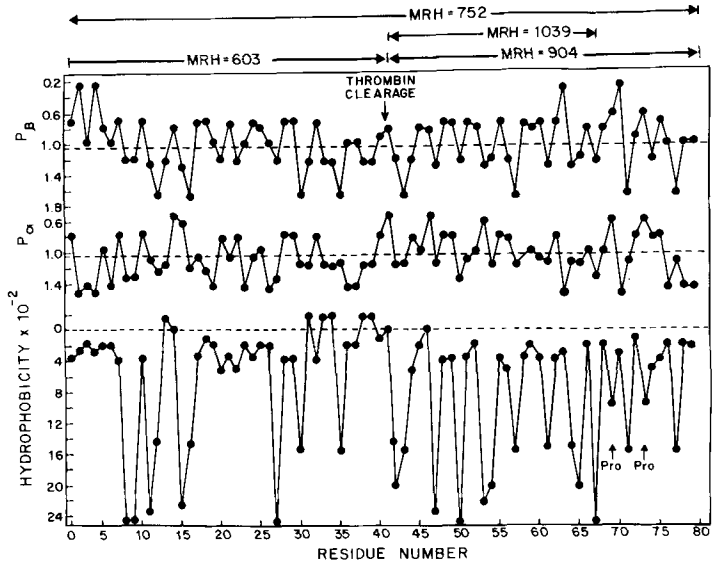

FIG. 9. Correlation of the hydrophobicity of each amino acid residue of apoC-III with its probability of appearing in a β-pleated sheet (P_β) or an α-helix (P_α). The probabilities and hydrophobicities are plotted as a function of the amino acid sequence. Values of P_α and P_β which are greater than those indicated by the dashed lines of the upper and middle panels, respectively, are predicted to exist in α-helical and β-pleated sheets. In the lower panel, the hydrophobicity values appearing below the dashed line are predicted to partition into a hydrophobic environment. The lines at the top refer to the fragments discussed in the text and contain their respective hydrophobicities. (Permission granted by Baylor College of Medicine).

apoC-II also suggest a localization of lipid-binding regions to specific segments of those proteins.

The activation of lipid metabolizing enzymes by apolipoproteins can also be localized to specific regions of the primary structure. ApoC-II is an activator of lipoprotein lipase, an enzyme at the plasma/endothelial cell interface which hydrolyzes triglyceride (5). Fragments of apoC-II have been obtained by cyanogen bromide cleavage or by solid phase peptide synthesis (15). The relative activation of lipase by these fragments is shown in Table I. Synthetic fragment apoC-II (55-78) activates as well as native apoC-II; both native and synthetic fragments of apoC-II (60-78) activate lipase nearly as well. Removal of the highly charged carboxyl terminal tripeptide destroys the ability to activate. In other studies, we have found that the amino terminal half of apoC-II binds to phospholipid, but the carboxyl half does not. Our present view of apoC-II structure-function correlation is summarized in Fig. 11. We assign the lipid-binding region to the amino terminal half of the apoprotein. The activating region is located in the carboxyl half of apoC-II. The charged tetrapeptide at the carboxyl terminus is hypothesized to interact electrostatically with lipoprotein lipase. Thus, we have localized both the enzyme-activating and lipid-binding determinants of apoC-II to specific but different regions of the primary structure. A similar view may emerge from detailed studies of the apoA-I and apoC-I activation of lecithin/cholesterol acyltransferase and the interaction of apolipoproteins with other as yet unknown plasma and cell membrane factors.

MECHANISM OF APOPROTEIN MOBILIZATION

Finally, we would like to speculate upon the mechanism of apoprotein movement between the HDL. It has been shown by Charlton and coworkers (16,17) that the hydrophobic fluorophore, pyrene, exchanges freely between the HDL. The reaction halftime is independent of HDL and pyrene concentration and is ca. 3 msec. The mechanism for transfer of this relatively insoluble substance is dissociation of pyrene into water and a diffusion-controlled collision with unlabeled HDL. Since the apoproteins of HDL are water soluble, it is possible that this mechanism is also valid for apoprotein transfer and for exchange between other lipoproteins, tissue sites and enzymes. Figure 12 shows initial data on the behavior of HDL labeled in vitro with ^{125}I-apoA-I and total apoC proteins containing a ^{131}I-label. In Fig. 12A, the elution profile of HDL labeled with ^{125}I-apoA-I from Sephacryl S-200 contains a

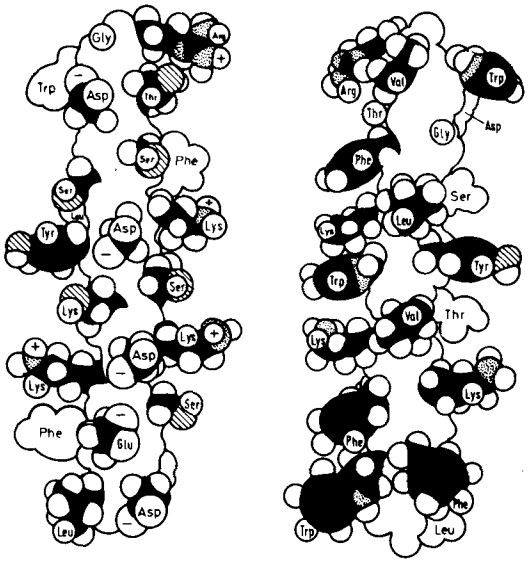

FIG. 10. Space-filling models of a fragment of apoC-III (40-72). Note that polar and nonpolar amino acid side chains appear on the opposite sides of the helix. (Permission granted by J.D. Morrisett).

TABLE I

Activation of Lipoprotein Lipase by Fragments of apo-II

Fragment	Activation
1-78	++
43-78	++
50-78	++
55-78	++
60-78	+
66-78	-
60-75	-

large peak at tube No. 45, demonstrating coelution of apoA-I and HDL phosphorus. In addition, a second small peak of free apoA-I elutes after HDL. This peak has an apoA-I concentration of ~ 0.003 mg/ml, a value comparable to that published by Scanu and coworkers for the critical monomer concentration of apoA-I (18). Thus, it is possible that monomer apoA-I exists in plasma in equilibrium with apoA-I bound to HDL. In Fig. 12B, the addition of apoC proteins to HDL can displace apoA-I to form a new structure enriched in apoC and devoid of most of its apoA-I. This new particle has a molecular weight of 400,000 and has the composition of LP-C (18). Since an apoC specie displaced an apoA specie, these results suggest that apoA and apoC cannot be assigned to different lipoprotein particles or families (19).

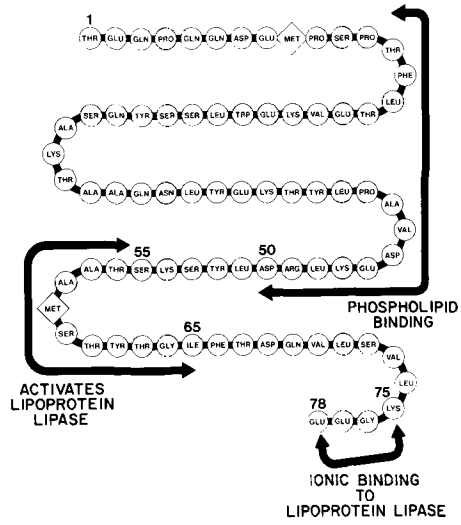

FIG. 11. Assignments of apoC-II functions to various regions of the peptide were based upon the activation of lipase and the lipid-binding studies conducted on synthetic and native fragments. (Permission granted by Baylor College of Medicine).

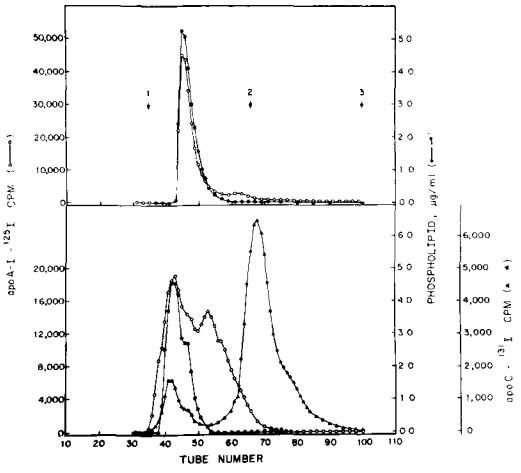

FIG. 12. (A). Gel filtration profile of HDL (6.5 mg) phosphorus and [^{125}I]-apoA-I. (B). Gel filtration profile of the products formed by the interaction of 6.5 mg HDL (containing [^{125}I]-apoA-I) and 5.5 mg total apoC proteins ([^{131}I]-labeled). The profile of the absorbance at 280 nm was approximately equal to the sum of the [^{125}I] and [^{131}I] profiles. (Permission granted by H.J. Pownall).

SUMMARY

We have shown that the apoproteins can bind rapidly to synthetic lipids and that the rate is a function of lipid structure. We have shown that the functions of HDL apoproteins can be localized to specific regions. Chemical

synthesis of these peptides is a powerful method to test theories of lipid-binding and enzyme activation. We suggest a mechanism for the mobilization of apolipoproteins in vitro; i.e. that the apoC of HDL may be transferred from the aqueous to the lipoprotein phase and that the reverse process can occur for apoA-I. The relative importance of these findings in a physiologic setting remains a challenging area of research.

ACKNOWLEDGMENTS

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Mitochondrial and Microsomal Phospholipid Phosphorus Metabolism during Postnatal Growth in Rat Heart and Liver

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ABSTRACT

The relative specific activities of phospholipids of hearts and livers of newborn and older rats were measured 2 hr after intraperitoneal ^{32}P-orthophosphate. There was a close linear correlation between the specific activity of cardiolipin and the net increase in the amount of this phospholipid. In one-day-old animals, the relative specific activities in the heart and liver were highest and exceeded by 7.3- and 3-fold, respectively, the corresponding activities in the adult. The apparent half-lives of cardiolipins were calculated on the basis of the linear correlation that was found between the net increase in cardiolipin and the rate of incorporation. These half-lives were 4.7 days in liver and 6.4 days in heart. Though the changes in the phospholipid composition of the organelles during neonatal development were small, the relative specific activities of the individual phospholipids varied considerably. In addition to cardiolipin, there was a good correlation between the specific activity and the net increase in mitochondrial phosphatidylinositol. At birth, the specific activity of mitochondrial phosphatidylcholine in liver was 2.9 times that in microsomes. During the 12 neonatal hours, the specific activity of microsomal phosphatidylcholine increased 6.3-fold and exceeded the corresponding mitochondrial activity. The relation between the activation of microsomal phosphatidylcholine synthesis and the induction of serum lecithin synthesis in newborn liver is discussed. The finding that at birth the specific activity of mitochondrial phosphatidylcholine unexpectedly was higher than that of microsomal phosphatidylcholine points out the difficulties in interpreting the in vivo evidence for precursor-product relationship.

INTRODUCTION

The neonatal period is characterized by growth and by adaptation to an abruptly changed environment. Many functions associated with the intracellular membranes, particularly with microsomes and mitochondria, increase in activity. In addition, the phospholipids of the membranes change during postnatal growth (1).

Labeled phosphate has been used to study phospholipid metabolism in a steady state (2,3) and during growth (4). The incorporation rates do not necessarily reflect the net membrane biosynthesis, since factors such as exchange phenomena, transport, changes in turnover rates and reutilization of the precursor may modify the activities measured. Cardiolipin is unique among the phospholipids, being specifically mitochondrial with respect to both localization and biosynthesis (5,6). By calculating the decay of the phosphate label, the apparent half-life of cardiolipin in rat liver was 11.5 days and even longer in the heart (7). However, proteins of the inner mitochondrial membranes of rat liver and heart had half-lives ranging between 5.5 and 6.2 days, when non-reutilizable precursors were used (8). In the present study, the apparent half-life of cardiolipin-P calculated during growth was not very different from that of the proteins bound to the inner mitochondrial membrane.

MATERIALS AND METHODS

Rats of the Sprague-Dawley strain were used. In labeling experiments using 0-hour-old animals, the fetuses were removed from the uterus by cesarean section at term (21.4-21.8 days from conception). Only litters with between 7 and 11 young were used. The individuals of a litter aged 10 days or less were killed at the same age. ^{32}P-orthophosphate was purchased from Radiochemical Centre, Amersham, and used within two weeks. The intraperitoneal dose of ^{32}P was 1.8 μCi/g body weight.

Liver mitochondria were isolated by differential centrifugation essentially as described earlier (9). The mitochondrial fraction was sedimented at 9000 g for 10 min. Mitochondria were washed twice in 10 ml of the isolation medium by resuspension and sedimentation at 7500 g for 10 min. Heart mitochondria were isolated by modification (9) of the method of Tyler and Gonze (10). Microsomes were obtained as follows: postmitochondrial supernatant was spun for 110,000 x g min. The new supernatant was spun for 60 min at 105,000 g and washed once by resuspension and sedimentation.

Inorganic phosphate in liver and heart homogenates was analyzed according to Ernster

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TABLE I

Heart Weights and Mitochondrial Phospholipid Pools during Postnatal Growth

Age (days)	Weight (mg)	Cardiolipin	Phosphatidylinositol	Phosphatidylcholine	Phosphatidylethanolamine
		(μg phosphorus)			
0	22.1	0.94	0.30	2.38	1.83
1	25.9	1.41	0.46	2.93	2.73
2	30.3	2.16	0.76	5.62	5.24
4	41.8	3.42	1.16	8.49	8.04
6	60.0	5.29	1.65	12.09	12.00
10	117.6	8.88	2.89	20.98	19.59
15	161.1	12.99	4.02	31.79	28.37
20	218.4	20.14	5.25	45.16	40.77

TABLE II

Liver Weights and Mitochondrial Phospholipid Pools during Postnatal Growth

Age (days)	Weight (mg)	Cardiolipin	Phosphatidylinositol	Phosphatidylcholine	Phosphatidylethanolamine
		(μg phosphorus)			
0	249.2	4.60	3.10	18.67	13.14
1	240.0	5.67	3.85	20.24	16.65
2	285.8	7.42	4.92	26.17	22.73
4	335.1	10.14	6.86	38.63	31.19
6	408.0	12.70	8.59	43.95	41.50
10	595.3	22.86	12.63	76.20	62.16
15	820.0	37.11	19.33	136.59	90.44
20	1219.6	49.54	29.44	207.04	139.74

and coworkers (11). The lipid fraction was extracted according to Folch et al. (12). Separation of phospholipids was performed by means of two-dimensional thin layer chromatography as described earlier (13). The individual phospholipids were detected by iodine vapor and quantified by measuring their phosphorus contents (14). Thereafter, the samples were decolorized and the radioactivity measured according to St. C. Palmer (15) using a Packard 2002 liquid scintillation spectrometer. The counting efficiencies were determined by internal standardization.

The relative rate of increase in the amount of phospholipid, (G), was measured as follows: the rate in the increase in the amount of the phospholipid was divided by the amount of the phospholipid at the time of the injection of the isotope. For instance, the relative rate of increase in phospholipid between the 12th and 14th neonatal hours was as follows: $G = (M_{24} - M_0)/ 1 \times M_{12}$ [μg/d x μg], where M_0, M_{12} and M_{24} are the amounts of phospholipids present at the ages of 0, 12 and 24 hr, respectively.

The amount of phospholipid was measured from 0 to 60 hr before ^{32}P-orthophosphate and from 12 to 60 hr after ^{32}P-orthophosphate, depending on the postnatal age. The calculation of the rate of increase in the amount of phospholipid was based upon these measurements. However, the growth rates were not quite linear (Tables I and II), causing some error in the calculation. This error is partly compensated for by the fact that at the age of less than two days, growth deviates positively and later negatively from the linear growth.

The electron microscopy was performed as described in the previous communication (9). The results are expressed as means ± SEM.

RESULTS

Purity of Organelles

The purity of the mitochondrial fractions was checked by electron microscopy. Liver and heart mitochondrial fractions from 0, 1, 4 and 8 day-old animals as well as from adults contained only small quantities of nonmitochondrial membranes. The contamination of microsomes by the inner mitochondrial membranes was estimated by measuring the ratio of cardiolipin in microsomes and in mitochondria. In most cases, the mitochondrial contamination was between 2 and 10%. However, in heart microsomes from animals 15 or more days old, mitochondrial contamination totalled 14 to 28%.

Amount of Phospholipids

The individual phospholipids in mitochon-

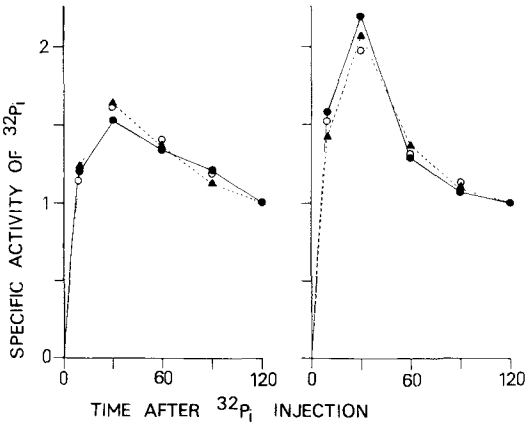

FIG. 1. Labeling of inorganic phosphate in heart (left) and liver (right) after intraperitoneal ^{32}P-orthophosphate into 2-4-hour-old (●), 3-day-old (▲), or adult (○) rats. For comparison, the specific activities 2 hr after the injection have been equalled. The results are the means of 3 to 6 individual measurements.

dria and in microsomes were expressed as a percentage of total phospholipid. There were only small changes during development, indicating that the net synthesis rates of individual phospholipids were rather constant (data not shown, *cf.* refs. 16 and 17). Tables I and II show the pool sizes of some mitochondrial phospholipids during postnatal development. It was assumed that cardiolipin is exclusively present in mitochondria. The other phospholipids were calculated on the basis of the percentages of mitochondrial phospholipids and of the total amount of cardiolipin. The relative growth rates of phospholipid pools were most rapid during the first neonatal days.

Labeling Experiments

We injected ^{32}P-orthophosphate intraperitoneally and measured the entry of the isotope into the nonlipid phosphate pools. Three different age groups were studied. Figure 1 presents data from 2-4 hour-old, 3-day-old and adult animals. There were only small differences in the labeling of phosphate in both liver and heart in the different age groups.

Figures 2 and 3 show the relative specific activities of mitochondrial and microsomal phospholipids as measured from liver and heart, respectively. Cardiolipins were assayed both in mitochondria and in the total homogenate. These measurements gave essentially the same result, suggesting that the *average* rates of cardiolipin synthesis did not differ in the mitochondrial fraction and in those mitochondria that sediment together with other cell fractions.

The relative specific activities of cardiolipin were about one order of magnitude lower than

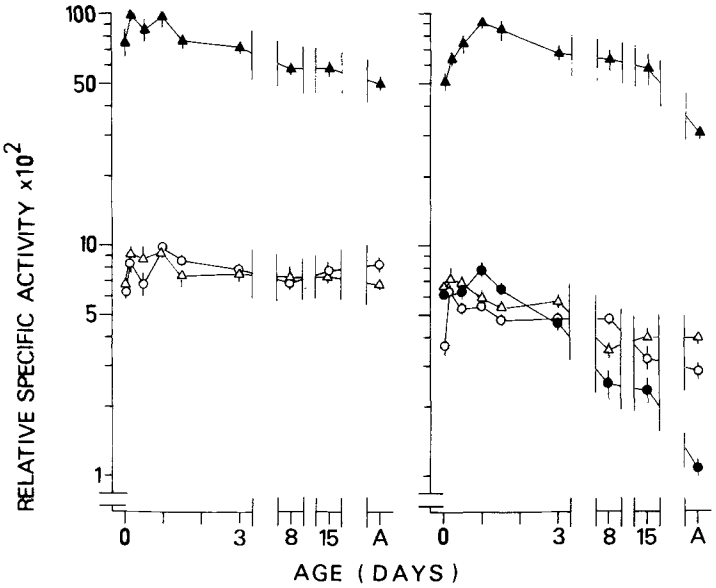

FIG. 2. Relative specific activities of phospholipid-P in heart microsomes (left) and mitochondria (right) 2 hr after the intraperitoneal ^{32}P-orthophosphate as a function of postnatal age. The relative specific activity was the ratio of the specific activity of the phospholipid fraction to the specific activity of the inorganic phosphorus. The results are expressed in a semilogarithmic scale as means ± SEM of 3 to 8 individual measurements. Cardiolipin (●), phosphatidylinositol (▲), phosphatidylcholine (○), phosphatidylethanolamine (△).

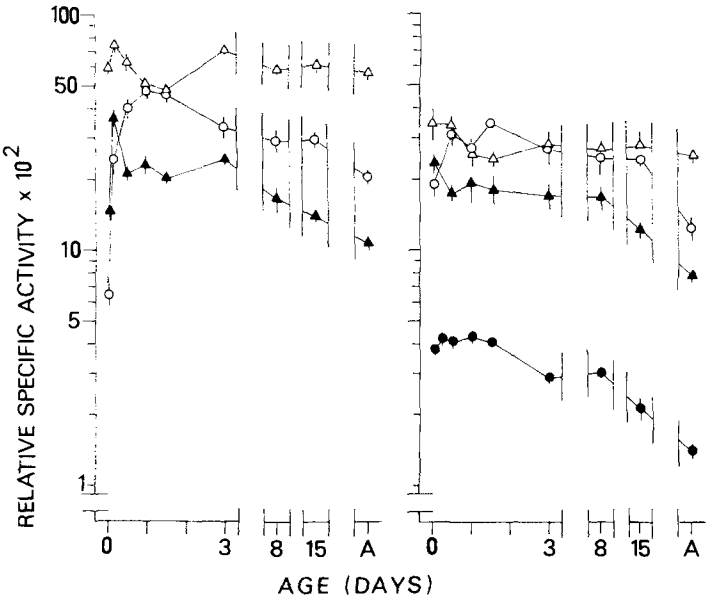

FIG. 3. Relative specific activities of phospholipid phosphorus in liver microsomes (left) and mitochondria (right) 2 hr after the intraperitoneal ^{32}P-orthophosphate as a function of postnatal age. Cardiolipin (●), phosphatidylinositol (▲), phosphatidylcholine (○), phosphatidylethanolamine (△). For other details see Figure 2.

those of phosphatidylinositol, and the specific activities of these two phospholipids changed during development in a similar way. In general, the activities were higher during growth than in the adult.

The specific activity of phosphatidylcholine in liver microsomes at birth was lower than that in liver mitochondria. However, during the 12 neonatal hours, the microsomal activity increased 6.3-fold, and by that time exceeded the corresponding mitochondrial activity. The specific activities of phosphatidylethanolamines showed only small developmental changes.

Figure 4 shows the relation between the specific activities of cardiolipin and the relative rates of increase in this phospholipid in liver and heart. A good linear correlation was shown between these two parameters ($r = 0.961$ in liver, $r = 0.968$ in heart).

DISCUSSION

According to the present results, the rate of cardiolipin synthesis correlates with the relative specific activity of cardiolipin labeling using ^{32}P-orthophosphate as a precursor. The fact that the corresponding correlation with the other phospholipids was worse or not present at all may be due to several factors.

Exchange Phenomenon

According to McMurray and Dawson, phosphate incorporates into phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol by a rapid exchange mechanism (2). The contribution of the exchange phenomenon to the specific radioactivities in the present results remains unclear. At least the rate of phosphatidylinositol incorporation bears a fairly good correlation to the growth, despite

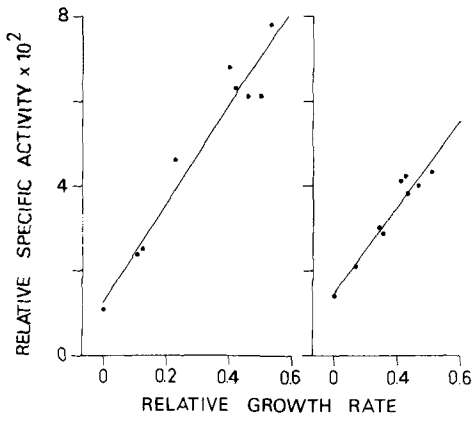

FIG. 4. Relationship between the relative specific activity of cardiolipin and the relative growth rate of cardiolipin pool (relative rate of increase in the amount of cardiolipin, G) in heart (left) and liver (right). The straight lines fitted to the data from heart and liver are $y = 11.603 x + 1.268$ ($r=0.968$); $y = 10.125 x + 1.499$ ($r=0.961$), respectively.

the exchange phenomenon. However, there is no evidence indicating the exchange of cardiolipin phosphorus.

Precursor-Product Relationship

The phospholipid may be either a biochemical precursor, such as phosphatidylethanolamine, or else transported to or from another compartment. According to the present evidence, mitochondrial phospholipids are mostly derived from microsomes (3,6,18). In contrast, cardiolipin is synthesized only in mitochondria (5,6).

On the basis of the assumed precursor-product relationship, it was unexpected that the specific activity of phosphatidylcholine was higher in mitochondria than in microsomes at birth. It is unlikely that a rapid mitochondrial biogenesis could cause such an abnormal relationship between mitochondrial and microsomal specific activities, since this relationship normalized shortly after birth, despite continuing rapid growth. According to the measurements of CDP-choline and S-adenosylmethionine incorporation into lecithins using mitochondria and microsomes isolated from newborn rat liver, mitochondrial phosphatidylcholine synthesis was not abnormally high at birth (unpublished results). On the other hand, there is evidence that plasma lecithin in the fetus comes from the placenta (19). It is tempting to speculate that the low rate of phosphate incorporation into phosphatidylcholine at birth and its rapid increase reflects an induction of plasma lecithin synthesis in liver microsomal fraction. This possibility remains to be studied further. Nonetheless, the finding that at birth the specific activity of mitochondrial phosphatidylcholine exceeds that of microsomes points to the difficulties in interpreting the in vivo data in favor of or against the evidence for a precursor-product relationship.

Rate of Phosphate Uptake and Labeling of Intermediates of the Biosynthetic Pathway

As shown in Figure 1, the pattern of phosphate uptake was affected very little by age. We additionally measured the specific activities of phosphatidic acid and found that they resembled those of inorganic phosphate, though low concentrations of phosphatidic acid rendered these measurements difficult. The analysis of the specific activities of the precursors of cardiolipin, CDP-diglyceride and phosphatidylglycerol was associated with even greater problems of quantitation. Therefore, we expressed the relative specific activities of the phospholipids on the basis of the specific activities of inorganic phosphate.

Stability of Half-Life during Growth

There is evidence that, during logarithmic growth, tissue breakdown is minimal (1). However, the linear correlation between the actual rate of increase in cardiolipin and the relative specific activity of cardiolipin synthesis (Fig. 4) does not support the alternative that the half-life varies during linear growth.

On the basis of the previous discussion, it seems that in the present conditions the relative specific activity of cardiolipin closely correlates with the total de novo synthesis (*i.e.*, the turnover) of this phospholipid. This association enables the calculation of the apparent half-life.

In the nongrowing rat, the relative specific activity of cardiolipin (RSA_0) represents the "prepair synthesis" (P). The "prepair synthesis" can be defined as the derivative of the exponential function ($y=k^x$, where y = the amount of cardiolipin, x = time, k = constant): P = 1n k (x = 0, y = 1).

During growth, the total synthesis is the sum of the "prepair synthesis" and the "growth synthesis." The latter equals the relative rate of increase in cardiolipin, (G). The following correlation exists between the specific activities and the actual biosynthesis rates of cardiolipins:

$$1n\ k/RSA_0 = (1n\ k + G)/RSA_1, \text{ where}$$

RSA_1 = relative specific activity during growth.

The half-life of cardiolipin could be expressed as follows:

$$T_{1/2} = \log 0.5/\log k = 0.693\ (RSA_1 - RSA_0)/RSA_0 \times G$$

The calculated half-lives for cardiolipins in the liver and the heart were 4.7 and 6.4 days, respectively. The corresponding values as measured by Gross and associates on the basis of the decay rate of ^{32}P-orthophosphate were considerably longer (7). As pointed out by these authors, isotope reutilization could artificially prolong the half-lives, whereas in the present method this is not a likely source of error. On the other hand, particularly the difficulty in measuring the actual relative rate of increase in cardiolipin may cause our calculations to deviate. Half-lives of cardiolipins remain to be further measured using other techniques.

The similarity in the apparent turnover rates as compared between cardiolipin and other components of the inner mitochondrial membrane (8) suggests that the biogenesis and the breakdown of the inner mitochondrial membrane takes place as a unit. This view does

not exclude the possibility that the membrane biosynthesis takes place stepwise and that some of the membrane components, such as the fatty acids of the phospholipids, have additional turnover mechanisms (20).

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Biliary Cholesterol Absorption in Normal and L-Thyroxin-Fed Rats

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ABSTRACT

Infusion of bile containing labeled cholesterol into bile fistula rats has permitted an in vivo study of the movements and of the absorption of biliary cholesterol in the digestive tract. The specific activities of cholesterol were similar in the micelles and the sediment of the luminal content after a 6 hr infusion, indicating rapid exchange of cholesterol between these fractions. In animals fed a basal diet, the biliary cholesterol absorption was higher (83%) than that of dietary cholesterol (70%). Bile cholesterol is essentially absorbed in the jejunum while the absorption of cholesterol from the diet takes place all along the small intestine but preferentially in its second and third quarters. Both alimentary cholesterol and bile cholesterol enter the top cells of the villi in preference to those of the crypts. In L-thyroxin-fed rats, a parallel decrease in biliary and dietary cholesterol absorption was observed. The increase in the intestinal transit of cholesterol and epithelium cell renewal of the jejunum accounted for this observation.

INTRODUCTION

Many authors have studied the various aspects of dietary cholesterol absorption; i.e., movements of sterols in the gut (1,2), physicochemical characteristics and localization of its absorption (3-5), and formation and transport of chylomicrons (5-8). We know that dietary cholesterol is not homogenously mixed with endogenous (biliary plus intestinal) cholesterol in the intestinal lumen. There are, in fact, two cholesterol compartments ("intermediary" and "axial") with a transfer of endogenous cholesterol from the intermediary to the axial compartment (2). Since the intestinal content is a nonhomogenous mixture of exogenous and endogenous cholesterol, the behavior of each component can be different, in particular the localization and the rate of biliary vs. dietary cholesterol absorption. Therefore, we were interested in the in vivo absorption of biliary cholesterol and set up experiments in which the movements of biliary cholesterol in the digestive tract could be differentiated from those of plasma cholesterol. Furthermore, we sought to compare rats fed L-thyroxin to ones fed a basal diet because L-thyroxin administration strongly increases fecal cholesterol excretion and decreases dietary cholesterol absorption (9).

EXPERIMENTAL PROCEDURES

Experiments were done either with bile fistula rats receiving a compensatory infusion of bile (infusion experiments) or with animals who did not undergo any surgical stress (isotopic equilibrium experiments).

Radioactive Cholesterol

[4-^{14}C]cholesterol and [^{3}H]G-cholesterol (40-50 mCi/mmole) were purchased from C.E.A. (France). Purity was checked by thin layer chromatography on Silica Gel G with hexane/ethylacetate (80:20, v/v) and was found to be $\geqslant$ 97%.

Animals and Diets

Control animals were 3 month-old male Wistar rats weighing an average of 250 g. The composition of semipurified basal diet was: casein 23%, sucrose 53%, yeast 2.5%, skim milk 4%, walnut oil 0.8%, lard 9.2%, salt mixture 5%, vitamins 0.2%, and agar-agar 2.3% (9). This diet was nearly free of cholesterol and phytosterols (0.015%). L-thyroxin-fed rats received the control diet supplemented with 5 mg L-thyroxin/kg for 11 days. Since these rats lost an average of 15 g in weight during the treatment, we chose proportionately bigger rats for these experiments. After thyroxin ingestion, the final weight was 370 to 400 g (Table I). The housing and the strict lighting schedule have been described (10). All rats were fed ad libitum. Bile donors were grown in the same conditions as experimental rats except that their diet contained trace amounts of [4-^{14}C]-cholesterol (300 μCi/kg). The day before an infusion experiment, the labeled bile was collected for 2 hr and kept at 4 C overnight.

Infusion Experiments

The common bile duct was doubly catheterized: first, to collect bile (PE 10 intramedic); and second, the perfuse (Biotrol n° 3) bile

containing [4-^{14}C]cholesterol at the rate of 0.9 ml/hr. This type of bile perfusion circumvents the interruption of bile acid enterohepatic circulation. It was always begun at 10 a.m., and the rat was placed in a restraining cage for 6 hr. As the experiment took place during a period of the day when rats do not normally eat (11), they just received water. At the end of the infusion (4 p.m.), rats were anesthesized with pentobarbital and killed by aortic puncture. After 3 to 4 washings with saline, the liver and the digestive organs (stomach, intestine divided into two equal fractions, caecum-colon) were removed. The intestinal contents were collected and the walls were washed with a citrate buffer (NaCl, 96 mM; KH_2PO_4, 8mM; Na_2PO_4H, 14 mM; KCl, 15 mM; $Na_3C_6H_5O_7$, 20 mM; pH = 7.4). The intestinal contents were then centrifuged at 54,000 g for 30 min in order to separate the micellar fraction from the sediment (1). The infused bile carried through the same centrifuging procedure showed no radioactivity in the sediment. Jejunal and ileal epithelial cells were also collected with the citrate buffer (12). With this method, we obtained essentially the epithelial cells of the top of the villi, as confirmed by optical microscopy.

Isotopic Equilibrium Experiment

An isotopic equilibrium experiment as described earlier (13) was performed in three rats fed the basal diet. These animals received a daily subcutaneous injection of [^{3}H]cholesterol (0.1 mg 1 μCi) and trace amounts of [4-^{14}C]cholesterol in their diet (2.5 μCi/kg). After 4 weeks, the specific activity of cholesterol in the plasma, red cells, liver, and bile reached the same value. Under these conditions the value (x) of cholesterol-specific activity in an organ (expressed as a percentage of the blood cholesterol specific activity) indicates that a fraction (equal to x %) of cholesterol present in that organ has been exchanged with plasma cholesterol (14). In this experiment, the rats were allowed to move freely about in their cages and were sacrificed at 4 p.m. The technique used for tissue collection was the same as above, except for the epithelial cells in the intestine. In this case, the method of Weiser (15) was used which permits the collection of cell fractions beginning at the top of the villi and ending with the crypts.

Analytical Methods

Tissue lipids were extracted overnight with boiling ethanol (16) or with chloroform/methanol (2:1, v/v). The extracts were washed and dried, and the free cholesterol was separated from its ester on a silicic acid column, following the modified method of Hirsch and Ahrens (17). After hydrolysis of the esterified cholesterol, cholesterol was extracted with petroleum ether and quantified by the Liebermann-Burchard reaction with the digitonin complex (18) or by gas liquid chromatography (19). The bile and intestinal contents were directly saponified 2N KOH in ethanol for 3 hr). Neutral sterols were extracted by petroleum ether from the ethanol/water (1:1, v/v) phase, and quantified as above. The bile acids present in the water phase were discarded.

Radioactivity was measured in a toluene solution containing PPO and dimethyl-POPOP with a liquid scintillation spectrometer (Intertechnique, Plaisir, France).

RESULTS

Mean body weights, cholesterol concentrations in bile, plasma, liver and digestive tract, and quantities of cholesterol in the intestinal contents are given in Table I. As the relative concentration of esterified cholesterol in the digestive tract walls was very low (1-5% of the free), only total cholesterol levels are reported in Table I. In this study, "control" rats are the bile fistula rats of the infusion experiments fed the basal diet, as opposed to those fed L-thyroxin. On the other hand, "normal" rats are the isotopic equilibrium animals who did not undergo any surgical stress. Cholesterol concentrations in the various digestive tract walls were the same in control bile fistula and in normal (isotopic equilibrium) rats (Table I). Also, the quantities of cholesterol found in the intestinal contents were similar.

Cholesterol Radioactivity of Digestive Contents

The total amount of biliary [^{14}C]cholesterol radioactivity infused over the 6 hr period was 25,000 to 90,000 dpm. No radioactivity was found in the contents of the stomach. However, bile cholesterol was found in the caecum and colon. In the control rats, one-third of the total infused cholesterol radioactivity (32 ± 6%) was found in the lumen of the digestive tract of control rats at their sacrifice. Almost all of it was localized in the intestine (95 ± 2%). In L-thyroxin-fed rats, more than half of the infused biliary cholesterol radioactivity (52 ± 10%) was still present in the contents of the digestive tract. Proportionately less radioactivity was found in the intestine (66 ± 9%) but more in the caecum and colon (28 + 12%). Also, the mean relative proportions of

TABLE I

The Cholesterol Content of Bile, Plasma, Liver and Digestive Tract of Control and L-Thyroxin-Fed Rats with a Bile Fistula (Infusion Experiments) and of Normal Rats (Isotopic Equilibrium Experiment)

Type of experiment		Infusion		Isotopic equilibrium
Diet		Basal	Basal L-thyroxin	Basal
Number of rats		5	4	3
Mean weight of rats (g)		352 ± 4[a]	384 ± 6	446 ± 5
Cholesterol concentrations in:				
Bile free (mg/ml)		0.13 ± 0.01	0.15 ± 0.03	—
Plasma free (mg/ml)		0.16 ± 0.02	0.16 ± 0.01	0.18 ± 0.02
Plasma esterified (mg/ml)		0.38 ± 0.05	0.38 ± 0.05	0.38 ± 0.04
Liver free (mg/g wet wt)		1.64 ± 0.05	1.68 ± 0.05	1.74 ± 0.06
Liver esterified (mg/g wet wt)		0.42 ± 0.11	0.28 ± 0.08	0.53 ± 0.10
Stomach total (mg/g wet/wt)		2.10 ± 0.14	2.02 ± 0.14	2.04 ± 0.07
Intestine				
first half total (mg/g wet/wt)		1.89 ± 0.06	1.98 ± 0.07	1.95 ± 0.05
second half total (mg/g wet wt)		1.95 ± 0.05	1.91 ± 0.06	1.92 ± 0.04
Caecum-colon total (mg/g wet/wt)		1.98 ± 0.11	2.02 ± 0.05	2.01 ± 0.06
Cholesterol in intestinal contents:				
first half (mg)	Micelles	0.06 ± 0.02	0.10 ± 0.04	0.10[b]
	Sediment	0.42 ± 0.08	0.32 ± 0.10	0.12[b]
second half	Micelles	0.08 ± 0.03	0.05 ± 0.02	0.05[b]
	Sediment	0.62 ± 0.14	0.54 ± 0.16	0.65[b]

[a]Mean ± SEM.

[b]Average of three pooled samples.

bile radioactivity found in the ileal vs. the jejunal contents was greater (157:100) than in the controls (69:100).

Both the sediment and the micelles in the first and second part of the intestine contained biliary cholesterol. This is shown by the relative cholesterol-specific activities of these fractions (Table II). This table contains also the results obtained from the isotopic equilibrium experiment. Plasma [^{3}H]cholesterol-specific activity was very similar in the micelles and in the sediment from jejunal or ileal contents. For dietary [^{14}C]cholesterol, the specific activity values are also very close in the sediment and micelles of the ileal content. The difference between the two values was slightly larger in the jejunum. We noted that the cholesterol specific activity was greater in the intestinal contents of the ileum.

Cholesterol Radioactivity of Intestinal Walls

Neither the wall of the stomach nor that of the caecum or the colon contained any radioactivity. In the control rats, 60 ± 5% of the infused biliary cholesterol was present in the intestinal wall. Most of it (95 ± 3%) was in the first half of the intestine at the end of the infusion (Fig. 1). In the thyroxin-fed animals, 42 ± 5% of the infused biliary cholesterol was found in the intestinal wall, and again the major part (90 ± 3%) in the first half.

In the isotopic equilibrium experiment, the [^{14}C]cholesterol radioactivity in the intestinal wall was due to its exchange with plasma and with the recently absorbed alimentary cholesterol. The [^{3}H]cholesterol radioactivity was due only to exchanges with plasma. After subtracting the [^{14}C]cholesterol exchanged with plasma, it was possible to calculate the quantity of dietary cholesterol present in the walls at the time of sacrifice. It has been expressed as the percent of total dietary cholesterol present in the intestinal wall (Fig. 1).

The mean [H^3]cholesterol-specific activity from plasma origin was the same in the crypts and in the top cells of the villi during the isotopic equilibrium experiment (Table II). It was different for [^{14}C]cholesterol where the mean cholesterol-specific activity appeared to be higher in the cells at the top versus those of the crypts in the second half of the intestine.

DISCUSSION

The fact that all results concerning the

TABLE II

Relative Cholesterol Specific Activities in Plasma and Intestine (Contents and Walls)[a]

Type of experiment			Infusion	Isotopic equilibrium	
Diet			Basal	Basal	
Radioactive cholesterol	Label Route administered		[4 ^{14}C] bile	[4 ^{14}C] diet	[^{3}H] subcutaneous
Cholesterol specific activity in:					
Bile			100	b	b
Plasma			<0.1	100 ± 2 [c]	100 ± 3
First half of intestine	Content	Micelles	21.1 ± 6.6[d]	131[e]	88
		Sediment	21.1 ± 4.5	119	83
	Wall		3.5 ± 0.7	84	59
	Epithelial	Villi	13.8 ± 4.6	121	56
	Cells	Crypts	n.d.	114	55
Second half of intestine	Content	Micelles	10.9 ± 5.0	224	85
		Sediment	11.6 ± 3.7	205	72
	Wall		0.2 ± 0.1	97	61
	Epithelial	Villi	2.2 ± 1.2	180	60
	Cells	Crypts	n.d.[f]	144	54

[a]The intestine was divided into two parts. Results are expressed as a percentage of cholesterol specific activity in bile (infusion experiments) or as a percentage of plasma cholesterol specific activity (isotopic equilibrium experiment).

[b]The specific activity of bile cholesterol is identical to that of plasma cholesterol in these rats (20).

[c]*Mean ± SEM (3 rats).*

[d]Mean ± SEM (5 rats).

[e]Value of three pooled samples.

[f]n.d. = Not determined.

concentrations of cholesterol in various tissues of rats submitted to the infusion experiment came out about the same as the normal rats used in the isotopic equilibrium experiment suggests that the surgical stress experienced by the bile fistula rats did not cause major perturbations in cholesterol turnover. In control rats, the mean concentration of bile cholesterol (0.13 ± 0.01 mg/ml) as well as its excretion rate (0.11 ± 0.02 mg/hr) in the 6-hr infusion experiments agree with previous results from our laboratory and with those obtained from bile fistula rats without reinfusion (21-23).

When rats were fed an L-thyroxin diet, neither cholesterol concentration in the walls of the digestive tract nor its quantity in the intestinal contents was significantly affected. Although hyperthyroidism is known to lower the cholesterolemia (5,24), its level was unchanged in our experiments (Table I). This may be related to the duration of the experiment (11 days), which perhaps is too short to produce a significant effect on plasma levels. Furthermore, the concentration of bile cholesterol was not affected by L-thyroxin. Although slightly higher on the average in thyroxin-fed animals, the mean hourly cholesterol excretion in bile was not significantly different from that of the control (0.15 mg ± 0.03 mg vs. 0.11 mg ± 0.02 mg). Therefore, we conclude that under our conditions L-thyroxin does not modify the rate of bile cholesterol excretion. As rats with chronic biliary drainage exhibit a circadian rhythm of the bile flow and of the excretory rates of bile salts, cholesterol and phospholipids (25), we measured the hourly rate of biliary cholesterol excretion between 10 p.m. and 4 a.m. in control (0.10 ± 0.03 mg/hr, 4 values) and in L-thyroxin-fed rats (0.13 ± 0.03 mg/hr, 4 values). Again, there was no significant difference.

Biliary Cholesterol Distribution in the Digestive Contents

The data presented here show that the duodenal content does not reflux into the stomach. Bile cholesterol found in the caecum and colon indicates that not all of the cholesterol is absorbed in the intestine.

The presence of biliary cholesterol in the sediment could be due to an exchange between micelles and sediment, or to desquamated epithelial cells containing recently absorbed biliary cholesterol, or to both mechanisms. Since the specific activity of cholesterol in the cells was much lower than in the adjacent content, particularly in the ileum, the role of cell sloughing is small compared to that of the exchange of cholesterol between the two fractions. This conclusion is consistent with the

results of MacIntyre et al. (1). They found a similar sterol pattern in the micelles and in the corresponding sediment. Moreover, the similarity of the cholesterol-specific activities in the sediment and the micelles in both halves of the intestine (Table II) suggests that the biliary cholesterol exchanges very rapidly between the two fractions. Results obtained from the isotopic equilibrium experiment as well as from the L-thyroxin-fed rats lead to this same conclusion. However, the differences found in the distribution of the infused biliary cholesterol at the end of the infusion experiments show that there is an accelerated intestinal transit in the L-thyroxin-fed rats compared to the controls.

In the isotopic equilibrium experiment, the higher cholesterol-specific activity of the ileal content (Table II) compared to that of jejunum is due to the increasing proportion of exogenous versus endogenous cholesterol, as shown in a previous report (2).

Biliary Cholesterol Distribution in the Intestinal Wall

The absence of radioactivity in the walls of the stomach, caecum, and colon is additional supportive evidence that cholesterol absorption takes place only in the intestine (2).

Since 68% of the infused biliary cholesterol had been absorbed at the end of the infusion experiment, the absorption coefficient of bile cholesterol has to be at least 68%. To obtain a more precise value for this coefficient, we infused bile fistula rats with bile containing labeled cholesterol for 6 hr and then re-established normal bile circulation. The unabsorbed biliary cholesterol was measured from the unsaponifiable fraction of feces collected during the following 3 days. Under these conditions, the absorption coefficient of bile cholesterol averages 83± 4% (4 rats), which is higher than that of diet cholesterol, found to be 69.9 ± 1.1% (5 rats) by an isotopic equilibrum method (26).

As shown by Figure 1, the biliary cholesterol is essentially absorbed in the first half of the intestine. In contrast, the absorption of dietary cholesterol takes place all along the intestine. In a complementary isotopic equilibrium experiment of four rats fed the basal diet, the percent distribution of the total alimentary cholesterol present in the wall of the intestine was: 15± 3% (first quarter), 33 ± 4% (second), 38 ± 4% (third) and 14 ± 2% (fourth). Similar data were also obtained in our laboratory with other dietary conditions. These results agree with previous data of Swell et al. (27), who obtained similar results in lymph fistula rats fed a test

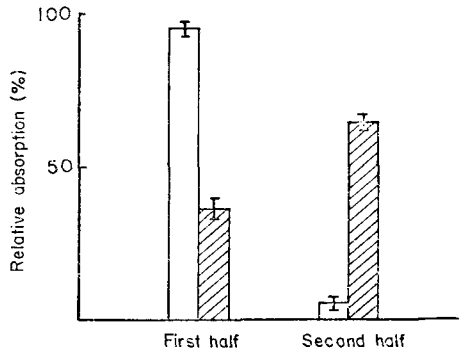

FIG. 1. Percent of absorbed biliary (□) cholesterol (infusion experiments) and dietary (▨) cholesterol (isotopic equilibrium experiment) present in the first and the second halves of the intestinal wall.

meal containing 40-44 mg [4-^{14}C] cholesterol but concluded that the upper half of the intestine is the chief site of cholesterol absorption.

Location of Absorption Activity in the Villi

Since the specific activity of [^{14}C] cholesterol in the cells was obtained on pooled samples (isotopic equilibrium experiment, Table II), no definite statistical conclusions can be drawn. However, the variance of specific activity of plasma cholesterol for each rat was less than 10%. Also, the higher mean specific activity of [^{14}C] cholesterol in the top cells of the ileal villi than those of the crypts suggests that dietary cholesterol absorption takes place preferentially in the villus, as proposed by Sylven and Nordström (4). Biliary cholesterol is also probably preferentially absorbed in the top cells since three to four times more radioactivity was found in the tops of the villi than in the crypts (jejunum) 3 hr after a duodenal infusion of bile containing labeled cholesterol (2 rats, data not included).

L-Thyroxin Influence on Biliary Cholesterol Absorption

The minimal absorption coefficient of the biliary cholesterol calculated for the thyroxin fed rats after the 6 hr infusion experiments reached only 47 ± 5%. The rest of the cholesterol was found in the luminal contents. If feces are collected during the three days following an infusion of labeled bile, as discussed above for control rats, the absorption coefficient of cholesterol reaches 59 ± 2% (4 rats). Therefore, L-thyroxin ingestion has a negative effect on bile cholesterol absorption. This result is to be expected, since a decrease in dietary cholesterol absorption under the same conditions has been

described previously (9). After a 6-hr infusion of labeled bile, there is proportionately more bile cholesterol in the ileum and colon contents and less in the jejunum than for the controls. Moreover, a higher amount of biliary cholesterol can be found in the ileal sediment. The lowered cholesterol absorption and the modified location of the biliary cholesterol in the L-thyroxin-fed rats agree with the accelerated intestinal transit and the higher turnover of intestinal epithelium described previously for hyperthyroid rats (28).

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Perinatal Development of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Rat Lung, Liver and Brain

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ABSTRACT

The developmental pattern of microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34), which catalyzes the rate-limiting step of cholesterol biosynthesis, was studied in lung, liver and brain of Sprague-Dawley rats. Each tissue exhibited a distinct pattern. Reductase activity in the fetal lung reached a peak at 19 days of gestation, which corresponds to the onset of active surfactant production. This observation is consistent with the suggestion that the fetal lung synthesizes all surfactant components including cholesterol. In the liver, reductase activity varied in a reciprocal fashion with serum cholesterol levels. The peak of brain reductase activity occurred at 3 days after birth at the onset of rapid brain growth despite rapidly rising serum cholesterol levels.

A deficiency in the production of lung surfactant is presently considered a principle cause of the Respiratory Distress Syndrome seen in the newborn (1). Since surfactant contains large amounts of fully saturated phosphatidylcholines, mainly dipalmitoyl phosphatidylcholine, the synthesis of this phospholipid by alveolar cells has been extensively investigated (1). The chemical composition of surfactant shows that this material is not solely dipalmitoyl phosphatidylcholine but rather a complex mixture of several species of phospholipids, neutral lipids and proteins (2). The neutral lipid is mostly cholesterol which on a molar basis comparises 10-25% of the total lipid of surfactant (2) and may serve an extremely important physiochemical function. Although dipalmitoyl phosphatidylcholine would by itself provide a sufficiently low alveolar surface tension, its phase transition temperature is 41 C which means that it would be in a "solid gel" state at body temperature (3). The addition of cholesterol to dipalmitoyl phosphatidylcholine lowers the transition between the gel and liquid crystalline phase to below 37 C (3). Monoenoic phospholipids exhibit a similar effect. The presence of these lipids in surfactant would be expected to lower the phase transition temperature of the complex and thus insure proper adsorption of the surface active material at the alveolar interface (2).

Surfactant is produced and secreted by alveolar type II epithelial cells (4,5). These cells have the capability to synthesize monoenoic and fully saturated phospholipids (1,6). Developmental studies indicate that the enzyme activities responsible for the synthesis of these lipids reach maximal levels at 85 to 90% of term which corresponds to the onset of lung surfactant production (1). It is implied that all surfactant components are synthesized in the lung. However, no information is presently available concerning the levels of the enzyme activities involved in lung cholesterol biosynthesis during the fetal and neonatal periods.

In view of these observations, we have investigated the development of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) activity in lungs from fetal and neonatal rats. This enzyme catalyzes the rate-limiting reaction of cholesterol biosynthesis (7). For the purposes of controls and comparison, we also measured HMG-CoA reductase in livers and brains during development.

MATERIALS AND METHODS

Timed, pregnant Sprague-Dawley rats were purchased from ARS/Sprague Dawley, Madison, WI. The animals were housed in individual cages in a windowless room with lights on from 600 hr to 1800 hr and were fed Wayne Lab Blox and water ad libitum. Pregnant females at 16.5 to 21.5 days of gestation were killed by decapitation at 900 hr. The fetuses were removed by hysterotomy and immediately decapitated. A blood sample was obtained. The lungs, livers and brains from littermates were pooled and weighed. Suckling rats were killed at 900 hr also. For postweanling studies, the pups were removed from their mothers at 18 days of age and fed solid food in a windowless room with lights on from 2200 hr to 1000 hr the following day. All postweanling animals were killed at 1300 hr.

The tissues were minced and then homogenized with a motor-driven Teflon-glass Potter Elvehjem homogenizer using 10 ml of ice cold 0.3 M sucrose containing 1 mM dithiothreitol (SD) per gram of tissue. The broken cell preparation was centrifuged at 10,500 x g for 15 min. The resulting supernatant fraction was

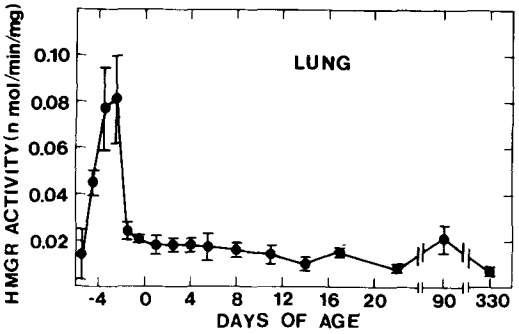

FIG. 1. Rat lung microsomal HMG-CoA reductase activity during development. The means and standard deviations are presented. Each point represents at least 3 litters or in the case of suckling pups at least 6 animals.

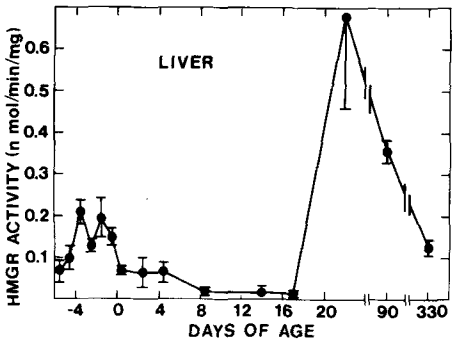

FIG. 2. Rat liver microsomal HMG-CoA reductase activity during development. The means and standard deviations are presented. Each point represents at least 3 litters or in the case of suckling pups at least 6 animals. Rat pups were weaned at 18 days of age.

centrifuged for 45 min at 145,000 x g. The pellet which contained the microsomes was resuspended in cold SD media using 1.5 ml per gram of tissue.

HMG-CoA reductase activity of these microsomal preparations was determined as described by Shapiro et al. (8). This method measures the formation of radioactive mevalonate from [^{14}C]HMG-CoA. The labeled mevalonate was converted to mevalonolactone and separated from the substrate by thin layer chromatography using acetone/benzene (1:1). Separation of labeled mevalonolactone from HMG-CoA by the frequently used anion exchange method (9) leads to erroneous values for HMG-CoA reductase activity caused by interfering enzyme activities present in isolated microsomes (10). Reductase activity was expressed in terms of nmol of mevalonate formed per min per mg of microsomal protein.

Microsomal protein was determined by a biuret method (11). Serum cholesterol levels were determined by the method described by Abell et al. (12).

RESULTS

If the cholesterol needed for the production of surfactant is derived from synthesis by the fetal lung, one would expect the development of cholesterol biosynthesis to parallel that of the other surfactant components. It has been shown that lung phospholipid synthesis and surfactant production increase sharply at ca. 90% of term (13). The developmental pattern of HMG-CoA reductase activity in rat lung microsomes is shown in Figure 1. A pronounced peak of reductase activity occurred at ca. 19 days of gestation or 86% of term (full term is 22 days). The activity then fell sharply before birth and remained at low levels. This pattern closely parallels that of fetal lung lecithin biosynthesis by the CDP-choline pathway (1). The peaks of lung cholesterol and phospholipid biosynthesis both occur at the onset of lung surfactant production.

Since the liver is considered to synthesize most body cholesterol, the development of hepatic HMG-CoA reductase was examined. As shown in Figure 2, hepatic microsomal HMG-CoA reductase activity is elevated during the last 4 days of gestation, when surfactant is being actively produced. Liver reductase activity declines after birth and reaches very low levels at 8 to 17 days of age. The activity then rises sharply as the rat pups are weaned onto solid food. These levels are considerably higher than those observed in adult rats. This general pattern for hepatic reductase activity agrees with that reported by McNamara et al. (14).

Since rat milk contains cholesterol and since it has been established that hepatic reductase activity decreases markedly in adult rats fed cholesterol (15), serum cholesterol levels were determined as a function of development (Fig. 3). Serum cholesterol levels increased 2- to 3-fold during the suckling period, when liver reductase levels reached their lowest values. Upon weaning, serum cholesterol levels fell concomitant with the sharp rise in hepatic reductase activity.

In contrast to the pattern in lung and liver, brain microsomal HMG-CoA reductase activity increases after birth reaching a peak at 3 days of age (Fig. 4). The time at which peak activity occurs corresponds to the onset of rapid brain growth. After 3 days of age, the specific activity of brain reductase steadily declines and does not increase when the rat pups are weaned

onto solid food as does the liver enzyme. Thus, brain microsomal reductase appears to be regulated independent of changes in serum cholesterol levels.

DISCUSSION

The essentiality of cholesterol biosynthesis to normal cell growth and function has been emphasized by the experiments of Kandutsch and Chen (16). These investigators have shown that when cholesterol biosynthesis in cultured cells is inhibited by the addition of oxygenated cholesterol derivatives, a number of consequence follow. These include: a decrease in intracellular sterol concentrations; followed by a decline in DNA synthesis; decreased rate of cell growth and ultimately cell death (16). These consequences can be prevented by adding mevalonate or cholesterol to the culture media. In experiments with phytohemagglutinin-stimulated lymphocytes, it was shown that a period of increased sterol synthesis must precede the synthesis of DNA and blastogenic transformation (17). Thus, it is of particular interest that HMG-CoA reductase, which catalyzes the rate-limiting reaction in cholesterol biosynthesis, reaches its peak activity in brain at 3 days after birth which corresponds to the onset of rapid brain growth. The high levels of brain HMG-CoA reductase observed in suckling rats (Fig. 4) are consistent with the previous suggestion (18) that the cholesterol required for myelin formation is derived from synthesis within the neural tissue.

The developmental pattern of brain cholesterol biosynthesis has been examined in mice by Kandutsch and Saucier (19). These investigators found a peak of synthesis at ca. 11 days after birth followed by a steady decline. In the only previous study of the developmental pattern of HMG-CoA reductase activity in rat brain, Aragon et al. (20) reported two peaks of enzymic activity; one at 10 days of age and the other at 20 dys of age immediately following weaning. These invetigators (20) also report only a 2- to 3-fold decrease in HMG-CoA reductase specific activity in the adult as compared to the newborn rat. These results differ considerably from those reported in Figure 4. The very low levels of HMG-CoA reductase activity found in adult brain in the present study are in good agreement with previous reports (21,22) of the rate of acetate incorporation into cholesterol. In these earlier studies, cholesterol synthesis was found to be markedly reduced with age and in some cases was not detectable.

In comparing the developmental patterns of

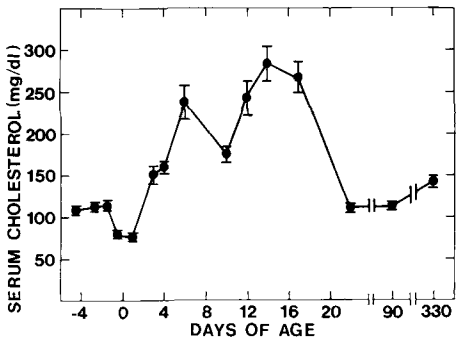

FIG. 3. Changes in serum cholesterol levels during development. The means and standard deviations are presented. Each point represents at least 3 liters or in the case of suckling pups at least 6 animals.

microsomal HMG-CoA reductase from lung, liver and brain, it is evident that the enzyme is not coordinately regulated. Rather, the enzyme activity appears to be responsive to the physiological demands of each tissue for cholesterol synthesis. For example, hepatic HMG-CoA reductase activity appears to vary in response to dietary intake of cholesterol. In the adult rat, these adaptive changes in hepatic HMG-CoA reductase activity appear to be mediated by changes in the intracellular concentrations of cholesterol and cholesterol esters (23,24). In the suckling rat, McNamara et al. (14) showed that liver cholesterol levels did not differ from those of the adult, but yet hepatic reductase activity was markedly lower. From the data presented in Figures 2 and 3, it is apparent that in the suckling rat hepatic reductase activity is inversely related to serum cholesterol levels.

The observation that in fetal lung the

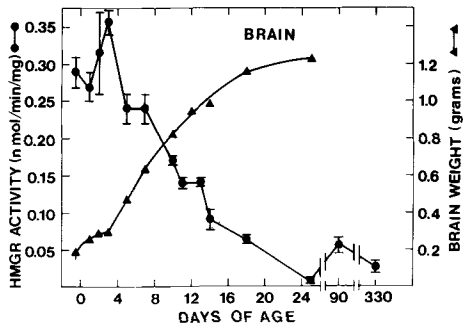

FIG. 4. Rat brain microsomal HMG-CoA reductase activity during development. The means and standard deviations are presented. Each point represents at least 3 litters or in the case of suckling pups at least 6 animals. Brain weight is presented in terms of wet weight. The values represent the means of at least 4 individual brains.

enzyme, which catalyzes the rate-limiting reaction in cholesterol biosynthesis, undergoes a sharp increase in activity at the time when surfactant production begins provides support for the view that the fetal lung has the capability of synthesizing each of the major components of surfactant including cholesterol. Studies on the development of surfactant production by the fetal lung have centered on the enzymes involved in phospholipid biosynthesis, particularly those responsible for the synthesis of dipalmitoyl phosphatidylcholine. As pointed out by Farrell and Avery (1), relevant data on the synthesis of apoproteins and other lipid constituents by the fetal lung is presently unavailable. A defect in the biosynthesis of any of the constituents of surfactant could result in a deficiency of surfactant and in impaired respiratory function.

ACKNOWLEDGMENTS

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De novo Fatty Acid Synthesis and Fatty Acid Elongation Catalyzed by Subcellular Fractions from Hog and Human Aorta

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ABSTRACT

De novo synthesis and mitochondrial elongation of fatty acids have been demonstrated in subcellular fractions from hog and human aorta. Microsomal fatty acid elongation has been shown in hog aorta. The activity catalyzing the formation of fatty acids from acetyl and malonyl CoA was associated with a high molecular weight complex in the 6 x 10^6 g x min supernatant fraction. The principal product was palmitic acid. Some myristic and stearic acids were also formed. One elongation system was associated with protein which sedimented between 4500 g x min and 150,000 g x min. It used acetyl CoA but not malonyl CoA, and NADH was the preferred reducing agent. Radioactivity from acetyl CoA was incorporated into many fatty acids. In hog aorta a second elongation system was found associated with protein which sedimented at 6 x 10^6 g x min. It used malonyl CoA preferentially as substrate and either NADH or NADPH as reducing agent.

INTRODUCTION

Studies from several laboratories have shown that the arterial wall can incorporate ^{14}C from acetate or glucose into fatty acids. Chernick et al. (1) showed in 1949 that [^{14}C]acetate is incorporated into fatty acids in rat aorta. Vost (2) and Howard (3) showed that [^{14}C]acetate is incorporated into fatty acids by the perfused rabbit aorta, and that both *de novo* synthesis and elongation were occurring. Chobanian and Manzur (4) have shown that segments of human aorta incorporate radioactive acetate into lipid. The major product corresponded in equivalent chain length to a fatty acid 22 carbons long with 4 double bonds. Whereat (5) isolated functioning mitochondria from both normal and atherosclerotic rabbit aorta, and showed that they carry out elongation of fatty acids with acetyl CoA. He did not find *de novo* synthesis, although the work of Vost (2) and Howard (3) had suggested that the rabbit aorta carries out this process. Howard (6), using subcellular fractions of monkey aorta, found that the cytoplasmic fraction catalyzed *de novo* synthesis and that both the mitochondrial and the microsomal fractions catalyzed elongation of fatty acids. Wilson et al. (7) have partially purified the fatty acid synthetase of chicken aorta. They also measured *de novo* fatty acid synthesis, and the rates of elongation catalyzed by mitochondrial and microsomal fractions from human aorta.

Changes in fatty acid composition in aorta lipids which suggest significant *in situ* lipid synthesis are typical of the early fatty lesions of atherosclerosis (8-10). Therefore, it seemed useful to examine more closely the enzymes capable of modifying the composition of the fatty acid pool in human arterial wall. Subcellular fractions from hog aorta were used to determine optimum experimental conditions, and then experiments were performed on human aorta samples as they became available from autopsy. *De novo* fatty acid synthesis and mitochondrial and microsomal elongation were examined. Substrate and cofactor requirements and product composition are described.

METHODS

Materials

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, Coenzyme A, nicotinamide, NADP, NADH, NADPH, ATP, methyl benzethonium hydroxide, dithiothreitol and oleic acid were purchased from the Sigma Chemical Co., St. Louis, MO. Solutions of Coenzyme A and dithiothreitol were prepared fresh for each experiment, and the concentration of free sulfhydryl groups determined using Ellman's reagent. [1,3-^{14}C]Malonyl CoA, [1-^{14}C]acetyl CoA, [1-^{14}C]palmitic, [1-^{14}C]oleic and [1-^{14}C]linoleic acids were purchased from New England Nuclear Corp., Boston, MA. The labeled acetyl CoA and malonyl CoA were purified by descending chromatography on Whatman no. 3 MM paper in a solvent system composed of isobutyric acid/ammonia/0.1 M EDTA, pH 4.5/water, 124:4.9:2.0:0.75. Fatty acid methyl ester standards for gas chromatography were purchased from Applied Science Corp., State College, PA.

Purified fatty acid synthetase from pigeon liver was a gift from Dr. Richard A. Muesing. Hog aortas were a gift from the Oscar Meyer

Company, Madison, WI, or were purchased from the Szala Slaughterhouse, Amherst, MA. Segments of human aorta removed during postmortem examination were obtained from the Departments of Pathology of the Veterans Administration Hospital and the University of Wisconsin Hospital, Madison, WI, and from Dr. William Doyle of Franklin County Hospital, Greenfield, MA.

Preparation of Subcellular Fractions

Hog aortas were obtained at the time of slaughter and kept on ice until homogenized. Segments of human aorta were obtained during postmortem examination. Only samples which could be obtained within 12 hr of death were used; most samples being obtained within 6 hr of death. Homogenates were prepared in a buffer of 0.10 M potassium phosphate, pH 7 and 0.001 M EDTA.

The aorta was opened longitudinally and the inner surface rinsed with buffer. A layer consisting of the intima and part of the media was stripped from the remaining media plus adventitia. Buffer, 4 ml, was added to 2 g portions of the intima-media and the tissue was finely minced with scissors. The mince was homogenized in a Kontes Duall ground glass, motor-driven homogenizer. The homogenate was transferred to a centrifuge tube, and the homogenizer rinsed with an additional 2 ml of buffer. The homogenate was centrifuged for 4,500 g x min and the pellet discarded. Mitochondria were collected by centrifugation for 150,000 g x min. The 150,000 g x min pellet was resuspended in buffer and again collected by centrifugation for 150,000 g x min. This pellet was resuspended and the suspension centrifuged for 4,500 g x min. The sedimented material was discarded, and the mitochondria again collected by centrifugation for 150,000 g x min.

The microsomal fraction was collected by centrifugation of the first 150,000 g x min supernatant for 6 x 10^6 g x min. The microsomes were washed by resuspending them in the original volume of buffer and collecting them again by centrifuging for 6 x 10^6 g x min.

Measurement of Enzyme Activities

The activity of cytoplasmic fatty acid synthetase was measured by a modification of the procedure described by Hsu et al. (11). The incubation mixture contained potassium phosphate buffer, pH 7, 50 μmoles; EDTA, 1.5 μmoles; DTT, 2.5 μmoles; NADP, 0.5 μmole; glucose-6-phosphate, 1 μmole; glucose-6-phosphate dehydrogenase, 0.6 units; acetyl CoA, 42 nmoles, [2-^{14}C] malonyl CoA or [1,3-^{14}C]-malonyl CoA, 100 nmoles, 0.5-1 mCi/mmol; and up to 1.5 mg protein from the 6 x 10^6 g x min supernatant in a total volume of 0.50 ml. Protein solutions, whether freshly prepared or stored frozen, were made 0.01 M in DTT and preincubated at room temperature for 30 min to insure that the fatty acid synthetase was completely activated. The mixture was incubated at 37 C for 15 min and the reaction stopped by the addition of 0.015 ml of 68% $HClO_4$. Ethanol, 0.50 ml, was added, and the product fatty acids extracted with three 1.2 ml aliquots of petroleum ether (b.p. 30-60 C). The petroleum ether extracts were washed with a solution containing 0.05 M malonic acid and 0.05 M acetic acid. The radioactivity in the combined petroleum ether extracts was measured, after evaporation of the solvent, as described below. The rate is expressed in nmol or pmol of malonyl CoA incorporated.

For the measurement of the rate of elongation of fatty acids catalyzed by mitochondria, incubation mixtures contained ATP, 2.5 μmoles; $MgCl_2$, 5 μmoles; nicotinamide, 0.6 μmole; NADH, 0.15 μmole; [1-^{14}C] acetyl-CoA, 50 nmoles and 2-3 mCi/mmole; and up to 1 mg of mitochondrial protein in potassium phosphate buffer, pH 7, 20 μmoles containing 0.2 μmole EDTA. The total volume of the incubation mixture was 0.50 ml.

The reaction mixture for the measurement of the rate of fatty acid elongation in microsomes contained potassium phosphate buffer, pH 7, 70 μmoles; EDTA, 0.2 mole; ATP, 2.5 μmoles; $MgCl_2$, 5 μmoles; NADPH, 0.15 μmole; Coenzyme A, 2.5 nmoles; [1,3-^{14}C]-malonyl CoA, 50 nmoles, 1-3 mCi/mmol; and up to 1.5 mg microsomal protein, in a total volume of 0.50 ml.

Reaction mixtures containing mitochondria or microsomes were incubated for 30 min at 37 C and the reaction stopped by addition of 0.50 ml of 10% KOH in ethanol/water (9:1). The mixture was heated at 75 C for 1 hr, cooled, and while still alkaline, extracted twice with 3 ml portions of petroleum ether. This petroleum ether extract was discarded. The reaction mixture was then acidified, and extracted for fatty acids as described above.

When the product fatty acids were to be isolated for further characterization, the substrates used had a specific radioactivity of 50 mCi/mmol. For the particulate systems, amounts of protein up to 10 mg were used, and both NADH and NADPH (0.15 μmoles of each) were included in the reaction mixtures. Cytochrome oxidase was measured as described by Magargal et al. (12).

Partial Purification of the Fatty Acid Synthetase Complex

The 6 x 10^6 g x min supernatant solution was collected from a homogenate prepared from hog or normal segments of human intima-media. An aliquot of 15 ml was treated with solid ammonium sulfate. The protein fraction which precipitated between 20 and 40% saturation was collected, resuspended in 1.5 ml of potassium phosphate buffer, 0.10 M, pH 7, containing 0.001 M EDTA and 0.001 M dithiothreitol and dialyzed against the same buffer for 24 hr. A small aliquot of the dialyzed protein solution was taken for analysis, and the remainder was layered on a 5-20% sucrose gradient prepared in the same buffer. Purified fatty acid synthetase from pigeon liver was layered on an identical gradient. The gradients were centrifuged at 25,000 rpm in a Beckman SW_{27} rotor for 36 hr at 10 C.

Product Identification

Free fatty acids were separated from other lipids on Silica Gel H plates with a solvent system containing petroleum ether/diethyl ether/acetic acid (70:30:1). Methyl esters of fatty acids were separated from other lipids on Silica Gel G plates using a solvent system containing 3% diethyl ether in petroleum ether.

Gas liquid chromatography was carried out with Barber Colman model 10 or model 5000 instruments. Columns (6 ft x 6 mm) were packed with diethylene glycol succinate (12% by weight on Gas-Chrom P). The column temperature was 198 C; the carrier gas nitrogen, and the effluent gas flow 60-70 ml/min. The model 5000 was equipped with a gas flow counter for monitoring radioactivity.

Methyl esters of fatty acids were prepared using diazomethane. Decarboxylation of fatty acids was carried out as described by Brady et al. (13). Since Dahlen (14) has shown that unsaturated acids may not be completely decarboxylated, fatty acids from incubation of particulate fractions were hydrogenated before decarboxylation. Reduction was done by the microhydrogenation procedure described by Appleqvist (15). [1-^{14}C]Palmitic acid was used as a standard for the decarboxylation procedure alone. [1-^{14}C]Linoleic acid was used to verify that recoveries were complete for the reduction followed by decarboxylation. Recovery of [^{14}C] from standards varied from 91-109% in 12 analyses.

Radioactivity was determined in either toluene-phosphor solution (4 g of 2,5-diphenyloxazole and 0.1 g of 2,5-bis[5-*tert*-butylbenzoxazole)] 2-thiophene per liter of toluene), or dioxane-phosphor solution (60 g of napthalene, 10 ml of ethylene glycol, 4 g of 2,5-diphenyloxazole, and 0.2 g of 2,5-bis[5-*tert*-butylbenzoxazole)]-2-thiophene per liter of dioxane) in a Packard Tri-Carb model 3365 or Beckman LS-250 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Preliminary experiments were done using homogenates of normal hog artery. Subsequent experiments were carried out with human tissue from autopsy as it became available. The cytoplasmic fatty acid synthetase and the mitochondrial elongation system behaved similarly whether hog or human tissue was used. For brevity, and since the human systems are of greater interest, only the data obtained with human aorta are presented for most experiments with these two systems.

Fatty Acid Synthetase

When protein in the 6 x 10^6 g x min supernatant fraction was incubated with [^{14}C]-malonyl CoA as described above, the incorporation of radioactivity into fatty acid was proportional to time for 15 min and to protein up to at least 1.5 mg. The fraction of supernatant protein which precipitated between 20 and 40% saturation with ammonium sulfate contained 58% of the total activity, and the specific activity was twice that of the crude supernatant solution. This fraction was examined by sucrose density gradient centrifugation (Figure 1). The fatty aicd synthetase activity was separated from most of the rest of the protein by this procedure, and its location coincided with that of purified pigeon liver fatty acid synthetase centrifuged on an identical gradient. The recovery of total activity placed on the gradient was 70%.

The fractions from the gradient which contained fatty acid synthetase activity were combined, and the protein concentrated using an Amicon PM 10 filter. This partially purified fatty acid synthetase was incubated with [1,3-^{14}C]malonyl CoA, and the product fatty acids isolated. The methyl esters of the product were prepared and purified by thin-layer chromatography (TLC). The radioactive product was added to a mixture of nonradioactive methyl esters of the fatty acids 12:0, 14:0, 16:0, 18:0 and 20:0 and chromatographed on the Barber-Colman model 5000. Of the radioactivity detected, 78% coincided with methyl palmitate, 20% with methyl stearate, and 2% with methyl myristate. The peak of radioactivity associated with methyl myristate

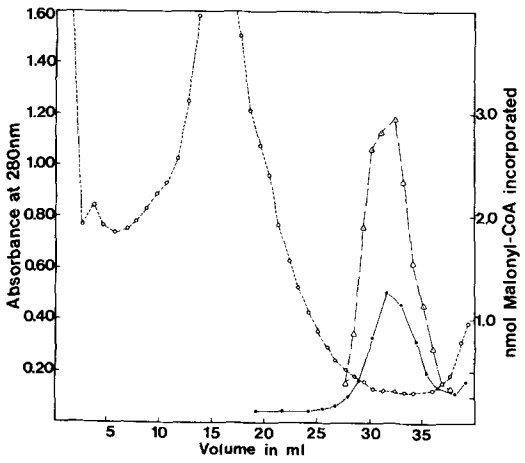

FIG. 1. Distribution of protein and fatty acid synthetase activity from human aorta ammonium sulfate fraction on a 5-20% sucrose density gradient. Protein was estimated by measuring the absorbance at 280 nm. Enzyme activity was measured as described in the text. Total activity in each fraction is shown. ●—●, protein in purified fatty acid synthetase from pigeon liver; ○- - -○, protein, aorta; △—△, fatty acid synthetase activity, aorta.

was small, but occurred reproducibly. No radioactivity appeared after methyl stearate in chromatograms monitored for as long as 30 min after the appearance of methyl arachidate. Radioactive products were also purified as free fatty acids by TLC and decarboxylated. The carboxyl carbon was found to contain 14.7% of the total radioactivity.

From the distribution of radioactivity among product fatty acids, the fraction of total radioactivity found in the carboxyl carbon, and the behavior of the catalytic activity on sucrose density gradients, we conclude that human and hog aorta contain a cytoplasmic fatty acid synthetase complex of high molecular weight like that described from avian and mammalian liver by many other workers. Howard (6) found such an activity in the high-speed supernatant of monkey aorta, and Wilson et al. have shown that chicken aorta has a fatty acid synthetase complex like the one in chicken liver (7). The apparent absence of such an activity in homogenates of rabbit aorta reported by Whereat (5) could have been due to use of NADH as a reducing agent, or to lack of added malonyl CoA, even though the supernatant contained significant acetyl CoA carboxylase activity. Katiyar and Porter (16) have shown that the rate of the FAS reaction is quite sensitive to the ratio of the acetyl to malonyl CoA present in the reaction mixture. At an acetyl/malonyl ratio of 4:1, the reaction is 75% inhibited. In a reaction mixture which contained 75 nmoles acetyl CoA and in which at most 5-10 nmoles of malonyl CoA could be generated in the time period of incubation (estimated from ref. 6), the observable fatty acid synthetase activity should be negligible.

Segments of intima-media of six of the human aortas used in this study were dissected according to their degree of atherosclerotic involvement. They were judged by visual inspection, and classified as normal, fatty streaked, or an involvement greater than fatty streak. The fatty acid synthetase activity was measured in homogenates prepared from the separated classes. In general, both specific and total activities were slightly lower in more diseased segments than in less diseased segments from the same individual, but the differences were small, less than 2-fold in the greatest case. The range of amounts of activity for the six individuals was 8-44 pmol substrate incorporated/min/mg protein. Similar observations have been made by Wilson et al. (7).

Fatty Acid Elongation

The microsomes (fraction which sediments between 1.5 x 10^5 and 6 x 10^6 g x min) isolated in this study contained up to 30% of the total tissue cytochrome oxidase activity, indicating contamination with mitochondrial fragments. Thus, when the terms "mitochondrial elongation system" and "microsomal elongation system" are used below, they refer to fatty acid elongation systems of particular characteristics which have been shown by others to be associated with mitochondria (17) and microsomes (18), respectively. It is possible to establish the presence of each of these systems in preparations which contain both, because of differences in their properties, as summarized below.

Washed mitochondria were incubated with [1-^{14}C] acetyl CoA, and fatty acids were recovered after saponification. The incorporation of radioactivity into total fatty acids was proportional to time for 30 min, and proportional to protein for amounts up to 1 mg. Washed microsomes from hog aorta or human aorta were incubated with [^{14}C] malonyl CoA, and total fatty acids were recovered after saponification. The incorporation of radioactivity into total fatty acids was proportional to time for 40 min and proportional to protein for amounts up to 1.5 mg. This activity was found in every preparation of hog aorta microsomes examined. Human aorta microsomes were active if the microsomes were used without washing. However, only two preparations out of twelve tested were active if the micro-

TABLE I

Properties of Fatty Acid Elongation Systems

	Relative rate of reaction[a]		
	Mitochondria		Microsomes
	Hog	Human	Hog
Substrate specificity			
Acetyl CoA	1.0	1.0	0.2
Malonyl CoA	0.05	---	1.0
Reducing agent			
None	---	---	0
NADH	1.0	1.0	1.0
NADPH	0.16	0.19	1.0
ATP Requirement			
No ATP	---	0.06	0.09
Specific activity pmoles/min/mg, standard reaction mixture	6.8-16.3(3)[b]	4.6,35.0	0.7-6.8(5)

[a]The data are reported as the ratio of the rate observed under the specified condition to the rate with the standard reaction mixture for the particulate fraction used. The standard reaction mixture for mitochondrial incubations contained [1-^{14}C]acetyl CoA, NADH, ATP, $MgCl_2$, and buffer and mitochondrial protein. The standard reaction mixture for microsomal incubations contained [1,3-^{14}C]malonyl CoA, NADPH, ATP, $MgCl_2$, Coenzyme A, buffer and microsomal protein.

[b]The number in parentheses is the number of different enzyme preparations for which this measurement was made.

somes were washed. Addition of oleic acid or oleyl CoA as acceptor did not restore activity. Microsomal data below are for hog microsomes.

The substrate specificity, ATP requirements, and reducing agent specificity of the particulate systems are shown in Table I. Mitochondria utilized acetyl CoA, and not malonyl CoA. Microsomes utilized malonyl CoA in preference to acetyl CoA. Mitochondria utilized NADH more efficiently than NADPH as a reducing agent. Microsomes did not distinguish between the reducing agents. Endogenous fatty acids were used as acceptors for both particulate systems. Apparently, activation of the fatty acid is required, since very little elongation occurred in the absence of ATP. However, neither system shows an absolute requirement for added Coenzyme A (Fig. 2). For the microsomal system, the highest activity is observed in the presence of 5 x 10^{-6} M Coenzyme A. The mitochondrial system is inhibited by concentrations of free Coenzyme A greater than 10^{-6} M. The activities of both systems are strongly inhibited by 10^{-3} M Coenzyme A (not shown). The mitochondrial system is also inhibited by 10^{-3} M glutathione or mercaptoethanol.

Radioactive fatty acids formed by each system were purified by TLC, hydrogenated, and decarboxylated. The carboxyl carbon contained 67% of the total radioactivity found in long chain fatty acids from human mitochondrial incubations and 100% of the radioactivity found in acids from hog microsomal incubations. The distribution of radioactivity among product fatty acids was determined for incubations using human mitochondria. The distributions differed from preparation to preparation, which presumably reflects differences in the pool available for elongation. More than 50% of the radioactivity was always found in acids 18 carbons or longer. Ten percent or more was always found in acids 20 or 22 carbons long containing 3 or more double bonds.

The substrate specificity, NADH requirement, the distribution of ^{14}C in product fatty acids, and the fraction of total ^{14}C found in the carboxyl carbon for product formed using washed mitochondria confirm that the mitochondria from hog and human aorta contain a fatty acid elongating system like that described for liver (17) and heart (19) and for rabbit and monkey aorta (5,6). Harlan and Wakil (17) and Dahlen and Porter (19) found that the elongation required ATP if free fatty acid was used as substrate, but not if acyl CoA was used. The mitocondrial system reported here also required ATP, using endogenous fatty acids as primers. However, free Coenzyme A at concentrations greater than 10^{-6} M dramatically inhibited the reaction. Both Howard (6) and Whereat (5) used endogenous fatty acids with added ATP as acceptors

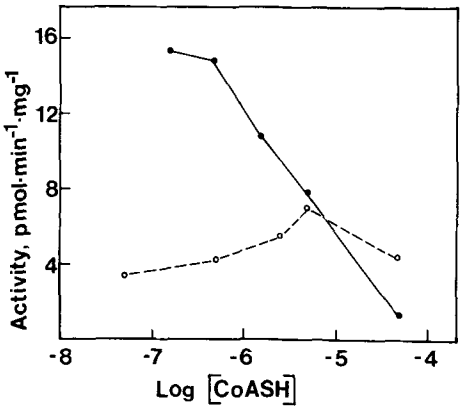

FIG. 2. Effect of Coenzyme A on the rate of elongation of fatty acids catalyzed by hog mitochondria or microsomes. Enzyme activity was measured as described in the text. Rates are given as pmol substrate incorporated/min/mg protein. •—•, mitochondria; o---o, microsomes.

for fatty acid elongation, and included free Coenzyme A in the reaction mixtures at concentrations which this study suggests may have been inhibitory. The system described by Whereat required Coenzyme A; however, he used acetate rather than acetyl CoA as a 2-carbon source. Presumably, the Coenzyme A was required for acetyl CoA formation. Wilkinson (20) has reported that incorporation of acetyl CoA into fatty acids by mitochondria from mouse skin is inhibited by free Coenzyme A. The dependence of inhibition on the concentration of Coenzyme A is similar to that reported here. Howard (6) also found some degree of inhibition by free Coenzyme A. Possibly the immediate substrate of mitochondrial fatty acid elongation is an activated acyl moiety other than the Coenzyme A ester, and the presence of free Coenzyme A tends to form acyl CoA and deplete the supply of other activated acyl groups. Other sulfhydryl compounds also inhibit elongation, but much higher concentrations are required. It may also be that free Coenzyme A and other sulfhydryl groups promote thiolytic cleavage of the β-ketoacyl intermediate formed in the elongation sequence.

The activity for incorporation of malonyl CoA into fatty acid observed using microsomes can be assigned unambiguously to a microsomal system for hog aorta. It can be distinguished from the mitochondrial system by substrate specificity, by its utilization of NADPH, and by the striking difference in response to Coenzyme A levels. It can be distinguished from supernatant fatty acid synthetase by its ATP requirement, its persistence in washed microsomes, and the fraction of total radioactivity found in the carboxyl carbon. The activity for incorporation of malonyl CoA into fatty acids catalyzed by hog aorta microsomes is similar to that first reported by Lorch et al. (21) in rat liver, and subsequently described in detail by several other laboratories (18,22-25).

The data obtained in this study using microsomes prepared from human aorta do not establish unequivocally the presence or absence of microsomal elongation. The activity observed could be accounted for by a mixture of mitochondrial fragments and trapped supernatant in the unwashed microsomal pellet. Assuming that the role of the microsomal elongation system is to modify the pool of fatty acids available to the cell (18,26), then the participation of this system in the conversion of linoleic and linolenic acids to acids which are prostaglandin precursors seems a particularly important aspect of this role. Supposing that human aorta really has a very low capacity for microsomal fatty acid elongation, the tissue would be dependent on exogenous sources for supplying prostaglandin precursor acids. This seems a serious disadvantage, given the stresses to which it is subjected. It is not practical to continue to study the problem using homogenates of whole tissue from autopsy. However, since both endothelial and smooth muscle cells from human blood vessels can be grown in culture (27), the question can be reexamined using cultured cells.

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The Effect of Steroids and Their Solubilizing Agents on Mycelial Growth of *Phytophthora cactorum*[1]

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ABSTRACT

Mycelia of *Phytophthora cactorum*, a fungus unable to synthesize sterols, were incubated with a series of sterols and several common sterol solubilizing agents. Cholesterol, campesterol, and sitosterol promoted growth, while carpesterol, saringosterol, 20α-hydroxy cholesterol, coprostanol, and 5α-cholestane inhibited growth. Cholestanol, epicholestanol, and 5α-cholestan-3-one had no significant effect on growth relative to the sterol-free control. All steroids were solubilized in ethanol for studying structure-activity correlations. The added steroids were reisolated from mycelia and their identity confirmed by gas liquid chromatography (GLC) or combined gas liquid chromatography-mass spectrometry (GLC-MS). Tween 80 promoted growth, Triton X-100 inhibited growth, and ethanol had no effect on growth relative to a control.

INTRODUCTION

Phytophthora cactorum, like other members of the Pythiaceae (fungi: oomycetes), is unable to synthesize sterols (1,2). Although these fungal pathogens have no requirement for sterols for vegetative growth, they have an obligatory requirement for sterols to induce sexual reproduction (3,4). A similar situation exists for mycoplasmas. Certain species require sterols or membranous sterol-like compounds. However, sterols have not been shown to induce sexual reproduction (5-7). It has been suggested that sterols play a dual role in pythiaceous fungi: as an architectural component of membranes (2,3,8), and as a precursor to steroidal fungal hormones (analogous to oogoniols and antheridiol in *Achlya*) (2,9,10). In support of the membranous role of sterols in pythiaceous fungi, *Phytophthora* and *Pythium* have been shown to incorporate sterols from the growth medium into mycelial subcellular fractions (11,12) resulting in altered membrane permeability (13,14).

In the present investigation, we were interested in whether mycelial growth rates vary in response to precise molecular features of added steroids. We assumed the primary effect of adding sterol to the growth medium of cultures growing vegetatively would be alterations in membrane structure and function. The study is an extension of the exploratory research by Elliott, Knights, and Hendrix and coworkers, who have shown that cholesterol- and sitosterol-supplemented cultures promote growth of *Phytophthora* and *Pythium* relative to a control (15-17). These authors have also shown that sterol metabolism occurs in liquid culture; i.e., cholesterol is esterified and also metabolized to some "polar" form (18-20).

MATERIALS AND METHODS

Culture Methods

Phytophthora cactorum, strain IMI 21168, was a gift from C. Elliott. Still cultures were grown over a period of 18 days at room temperature. The fungus was cultured on a synthetically defined medium as described by Elliott et al. and modified only by the inclusion of 0.1 g/liter of calcium chloride (21). Cultures were grown in 250 ml flasks containing 50 ml of autoclaved medium. The steroids (9 mg) were solubilized in 2 ml of ethanol and added to 1 liter of medium. Flasks were inoculated with a plug of agar 5 mm in diameter cut from a colony growing on basal medium. The weight of the plug varied in dry weight from 0.2 to 0.5 mg depending on the age or place from which the inoculum was cut from the agar plate. We were, however, able to reduce variation in apparent mycelial growth rates by inoculating liquid cultures with a plug of agar cut from 12-day cultures previously grown on solid medium. It was also important to cut on the same perimeter around the plate, preferably toward the outer edge of the colony. Eighteen flasks were inoculated in this manner for each steroid treatment. At 3-day intervals after inoculation, 3 flasks were harvested at random constituting 3 replicates for a given treatment. Each treatment was reproduced a minimum of 3 times, and the coefficient of variation at each harvest did not exceed ± 5% for any treatment throughout its growth period. The coefficient of variation between the growth rates for each particular sterol when the experiment was repeated did not exceed ± 5% in late log phase

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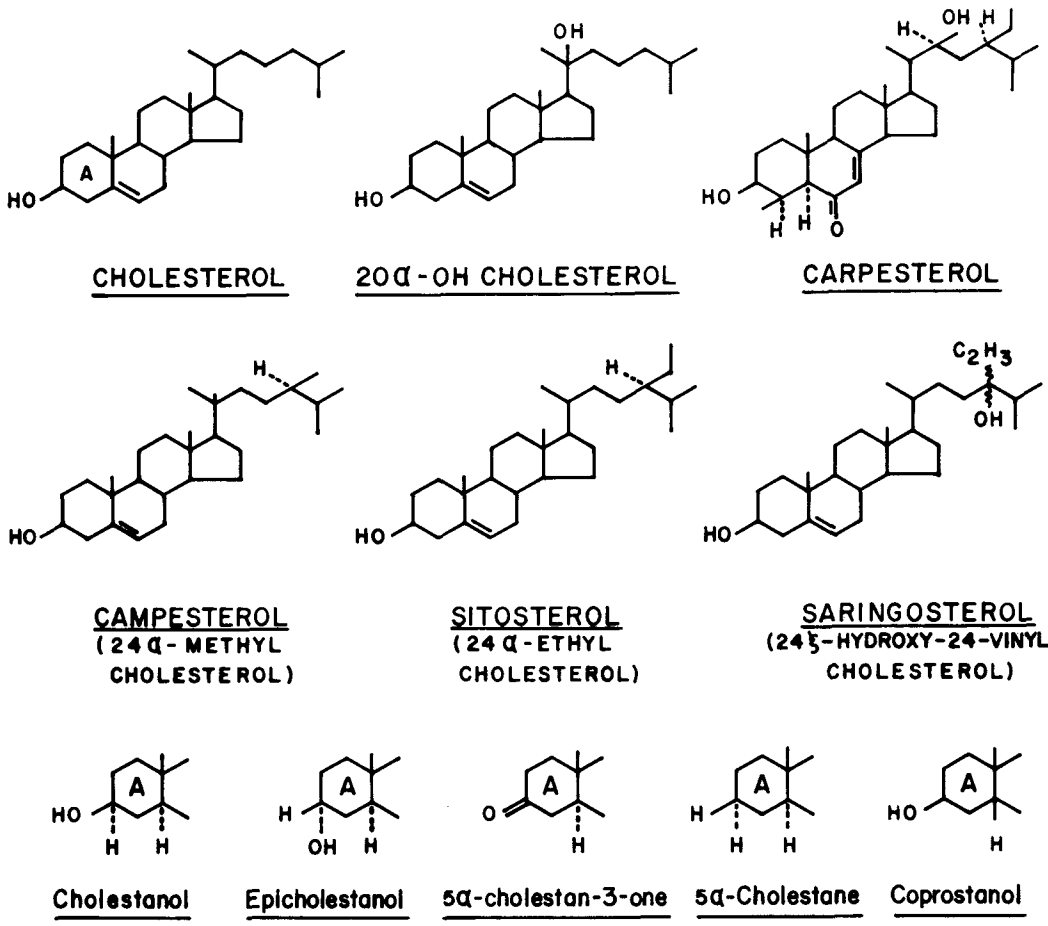

SCHEME 1. Structures of various steoids administered to growing mycelia of *Phytophthora cactorum.*

but was as high as ± 10% in early log phase. The cultures were routinely examined microscopically at each harvest.

Chemicals

Cholesterol, 5α-cholestan-3-one, 3-epicholestanol, 5α-cholestane, and coprostanol were recrystallized commercial samples. Sitosterol and 20α-hydroxy cholesterol were gifts from W.R. Nes. Campesterol and cholestanol were gifts from H. Kircher. Carpesterol was a gift from Y. Sato, and saringosterol, a probable autooxidation product of fucosterol, was previously isolated from *Laminaria* (22). Structure of the steroids incubated with *P. cactorum* are shown in Scheme 1. All compounds used were greater than 99% pure by gas liquid chromatography (GLC), and the retention times relative to cholesterol were in agreement with those of authentic standards reported previously from this laboratory (23). Mass spectra of the compounds used in the present study have also been reported (24,25). Tween 80 and Triton X-100 were purchased from Sigma Chemical Company, St. Louis, MO.

Recovery of Sterols from Mycelia

Mycelia were collected on filter paper, washed 3 times with distilled water, and lyophilized for 24 hr. The freeze-dried mycelia were extracted for 24 hr in chloroform/methanol (2:1). The chloroform/methanol extract was brought to dryness under a stream of nitrogen and the total weight of lipid obtained. The total lipid was chromatographed on a thin layer of Silica Gel G and developed in benzene/ether (9:1). Cholesterol was routinely placed on the edge of the plate to act as the desmethyl standard. Qualitative and quantitative analyses were made only for the free sterol because free sterol and not sterol ester is believed to be important in membranes. Visualization of the

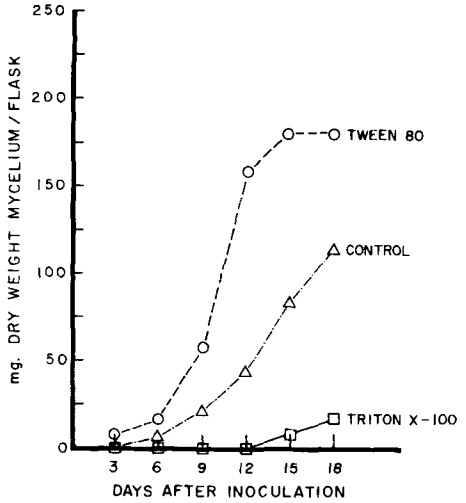

FIG. 1. The effect of the solubilizing agents TWEEN 80 and TRITON X-100 on mycelial growth of *Phytophthora cactorum* (at 5 ml/liter).

free sterol band was by use of a 2′,7′-dichlorofluoroscein spray. Sterols were identified by GLC on a Varian series 3700 gas chromatograph equipped with a flame ionization detector and a CDS 111 data system. A 1/4" (O.D.) x 2 mm (I.D.) x 200 cm column with a liquid phase of either 1% SP-1000 or 1% SE-30 (Applied Science Lab, State College, PA) was used with the carrier gas (helium) pressure at 35 psi and a column temperature of 245 C. Identification of 4 sterols reisolated from the fungus, campesterol sitosterol, cholesterol, and cholestanol, was by combined gas liquid chromatography-mass spectrometry (GLC-MS).

Addition of Sterol with Its Solubilizing Agent to Mycelium

Sterols were initially solubilized in Triton X-100, and no stimulation was observed relative to cultures containing no sterol and no detergent. This led us to study the effect of three common solubilizing agents, Tween 80, Triton X-100, and 100% ethanol, on the growh of *P. cactorum* with or without added sterols. For determination of absolute growth rates, the following formula was used (26):

$$\frac{79.7}{\Delta t} \log_{10} \frac{N}{No} = K$$

K = absolute growth rate in doublings per day
Δt = time period of growth in hours
N = mycelial dry weight at end of growth period
No = mycelial dry weight at beginning of growth period

RESULTS AND DISCUSSION

A marked difference in the initial growth was observed, depending upon whether the solubilizing agent was Triton X-100, Tween 80, or ethanol. When the mycelium was supplemented with different concentrations of ethanol (0.5-2 ml/l), its growth was essentially the same as without ethanol. Tween 80 added to the growth medium at various concentrations (1-5 ml/l) stimulated growth while Triton X-100 (1-5 ml/l) inhibited growth (Fig. 1). In previous studies where sterols were solubilized in Tween 80, Tween 20, or ether, it is unclear whether the surfactant or the sterol was responsible for promoting or retarding growth of the fungus. In the present investigation, the sterol was solubilized in ethanol because the latter had no significant effect on mycelial growth.

It has been known for over 15 years that sterols added to the culture medium of pythiaceous fungi alter growth (2). In order to assess whether the change in growth exhibited by fungal cultures supplemented with various sterols was due to a difference in the absolute growth rate "K" or to a shortened lag phase, we grew the fungus over an 18-day period. A typical growth curve of 3 representative steroid treatments and the control is shown in Figure 2. Examination of growth rates over the 18-day growth period shows that none of the steroids stimulates the absolute growth rate (expressed as K) during the log phase of growth. Cholesterol, however, apparently stimulates growth in *Phytophthora* mycelia by stimulating the growth rate in the lag phase in days 0-3, where absolute growth rates could not be measured. In spite of rigid measures taken to standardize the inoculum, cholesterol cultures still showed greater growth during the first 3 days. The absolute growth rates of all cultures were unaffected by steroids after the third day of growth. Thus, cholesterol stimulates the mycelial growth of *Phytophthora* by stimulating growth during the lag phase and not by increasing the absolute growth rate of the mycelium.

The combined mycelia from each treatment harvested from days 6-15 were extracted, and the added steroid was reisolated unchanged from the free sterol fraction. The identity of the compounds was confirmed by comparison with authentic compounds through the use of GLC on 2 liquid phases and in four cases, use of combined GLC-MS. The amount of mycelium which was extracted ranged from 50 to 450 mg depending on the steroid added to the culture medium. For mycelia incubated with added

sterol, the amount of steroid recovered did not vary significantly among the treatments and was ca. 0.01% free sterol/dry weight mycelium. When Tween 80-supplemented cultures and the control cultures were grown in large quantities (20 g dry weight mycelium) and the chloroform/methanol extract analyzed, a spectrum of compounds of unknown structure was isolated from the "sterol fraction." In both cases, the major compound in the spectrum had a molecular ion of m/e 442 and a retention time relative to cholesterol on 1% SE-30 of 1.34. It is tentatively identified as a polycyclic terpenoid, but definitely not a sterol. The unknown terpenoid recovered from 20 g of dry weight mycelia was 0.0001% unknown terpenoid/dry weight mycelium. The identity of the unknown is under investigation.

These data are in agreement with Knights and Elliott and coworkers, who found no sterol in *P. cactorum*(4) and that Δ^5-sterols were recovered from the mycelium unchanged (27). Knights and Elliott, however, have shown that $\Delta^{5,7}$ and Δ^7-sterols are converted to Δ^5-sterols (27). We have also examined the fatty acids of the polar lipids for each treatment. The fatty acid spectrum of the control was not altered by adding sterol(s) to the fungus. We will report on the fatty acid composition of *P. cactorum* in a subsequent communication.

From our present investigation, we could evaluate the effectiveness of precise molecular features of the sterol on growth of *P. cactorum*. Cholesterol, campesterol, and sitosterol treatments grew in synchrony, all promoting growth while cholestanol, 5α-cholestan-3-one, and epicholestanol-supplemented cultures had no significant effect on growth relative to a control. 5α-Cholestane and coprostanol treatments slightly inhibited growth while saringosterol, carpesterol, and 20α-hydroxy cholesterol strongly inhibited growth. Those sterols which promote growth have a Δ^5-bond in the nucleus with a 3β-hydroxyl group and a side chain with 8-10 carbon atoms, features which allow the molecule to pack well into a phospholipid layer. We assume that the stimulation of growth reflects the good fit of those sterols into the mycelial membrane, although we recognize that other possibilities could explain the enhanced growth.

The effect of highly oxygenated steroids on mycelial growth are not as easily explained. We have considered two possibilities. The hydroxylated side chain, when presented to the lipid bilayer, would by means of hydrogen bonding or steric hindrance act to disrupt Van der Waals' interactions, thereby causing a

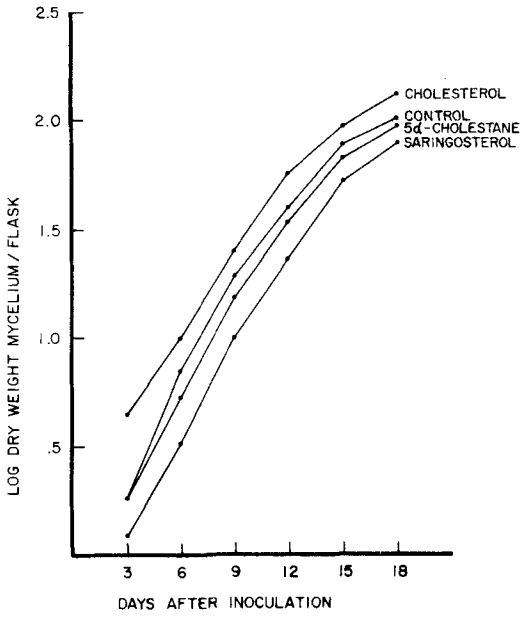

FIG. 2. Growth curves of *Phytophthora cactorum* mycelia as affected by various steroid additions to the growth medium (at 9 mg/liter).

packing constraint between itself and some other neighboring membrane component resulting in growth inhibition. There is some evidence for the latter hypothesis in studies with anaerobic yeast (28) and *Tetrahymena pyriformis*(29). An alternative explanation is that the highly oxygenated steroids are regulating HMG-CoA reductase, thereby regulating dolichol and glycoprotein biosynthesis. Recent evidence has shown that sitosterol, cholesterol, and stigmasterol have no effect on dolichol biosynthesis, but oxygenated sterols have an effect (30). It is likely there is more than one mycelial response mechanism (membranous-allosteric) which is involved when exogenous sterol is taken up from the culture medium and utilized by *P. cactorum*. Thus, the extent to which growth is influenced by the effect of certain sterols on membrane structure requires further study. The effect on mycelial growth of adding various detergents is not clear but may have to do with the mechanism of action of detergents; i.e., it has been shown in some cases detergents act as delipifying agents when cell-free systems are used (31).

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Age-related Changes in Glycerolipid Formation in Lean and Obese Zucker Rats

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ABSTRACT

Age-related changes in hepatic and adipose glycerolipid formation have been described in Zucker rats. Glycerolipid formation was measured in vitro in the presence of [^{14}C] glycerol-3-phosphate, palmitate, ATP, CoA, and Mg^{2+} by using liver and adipose tissue homogenates derived from various age groups of animals. Hepatic glycerolipid formation increased after birth to reach a peak value at 1 day of age. This period was followed by a decline in the rates of glycerolipid formation. Hepatic glycerolipid formation increased again at the time of weaning and continued to rise up to 32 days in lean rats and 42-44 days in obese rats. Obesity in rats was recognizable at the age of 32 days and was associated with increased rates of glycerolipid formation in both liver and adipose tissue. As far as the changes in hepatic glycerolipid formation and triglyceride accumulation are concerned, obese rats showed more resemblance to 1-day-old rats than to lean animals of similar age groups. Glycerolipid formation decreased in liver and increased in adipose tissue with age in both lean and obese rats. These studies suggest that hepatic and adipose tissue glycerolipid formation is significantly influenced by age and obesity in Zucker rats.

INTRODUCTION

Genetically transmitted obesity has been described in several strains of rodents (1). In Zucker obese rats, the abnormality is inherited as autosomal recessive. The phenotypic expression of this trait is not apparent at birth but can be recognized at 2-3 weeks of age. By this time, the animals are hyperphagic and show increased body fat and elevated levels of plasma insulin (1). Obesity in this animal model is accompanied by enhanced rates of triglyceride formation in both liver and adipose tissue (2,3). Recent studies from this laboratory demonstrate that the period between birth and 3 weeks of age is active in hepatic triglyceride formation in Sprague Dawley rats (4). In the present studies, age-related changes in hepatic and adipose tissue triglyceride formation have been investigated in Zucker rats to determine whether increased potential of triglyceride formation is responsible for the genetic expression of obesity in this animal model.

MATERIALS AND METHODS

sn[1,3-^{14}C] glycerol-3-phosphate (sp. radioactivity 30 mCi/mmol) was purchased from ICN Chemicals and Radioisotope Division, Irvine, CA. Most of the other chemicals were of A.R. grade quality and were purchased from the sources reported previously (3,4). Male and female obese (fa/fa) rats and their lean controls (FA/-) were either from our animal colony or were purchased from Harriet G. Bird Memorial Laboratory, Stow, MA. Birth dates and time of weaning (21 days of age) were recorded carefully. After weaning, all rats received Purina Chow diet, Ralston Purina Co., St. Louis, MO. The animal colony was maintained in a temperature-controlled room with a 12 hr on, 12 hr off light cycle. For developmental studies, animals from both sexes were selected. Animals were killed by decapitation and blood was collected in heparinized tubes. In some studies, blood was pooled from 2 or 3 animals (newborn animals) to obtain sufficient plasma for triglyceride determination. All animals were killed between 9 and 11 a.m.

Initial studies were conducted with liver and adipose tissue from albino rats to determine optimum conditions used in various assays. Sprague Dawley rats of the same age as the Zucker rats were from our animal colony. The incubation conditions developed in tissues from albino rats were satisfactory to measure the esterification rates in liver and adipose tissues from Zucker rats.

Preparation of Homogenates

Livers were removed, blotted free of blood, and weighed. They were homogenized (at 4 C) in a Teflon glass homogenizer with 4 vol of buffer (0.25 M sucrose, 1 mM dithiothreitol, 1 mM EDTA, and 1 mM Tris, pH 7.5) and the resultant homogenate was used in various assays. Gonadal adipose tissues were removed and homogenized with 3 vol of buffer. The homogenization was performed at speed 5 for 30 sec in the cold (4 C) with a Tekmar Tissumizer (Tekmar Instruments, Cincinnati, OH).

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TABLE I

Changes in Hepatic and Plasma Triglyceride Concentrations as a Function of Age[a]

Group	No. and age of animals	Body weight (g)	Liver weight (g)	Triglyceride Liver (mg/g)	Triglyceride Plasma (mg %)
	18 hr (12)	5.5 ± 0.5	0.23 ± 0.02	18 ± 3	110 ± 20
	1 day (8)	6.0 ± 0.5	0.21 ± 0.02	30 ± 4	
	4-6 days (23)	10 ± 1	0.30 ± 0.02	7 ± 4	140 ± 18
	11 days (11)	18 ± 1	0.50 ± 0.04	6.0 ± 0.5	141 ± 19
	15 days (5)	27 ± 3	1.2 ± 0.1	±	
	20 days (8)	33 ± 3	1.3 ± 0.2	1.2 ± 0.3	237 ± 85
	22 days (8)	42 ± 3	1.6 ± 0.1	3 ± 1	114 ± 52
Lean	32 days (4)	75 ± 8	3.0 ± 0.2	3 ± 1	76 ± 10
Obese	32 days (4)	83 ± 9	4.0 ± 0.4	6.2[b] ± 1.2	79 ± 20
Lean	42-44 days (8)	114 ± 17	5 ± 1	2.6 ± 1.2	90 ± 40
Obese	42-44 days (8)	111 ± 19	5 ± 1	6.5[b] ± 2.4	138 ± 22
Lean	67-69 days (5)	192 ± 45	8 ± 1	1.7 ± 0.8	71 ± 32
Obese	67-69 days (5)	268[b] ± 30	11[b] ± 2	5[b] ± 2	197[b] ± 38
Lean	75-79 days (4)	287 ± 11	12 ± 1	1.5 ± 0.4	75 ± 23
Obese	75-79 days (4)	326 ± 28	15[b] ± 1	9[b] ± 3	323[b] ± 104

[a]Each value is mean ± S.D. from the number of rats referred to in parentheses.
[b]P <0.05 †P <0.01.

The homogenate was centrifuged at 600 g for 15 min and separated into upper fat cake, a pellet (containing nuclei, cell debris, and other tissue fragments), and an intermediate layer. This intermediate layer, called the fat-free homogenate, was used to measure triacylglycerol formation.

Conversion of [^{14}C] Glycerol-3-Phosphate into Lipid by Liver and Adipose Tissue Homogenates

sn-Glycerol-3-phosphate acyltransferase (E.C. 2.3.1.15) was measured in the presence of palmitate, ATP, CoA, and $MgCl_2$ by using either liver or adipose tissue homogenates described earlier (3,4). For liver homogenates, the incubation mixture (0.7 ml) contained 17.5 mM Tris, pH 7.5, 50 mM KCl, 0.84 mM *sn*-glycerol-3-phosphate with 0.1 μCi of [^{14}C]*sn*-glycerol-3-phosphate, 0.7 mM dithiothreitol, 0.039 mM CoA, 3.60 mM $MgCl_2$, 3.55 mM ATP, and 1.4 mM NH_4-palmitate complexed with 1.25 mg of fatty acid-poor albumin. The reaction was initiated by the addition of 0.1-0.2 ml of homogenate (1.5-3 mg protein). To determine triglyceride formation by adipose tissue homogenates, the reaction mixture (0.75 ml) contained 24 mM Tris, pH 7.5, 50 mM KCl, 0.84 mM *sn*[^{14}C]glycerol-3-phosphate, 0.7 mM dithiothreitol, 1.05 mM ammonium palmitate, 3 mM ATP, 3.6 mM $MgCl_2$, 0.01 mM CoA, and 1.25 mg fatty acid-poor albumin. The reaction was initiated with 0.1-0.2 ml of homogenate containing 200-300 μg of protein. Incubation was under air at 37 C in a shaking water bath. The reactions were linear with time for 30 and 20 min for liver and adipose tissue, respectively.

The radioactive lipids formed were extracted as described by van den Bosch and Vagelos (5) and dried under N_2. The dry lipids were dissolved in 0.5 ml of benzene and stored at -30 C. Samples (0.1 ml) were applied to Silica Gel G plates impregnated with 0.1 M borate, and neutral lipids were separated with a solvent system of hexane/ether/acetic acid, 73:25:2 (by volume). Phospholipids were separated with chloroform/methanol/acetone/acetic acid/water, 100:20:40:20:10 (by volume) on silica gel HR plates slurried in 10 mM sodium carbonate (6). The different classes of lipids were identified by authentic standards. Various lipids were visualized by exposure to iodine. After sublimation of iodine, appropriate areas from the plates were scraped directly into scintillation vials containing 10 ml of Liquifluor in toluene, and the radioactivity was determined in a Beckman LS 250 scintillation counter.

The rates of incorporation of [^{14}C]glycerol-3-phosphate into lipid were expressed as nmoles of products formed/30 min/mg of homogenate protein for liver. In adipose tissue, these rates were expressed in relation to fat cell number. Previously, we have demonstrated that this consideration is particularly important in the studies with obese animals because obesity in animals is associated with a significant increase in the nonfat cell population, which causes a rise in the proportion of adipose tissue protein content derived from nonfat cells (7). Portions of the adipose tissue used for glycerolipid formation were processed for the determination of adipocyte number by the method

TABLE II

Glycerolipid Formation by Liver Homogenates as a Function of Age[a]

Group	No. and age of animals	Glycerolipid formation (nmoles/30 min/mg protein)	% Distribution of αGP into Phospholipid	Diglyceride	Triglyceride	NL/PL
	18 hr (12)	14 ± 3	33 ± 3	27 ± 4	40 ± 4	2.0 ± 0.3
	1 days (8)	22 ± 4	30 ± 3	26 ± 4	44 ± 6	2.3 ± 0.4
	4-6 days (23)	7 ± 1	53 ± 6	23 ± 3	24 ± 7	0.9 ± 0.2
	11 days (11)	4.2 ± 0.4	53 ± 3	30 ± 2	16 ± 1	0.9 ± 0.1
	15 days (5)	4 ± 1	59 ± 10	21 ± 4	20 ± 8	0.7 ± 0.3
	20 days (8)	7.0 ± 0.6	57 ± 4	27 ± 3	16 ± 3	0.8 ± 0.1
	22 days (8)	7 ± 1	34 ± 7	24 ± 14	38 ± 14	1.8 ± 0.6
Lean	32 days (4)	16.0 ± 2.3	46 ± 8	33 ± 8	21 ± 1	1.2 ± 0.4
Obese	32 days (4)	23.0 ± 3.4	34 ± 9	37 ± 3	29 ± 3	2.1 ± 0.1
Lean	42-44 days (8)	14.0 ± 2.4	55 ± 3	25 ± 2	20 ± 2	0.8 ± 0.1
Obese	42-44 days (8)	24†.0 ± 1.3	41 ± 8	30 ± 2	29 ± 8	1.5 ± 0.6
Lean	67-69 days (5)	10.5 ± 1.5	43 ± 5	30 ± 3	27 ± 3	1.3 ± 0.3
Obese	67-69 days (5)	17* ± 5	36 ± 6	31 ± 3	33 ± 5	1.8 ± 0.3
Lean	75-79 days (4)	6.0 ± 1.3	53 ± 1	26 ± 1	21 ± 6	0.9 ± 0.1
Obese	75-79 days (4)	12*0 ± 1.3	39 ± 5	32 ± 2	29 ± 5	1.6 ± 0.4

*P<0.05, †P<0.01

[a]Glycerolipid formation was measured in the presence of [^{14}C]glycerol-3-phosphate and palmitoyl CoA generating system by liver homogenates derived from various age groups of animals. Each value represents mean ± S.D. of assays conducted in duplicate using liver homogenates from the number of rats given in parentheses. αGP = α glycerophosphate, NL = neutral lipid (sum of di- and triglyceride), PL = phospholipid.

of Hirsch and Gallian (8).

Hepatic and plasma triglyceride concentrations were determined according to Haux and Natelson (9). Protein was determined by the procedure of Lowry et al. (10) with bovine crystalline albumin as standard.

RESULTS AND DISCUSSION

Changes in Hepatic and Plasma Triglyceride Concentration

Although the phenotypic expression of genetic obesity in Zucker rats is apparent at 2-3 weeks of age, the primary metabolic event(s) leading to obesity in this animal model is not yet identified (1). Since obesity in adult rats is associated with increased neutral lipid formation in both liver and adipose tissue (2,3), this parameter was examined in relation to genetic expression of obesity in Zucker rats. However, various measurements performed such as changes in body weight, plasma and hepatic triglyceride concentration, and hepatic glycerolipid formation in the early ages of Zucker rats did not appreciably differ from one animal to the other in the same litter (Tables I and II). In other words, preobese animals were not recognizable from their thin littermates on the basis of these measurements. It has been demonstrated recently that a decrease in O_2 consumption is a reliable indicator to predict obesity in mice at early ages (11). However, no such information is available for obese rats.

In the present study, obesity is recognized on the basis of visual examination of subcutaneous and other fat deposits. Animals which showed pronounced development of adipose tissue were considered obese and the earliest time period when obesity in animals was clearly recognizable was at 32 days. Therefore, results obtained before this age represent the changes in early growing period in Zucker rats. The results obtained at 32 days and thereafter represent the changes in triglyceride metabolism with age and the development of obesity in Zucker animals.

Table I shows the age-related changes in plasma and hepatic triglycerides in newborn, neonatal, adult lean and obese Zucker rats. Following birth, plasma triglyceride concentration reached a peak value at 20 days of age. Thereafter, during the postweaning period, plasma triglyceride began to decrease gradually. As obesity progressed with age, plasma triglyceride concentration increased again. Thus, at 75-79 days of age, obese rats showed 4 times higher plasma triglyceride concentration compared to lean animals.

The hepatic triglyceride concentration increased after birth to reach a peak level at 1 day of age and then decreased with age to an adult level (lean adult) by 20 days of age. As obesity progressed with age, hepatic triglyceride concentration increased again. Although the overall developmental changes of plasma and hepatic triglyceride concentrations in the early growing period of Zucker rats were comparable to that with Sprague Dawley rats (4), a close examina-

tion of these changes showed some differences. In Zucker rats, the rise of plasma triglyceride concentration after birth was sharper than found in Sprague Dawley rats. As far as the hepatic triglyceride concentration in these two species of rats are concerned, triglyceride level reached a peak level at 1 day of age. However, it dropped more slowly in Zucker rats compared to Sprague Dawley rats. Thus, an adult level of hepatic triglycerides was reached at 20 days of age in Zucker animals instead of at 10 days of age in Sprague Dawley rats (4). The reasons for these differences are not clear.

Changes in Hepatic Glycerolipid Formation with Age

As reported previously (4), the newborn Zucker rats also showed high rates of hepatic glycerolipid formation (Table II). This was followed by a decline in the rates of glycerolipid formation as the rats grow older. Hepatic glycerolipid formation increased again at day 20 and continued to increase up to 32 days of age in lean rats and 42-44 days in obese rats. This period was then followed by the gradual decrease in the rates of glycerolipid formation with age in both lean and obese animals. Obese rats showed significantly higher rates of glycerolipid formation compared to lean animals in various age groups of animals. In our previous studies (4), the early rise in glycerolipid formation after birth was related to increased synthesis of esterifying enzymes rather than the activation of preformed enzymes. It is likely that the early rise in glycerolipid formation observed in Zucker animals after birth may also be due to increased synthesis of esterifying enzymes.

In our earlier studies, measurements of the hepatic glycerophosphate concentration at various ages indicated that the changes in hepatic glycerolipid formation with age were related to changes in enzyme concentration rather than to variation in the glycerophosphate pool (4). In the present studies, glycerophosphate concentration was not measured. However, it is possible that the age-related changes reported here may also be due to changes in enzyme concentration involved in glycerolipid formation.

Liver homogenates derived from newborn, neonatal, lean and obese rats formed phospholipid (largely phosphatidate), diglyceride, and triglyceride from *sn*-glycerol-3-phosphate and palmitoyl CoA generating system (Table II). In the preweaning period, liver homogenates from 1-day-old rats were most active and formed more neutral lipids (sum of di-and triglyceride) compared to phospholipids. This was followed by a decline in the rates of glycerolipid formation. Following weaning, the rates of glycerolipid formation increased again, which were accompanied by the increase in neutral lipid and decrease in phospholipid formation. At any given age, obese rats showed higher rates of total and neutral lipid formation. These changes in the product formation are consistent with the alteration of both *sn*-glycerol-3-phosphate acyltransferase and phosphatidate phosphohydrolase activities in the liver homogenates as a function of age. In the present studies, individual enzyme activities involved in hepatic glycerolipid formation were not measured. However, previous studies demonstrate that the ratio of neutral lipid to phospholipid formed from *sn*-glycerol-3-phosphate and palmitoyl CoA generating system is a reliable index of phosphatidate phosphohydrolase activity (12). As indicated by this ratio of product formation, phosphohydrolase activity was high when neutral lipid formation was increased (in newborn, postweaning, and obese rats), and the activity of this enzyme decreased with age. It was suggested earlier that phosphatidate phosphohydrolase plays an important role in neutral lipid formation (12,13), and this was also apparent in the present studies.

It is apparent from the present as well as from the earlier studies (4) that there are certain periods in the life of a laboratory rat when hepatic glycerolipid formation is very active. These are: (a) the period between birth and 1 day of age and (b) the period between 15-25 days of age. Both of these periods are accompanied by the sudden changes in the dietary intake of growing animals. During the first period, high carbohydrate content of transplacental food is replaced by high-fat content of a milk diet. In the second period, the milk diet of suckling is replaced by the laboratory diet which has a high carbohydrate and low fat content. In addition to these dietary changes, hormonal changes are also occurring during development (14,15). Therefore, the interaction between both hormonal and dietary factors may be important in regulating the age-related changes in hepatic glycerolipid formation, possibly through alterations in the activities of enzymes involved in fatty acid esterification.

As far as the changes in hepatic glycerolipid formation in obese rats are concerned, obese rats showed more resemblance to newborn animals than to lean animals of similar ages. The presence of a factor(s) common to both newborns and obese animals may be responsible for the increased rates of hepatic triglyceride

TABLE III

Glycerolipid Formation by Adipose Tissue Homogenates as a Function of Age[a]

Group	No. and age of animals	Glycerolipid formation nmoles/min/10^6 adipocytes	% Distribution of αGP into Phospholipid	Diglyceride	Triglyceride	NL/PL
Lean	42-44 days (3)	3 ± 1	13 ± 3	6 ± 1	81 ± 4	6.8 ± 0.7
Obese	42-44 days (3)	23[b] ± 2	11 ± 3	7 ± 1	81 ± 4	8.5 ± 2.7
Lean	67-69 days (8)	8 ± 3	56 ± 6	9 ± 1	35 ± 5	0.7 ± 0.2
Obese	67-69 days (5)	22[b] ± 5	32 ± 5	14 ± 2	54 ± 5	2.0 ± 0.4
Lean	75-79 days (7)	10 ± 3	44 ± 13	11 ± 4	45 ± 13	1.3 ± 0.5
Obese	75-79 days (7)	38[b] ± 12	34 ± 12	15 ± 13	51 ± 13	2.0 ± 0.4

[a]Glycerolipid formation was measured in the presence of [^{14}C]glycerol-3-phosphate and palmitoyl CoA generating system as described in the Methods. Each value represents mean ± S.D. of assays conducted in duplicate using adipose tissue homogenates from the number of animals given in parentheses. Details of animals are given in Table I.

[b]$P<0.01$.

formation and hepatic triglyceride accumulation under these conditions.

Glycerolipid Formation by Adipose Tissue Homogenates

Although, on the basis of visual examination of subcutaneous and other fat deposits, obesity in Zucker animals was apparent as early as 32 days, sufficient adipose tissue was not available to conduct various measurements before 42 days of age. Therefore, changes in adipose tissue glycerolipid formation were measured between 42-79 days of age in lean and obese rats (Table III).

Adipose tissue glycerolipid formation increased with age in both lean and obese animals. As noted earlier (3), adipose tissue homogenates incubated in the presence of *sn*-glycerol-3-phosphate and palmitoyl CoA generating system formed phosphatidate, diglyceride, and triglyceride. The homogenates derived from 42-44-day-old lean and obese rats formed more neutral lipids compared to phosphatidate, and as the rats grew older, phospholipid formation increased with concomittant decrease in neutral lipid formation. Similar age-related changes in the product formation were also evident in the liver homogenates derived from various age groups of rats (Table II). As indicated by neutral lipid to phospholipid ratio (NL/PL ratio), the phosphatidate phosphohydrolase seems to decrease with age in both liver and adipose tissue (Tables II and III).

When the overall changes of glycerolipid formation in liver and adipose tissue were taken into consideration, glycerolipid formation in liver tends to decrease with age and that in adipose tissue increases with age. Thus, there was a shift in glycerolipid formation from liver to adipose tissue as a function of age. As far as the neutral lipid formation is concerned, it decreased with age in both liver and adipose tissue.

As noted in the earlier studies (2,3), obesity in Zucker rats was associated with an increase in glycerolipid formation in both liver and adipose tissue. However, changes in glycerolipid formation in the early ages of Zucker rats do not indicate that the increased potential of hepatic glycerolipid formation is a primary defect leading to obesity in these animals.

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Effects of Diets High in Saturated Fat and Cholesterol on the Lipid Composition of Canine Platelets

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ABSTRACT

The phospholipid composition of platelets from dogs on various experimental diets was determined. Thyroidectomized foxhounds were fed a control diet or the control diet supplemented with (1) beef tallow, (2) beef tallow and cholesterol, or (3) beef tallow, cholesterol, and safflower oil for 23 weeks prior to isolation of platelets. Platelets from animals fed the control diet contained 36.7% phosphatidylcholine (PC), 22.8% phosphatidylethanolamine (PE), 18.4% sphingomyelin (Sph), 11.8% phosphatidylserine (PS), 6.3% phosphatidylinositol (PI), and 2.2% lysophosphatidylcholine. The PE was 77.6% in the plasmalogen form. No highly significant changes in the phospholipid class composition resulted from the experimental diets. Cholesterol supplementation of the diets, however, caused consistent alterations in the fatty acid compositions of the platelet phospholipids including increases in the percentages of 18:1ω9 (oleic acid), 18:2ω6 (linoleic acid), and 20:3ω6 (homo-gamma linolenic acid) and a decrease in the percentage of 20:4ω6 (arachidonic acid). Addition of safflower oil to the tallow-cholesterol diet partially reversed these effects. These cholesterol-induced alterations in fatty acid composition could be due to exchange with plasma lipids, de novo synthesis, or altered platelet metabolism. The mechanism remains to be determined.

INTRODUCTION

Platelets and platelet factors may be implicated in the genesis of atherosclerosis (1), and alterations in platelet half-life in experimental animals have been correlated with intimal injury and degree of atherosclerosis (2). It has been suggested that the role of platelets in the disease process may be to release factors which lead to smooth muscle cell proliferation (3). This release is triggered by platelet aggregation.

Platelet aggregation can be stimulated by thromboxanes and other oxygenated products of fatty acids derived from platelet phospholipids (4). Since phospholipid has obvious importance in platelet function, it is of interest to study the effect of atherogenic diets on the phospholipid composition of platelets.

Composition of the platelet lipids as well as the lipid environment of platelets can be determinants of platelet function, and may thereby be factors in the atherosclerotic disease process. Both of these properties may be altered by diet. Epidemiologic studies with humans (5) and dietary studies with animals (6) have suggested that diets high in cholesterol and saturated fat may be associated with increased risk of atherosclerosis. Our laboratory has previously reported the occurrence of thrombosis in association with accelerated atherosclerosis in dogs fed saturated fat and cholesterol (7). By contrast, human consumption of polyunsaturated fatty acids seems to correlate with lower levels of circulating cholesterol (8) and possibly with lower risk of atherosclerosis and myocardial infarction. This paper reports a study of the effects of diet and particularly the effects of both saturated and unsaturated fats, and cholesterol, on the phospholipid composition of canine platelets. These results, combined with others, including those of a previous study on the fatty acid composition of sphingomyelin (Sph) from canine platelets (9), may give some insight into the role played by platelet lipids in the development of atherosclerosis.

MATERIALS AND METHODS

The experimental diets were initiated one week after thyroidectomy of nine-month-old foxhounds. The animals were fed a basal diet (Table I) [Zeigler Brothers, Inc., Gardners, PA] or the basal diet supplemented with one of the following: 0.75% taurocholic acid; 15% beef tallow; 12.0-14.5% beef tallow, 0.5-3.0% cholesterol, and 0.75% taurocholic acid or 0.75% cholic acid; or 10.5-13.0% beef tallow, 0.5-3.0% cholesterol, 0.75% taurocholic acid, and 1.5% safflower oil, as previously described (9). The cholesterol content of the cholesterol-supplemented diets was varied weekly in order to maintain a blood cholesterol level of ca. 1500 mg/dl. All dogs were surgically thyroidectomized and maintained in a hypothyroid condition by administering 500 mg of propylthio-

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TABLE I

Composition of Experimental Diets[a]

Basal (Control) Diet	
Ingredients	Amount
	Percentage by weight
Corn flakes	33.44
Beet pulp	11.80
Soybean meal (48.5% protein)	11.80
Corn gluten meal (60% protein)	5.85
Egg white solids	5.85
Brewer's dried yeast	2.95
Dried whey	5.85
Casein (lactic acid precipitated)	11.80
Dicalcium phosphate	5.50
Limestone	1.80
Salt	0.60
Choline chloride (50% choline)	0.64
Sodium tripolyphosphate	1.12
Potassium chloride	.90
Vitamin premix	.05
Mineral premix	.05
	100.00
Vitamin premix	Amount per kg premix
Vitamin A (IU)	34,750,000
Vitamin D_3 (IU)	1,600,000
DL-α-Tocopheryl acetate (50%) (g)	363.0
Vitamin B_{12} (mg)	80.0
d-Calcium pantothenate (g)	18.1
Pyridoxine hydrochloride (g)	28.8
Biotin 1% (g)	17.6
Cornstarch to	1000.0 g
Mineral premix	g per kg premix
Potassium chloride	619.6
Ferrous sulfate	134.0
Copper sulfate	26.4
Zinc oxide	220.0
	1000.0 g

[a]Diet formulated to supply 120 and 110% of the NRC recommended vitamin and mineral requirements, respectively, when test animals consumed 85% basal diet and 15% fat. The basal diet contained less than 2% crude fat.

uracil daily as described (7). The animals were maintained on diet for 23 weeks, after which platelets were isolated and lipids were extracted and purified as described previously (9). The general health of all animals was excellent and hematologic studies revealed no anemia (R.M. Jaffe, unpublished observations).

Cholesterol was determined by gas liquid chromatography (GLC) after separation of free and esterified cholesterol on a silicic acid-Celite column (10). Stigmasterol was used as an internal standard. Phospholipids were separated using two-dimensional thin layer chromatography (TLC), and the phospholipid spots were visualized by spraying with 0.6% $K_2Cr_2O_7$ in 50% H_2SO_4 and then charring for 20 min at 175 C or by spraying with 2′7′-dichlorofluorescein and then using UV light to visualize the spots (11,12). Phospholipids were quantitated by analyzing the phosphorus content of the spots scraped from the charred plates (13). All phosphorus analyses were performed in triplicate. Methyl esters and dimethylacetals (DMA) were prepared in the presence of the TLC adsorbant by heating the TLC spot in a sealed tube with 3N methanolic HCl for 1 hr at 70 C. Methyl heptadecanoate was added as an internal standard.

Phosphatidylethanolamine (PE) isolated by two-dimensional TLC was eluted from the adsorbant with chloroform/methanol/H_2O (65:35:2, v/v) containing 5 μg/ml of butylated hydroxytoluene. It was then hydrolyzed with phospholipase C (14). The diacyl and alkenylacyl glycerides formed enzymatically from the diacyl and plasmalogen PE, respectively, were extracted from the reaction mixture with diethyl ether. The free hydroxy groups were acetylated with acetic anhydride/pyridine (5:1, v/v), reagents were removed under nitrogen, and the products were separated by TLC on Silica Gel G plates using a solvent system of

TABLE II

Percentage of Cholesterol, Cholesteryl Ester, and Phospholipid in Platelets of Dogs on Experimental Diets

Component[b]	Diet[a] Control	Tallow	Tallow cholesterol	Tallow, SFO cholesterol
Phospholipid	79.4 ± 0.4[c]	(78.6,80.3)	77.3 ± 4.2	76.2 ± 0.9[d]
Cholesterol	19.7 ± 0.4	(19.7,19.3)	18.8 ± 2.5	21.6 ± 0.7[d]
Cholesteryl Ester	0.9 ± 0.5	(1.6, 0.4)	3.9 ± 2.1[e]	2.2 ± 0.5[e]
(n)[f]	(4)	(2)	(4)	(3)

[a]See text for composition of diets; SFO, safflower oil.
[b]Phospholipid determined by multiplying phosphorus X 25. Cholesteryl ester reported as weight of free cholesterol.
[c]Weight percent, reported as the mean ± SD (n= 3 or 4) or as the individual results (n=2).
[d]Significantly different from control group, $P < 0.01$.
[e]Significantly different from control group, $P < 0.05$.
[f](n) = number of animals.

TABLE III

Phospholipid Composition of Platelets from Dogs on Experimental Diets

Fraction	Diet[a] Control	Tallow	Tallow cholesterol	Tallow, SFO cholesterol
Phospholipids[b,c]				
PC	36.7 ± 1.3[d]	(35.6,36.2)	42.4 ± 3.2[e]	39.6 ± 1.4[e]
PE	22.8 ± 0.6	(22.4,23.4)	22.0 ± 2.3	23.6 ± 0.5
Sph	18.4 ± 0.7	(18.0,19.4)	16.4 ± 1.4[e]	18.0 ± 0.2
PS	11.8 ± 0.7	(11.3,12.8)	10.8 ± 1.3	12.2 ± 0.5
PI	6.3 ± 0.8	(6.4, 5.2)	5.2 ± 0.9	4.8 ± 0.2[e]
LPC	2.2 ± 0.6	(3.7, 1.9)	1.8 ± 0.8	1.2 ± 0.4
PA	0.3 ± 0.4	(1.1, 0.2)	0.3 ± 0.3	0.0 ± 0.0
unk	1.3 ± 0.4	(1.4, 0.8)	1.2 ± 0.7	0.5 ± 0.2[e]
PE				
Diacyl	22.4 ± 2.0	(18.9,19.0)	18.8 ± 3.9	19.8 ± 6.3
Alkenylacyl[f]	77.6 ± 2.0	(81.1,81.0)	81.2 ± 3.9	80.2 ± 6.3
(n)[g]	(4)	(2)	(4)	(3)

[a]See text for composition of diets; SFO, safflower oil.
[b]Determined by phosphorus analysis following two-dimensional thin layer chromatography.
[c]PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; PA, phosphatidic acid; unk, unknown.
[d]Reported as the mean ± SD (n=3 or 4) or as the individual results (n=2).
[e]Significantly different from control group, $P < 0.05$.
[f]Alkenylacyl = plasmalogen fraction.
[g](n) = number of animals.

toluene/methanol (100:0.5 v/v) (15). Separated bands were converted to methyl esters and DMA with methanolic HCl (3N) as described above. Plasmalogen and diacyl forms of PE were quantitated by GLC of the products derived from the alkenylacyl and diacyl acetates, respectively, and by reference to the internal standard.

A glass capillary column wall coated with SP 2340 was used to separate and quantitate fatty acid methyl esters and dimethylacetals by GLC (9). The chromatograph was equipped with a digital communications interface (Hewlett Parkard, Avondale, PA), and the data were recorded on cassette tapes using a Texas Instruments Silent 700 ASR Electronic Data Terminal. Relative retention times and sample weight were calculated by computer. Peak areas contributed by fatty acid contaminates observed in the enzymes and reagent blanks were automatically subtracted from the data. Statistical significance was calculated by use of the t-test (16).

Methyl esters were identified by comparison of relative retention times with those of authentic standards (NU Chek Prep, Inc., Elysian, MN).

TABLE IV

Fatty Acid Composition of Phosphatidylcholine from Platelets of Dogs on Experimental Diets

Fatty acid	Diet[a]			
	Control	Tallow	Tallow cholesterol	Tallow, SFO cholesterol
16:0	26.4 ± 0.7[b]	(22.5,22.9)	20.8 ± 0.6[c]	21.4 ± 1.2[c]
16:1	1.2 ± 0.1	(1.2, 1.4)	2.1 ± 0.6	1.7 ± 0.4
18:0	17.2 ± 1.3	(18.7,19.6)	18.6 ± 1.6	18.6 ± 0.7
18:1ω9	13.2 ± 0.3	(15.7,13.0)	19.0 ± 2.5[c]	11.8 ± 1.3[d]
18:1ω7	3.7 ± 0.2	(2.8, 2.5)	2.7 ± 0.6[e]	2.5 ± 0.4[c]
18:2ω6	11.5 ± 1.0	(9.0, 6.9)	14.7 ± 2.3	21.3 ± 2.0[c,f]
20:1ω9	1.4 ± 0.1	(1.6, 1.2)	1.4 ± 0.5	1.0 ± 0.1
20:3ω9	0.1 ± 0.0	(0.4, 0.2)	0.3 ± 0.2 [e]	---
20:3ω6	2.0 ± 0.1	(2.2, 1.6)	4.0 ± 0.7[c]	2.0 ± 0.1[d]
20:4ω6	21.9 ± 1.0	(23.3,29.3)	14.1 ± 3.5[c]	18.5 ± 2.3
20:5ω3	0.2 ± 0.1	(1.0, 0.4)	1.1 ± 0.5[e]	0.2 ± 0.0[f]
22:4ω6	1.0 ± 0.2	(1.1, 1.4)	0.5 ± 0.2[c]	0.6 ± 0.1[e]
22:xω3[g]	0.2 ± 0.1	(1.0, 0.7)	0.7 ± 0.3[e]	0.3 ± 0.1
(n)[h]	(4)	(2)	(4)	(3)

[a]See text for composition of diets; SFO, safflower oil.
[b]Weight percent, reported as the mean ± SD (n=3 or 4) or as the individual results (n=2).
[c]Significantly different from control group, $P < 0.01$.
[d]Significantly different from tallow-cholesterol group, $P < 0.01$.
[e]Significantly different from control group, $P < 0.05$.
[f]Significantly different from tallow-cholesterol group, $P < 0.05$.
[g]22:xω3 = 22:5ω3 plus 22:6ω3.
[h](n) = number of animals.

Dimethylacetal standards were prepared by heating aldehydes (synthesized from fatty alcohols (17)) with methanolic HCl. The DMAs liberated from the reaction mixture were extracted after saponification to remove traces of fatty acid methyl esters.

RESULTS

The addition of taurocholate to the basal diet had no effect on the composition of the phospholipid classes or on the fatty acid compositions as compared with the results obtained for the platelets of dogs on the basal diet; therefore, the data from these two groups were averaged (referred to as control in the tables). Likewise, the data from the dogs fed beef tallow, cholesterol, and either taurocholate or cholate were averaged (referred to as tallow-cholesterol group, Tables II-VIII). The contents of cholesterol, cholesteryl ester, and phospholipid found in the platelets of the animals on the various diets are reported in Table II. Incorporation of safflower oil into the tallow-cholesterol diet caused a decrease in platelet phospholipid content and an increase in the percentage of platelet cholesterol in comparison with platelets from control animals.

Phospholipid Composition

The phospholipid compositions of canine platelets are listed in Table III. No highly significant changes in the composition resulted from the experimental diets. Platelets from animals fed the tallow-cholesterol diet had more phosphatidylcholine (PC) and less sphingomyelin (Sph) than platelets from either the control or tallow-fed animals. Supplementing the tallow-cholesterol diet with safflower oil increased the Sph to control levels and reduced the PC slightly. The PC, however, remained significantly above the level in the control group, while phosphatidylinositol (PI) and an unidentified component were reduced. The unidentified component, which contained phosphorus, was observed in platelets from all animals. On two-dimensional TLC, this component migrated with PE in the first dimension (basic system) and slightly further than PE in the second dimension (acidic). Phosphatidylethanolamine isolated from the platelet phospholipids contained a high percentage of plasmalogen (Table III). No significant changes were caused by supplementing the control diet with tallow plus cholesterol or with tallow, cholesterol, and safflower oil. Alkylacyl PE was not observed in any of these samples.

Fatty Acid Composition of Platelets from Animals on Control Diets

Tables IV-VIII report the fatty acid compositions of the phospholipids from platelets of control animals. Components which comprised

TABLE V

Fatty Acid Composition of Phosphatidylinositol from Platelets of Dogs on Experimental Diets

Fatty acid	Diet[a] Control	Tallow	Tallow cholesterol	Tallow, SFO cholesterol
16:0	0.8 ± 0.2[b]	(1.1, 0.7)	1.0 ± 0.4	0.6 ± 0.1
18:0	40.5 ± 1.2	(49.5,48.4)	39.3 ± 1.4	41.0 ± 4.0
18:1ω9	4.8 ± 0.2	(4.5, 4.6)	7.6 ± 1.7[c]	5.6 ± 0.8
18:1ω7	2.1 ± 0.2	(1.1, 1.3)	1.7 ± 0.4	1.7 ± 0.3[c]
18:2ω6	0.4 ± 0.1	(0.2, 0.2)	0.6 ± 0.3	0.7 ± 0.1
20:1ω9	0.9 ± 0.1	(0.7, 0.6)	0.8 ± 0.3	0.8 ± 0.1
20:3ω9	0.1 ± 0.1	(0.3, 0.2)	0.6 ± 0.4[c]	---
20:3ω6	0.3 ± 0.1	(0.2, 0.1)	0.9 ± 0.4[c]	0.4 ± 0.1
20:4ω6	49.6 ± 0.7	(41.4,43.6)	46.1 ± 3.4	49.0 ± 4.5
20:5ω3	0.3 ± 0.3	(0.6, 0.1)	1.3 ± 0.6[c]	0.1 ± 0.1[d]
22:4ω6	0.2 ± 0.0	(0.4, 0.1)	---	0.1 ± 0.1
(n)[e]	(4)	(2)	(4)	(3)

[a]See text for composition of diets; SFO, safflower oil.
[b]Weight percent, reported as the mean ± SD (n=3 or 4) or as the individual results (n=2).
[c]Significantly different from control group, $P < 0.01$.
[d]Significantly different from tallow-cholesterol group, $P < 0.05$.
[e](n) = number of animals.

TABLE VI

Fatty Acid Composition of Phosphatidylserine from Platelets of Dogs on Experimental Diets

Fatty acid	Diet[a] Control	Tallow	Tallow cholesterol	Tallow, SFO cholesterol
16:0	0.5 ± 0.5[b]	(0.3, 0.2)	0.8 ± 0.9	0.2 ± 0.1
16:1	0.6 ± 0.5	(0.4, 0.3)	0.7 ± 0.6	0.3 ± 0.1
18:0	45.3 ± 1.2	(43.3,51.6)	42.8 ± 0.5[c]	46.4 ± 5.8
18:1ω9	7.6 ± 0.5	(10.8, 5.1)	11.4 ± 1.9[c]	5.4 ± 0.2[c,d]
18:1ω7	0.5 ± 0.1	(0.6, 0.5)	0.4 ± 0.3	0.4 ± 0.0
18:2ω6	12.8 ± 2.0	(11.5, 6.8)	17.8 ± 4.2	22.0 ± 5.5[e]
20:0	1.0 ± 0.1	(1.3, 0.9)	1.1 ± 0.0	1.3 ± 0.1[c]
20:1ω9	1.0 ± 0.1	(1.2, 0.7)	1.2 ± 0.3	1.1 ± 0.2
20:3ω9	0.1 ± 0.0	(1.0, 0.4)	0.5 ± 0.4	0.1 ± 0.0
20:3ω6	4.3 ± 1.5	(4.2, 2.2)	4.7 ± 1.4	2.3 ± 1.0
20:4ω6	25.0 ± 3.3	(23.4,29.3)	16.9 ± 5.7	19.5 ± 2.1
20:5ω3	0.1 ± 0.0	(0.5, 0.2)	0.7 ± 0.4[e]	0.1 ± 0.0
22:4ω6	1.2 ± 0.4	(1.7, 1.6)	0.8 ± 0.5	0.7 ± 0.2[c]
(n)[f]	(4)	(2)	(4)	(3)

[a]See text for composition of diets; SFO, safflower oil.
[b]Weight percent, reported as the mean ± SD (n=3 or 4) or as the individual results (n=2).
[c]Significantly different from control group, $P < 0.01$.
[d]Significantly different from tallow-cholesterol group, $P<0.01$.
[e]Significantly different from control group, $P< 0.05$.
[f](n) = number of animals.

less then 0.5% of the sample were omitted from the tables unless they were considered to be of possible metabolic importance. Each of the phospholipid classes had a distinct fatty acid pattern. The percentage of 16:0 in PC (Table IV) was at least eight times the percentage of 16:0 noted in any of the other phospholipids reported. The fatty acid composition of PI (Table V) was similar to that of phosphatidylserine (PS) (Table VI), with 18:0 and 20:4ω6 predominating. The amounts of 18:0 were similar in PI and PS, but the contents of 20:4ω6 were 49.6% and 25.0%, respectively. Furthermore, PS had higher percentages of 18:2ω6, 18:1ω9, and 20:3ω6 than PI. In diacyl PE (Table VII), 18:0 and 20:4ω6 also

TABLE VII

Fatty Acid Composition of Diacyl Phosphatidylethanolamine from Platelets of Dogs on Experimental Diets

Fatty acid	Diet[a]			
	Control	Tallow	Tallow cholesterol	Tallow, SFO cholesterol
16:0	2.9 ± 0.5[b]	(3.5, 3.0)	2.7 ± 0.4	4.2 ± 1.6
16:1	0.4 ± 0.1	(1.0, 0.5)	0.4 ± 0.2	0.5 ± 0.4
18:0	36.0 ± 2.9	(38.6,35.9)	33.9 ± 3.7	37.0 ± 3.7
18:1ω9	6.3 ± 0.7	(7.7, 5.2)	10.1 ± 2.0[c]	7.0 ± 1.1
18:1ω7	1.8 ± 0.2	(1.4, 1.5)	1.9 ± 0.5	1.6 ± 0.4
18:2ω6	6.8 ± 0.2	(4.6, 2.8)	11.0 ± 4.0	12.2 ± 4.1
20:3ω9	0.2 ± 0.0	(1.4, 0.5)	1.1 ± 0.5[c]	0.1 ± 0.1[d]
20:3ω6	2.4 ± 0.2	(1.8, 1.0)	3.4 ± 0.6[c]	1.7 ± 0.7[d]
20:4ω6	37.7 ± 3.4	(34.2,41.6)	31.3 ± 5.7	32.5 ± 0.8
20:5ω3	0.2 ± 0.1	(0.7, 0.3)	1.8 ± 0.4	0.1 ± 0.1
22:4ω6	5.5 ± 1.3	(5.1, 7.7)	4.3 ± 1.7	3.3 ± 1.0
(n)[e]	(4)	(2)	(4)	(3)

[a]See text for composition of diets; SFO, safflower oil.
[b]Weight percent, reported as the mean ± SD (n=3 or 4) or as the individual results (n=2).
[c]Significantly different from control group, $P < 0.01$.
[d]Significantly different from tallow-cholesterol group, $P < 0.01$.
[e](n) = number of animals.

TABLE VIII

Composition of Alkenylacyl Phosphatidylethanolamine from Platelets of Dogs on Experimental Diets

Fatty acid or aldehyde	Diet[a]			
	Control	Tallow	Tallow cholesterol	Tallow, SFO cholesterol
16:0[b]	4.0 ± 0.2[c]	(2.8, 3.6)	3.1 ± 0.3[d]	3.4 ± 0.9
18:0[b]	31.7 ± 2.4	(31.9,32.9)	32.0 ± 2.7	32.6 ± 1.7
18:1ω9[b]	5.6 ± 0.0	(5.5, 4.5)	5.6 ± 0.8	5.3 ± 1.3
18:1ω7[b]	2.2 ± 0.4	(1.5, 1.5)	2.7 ± 2.5	1.4 ± 0.5
18:1ω9	1.8 ± 0.6	(2.0, 0.8)	2.0 ± 0.7	1.2 ± 0.1
18:2ω6	1.0 ± 0.1	(1.6, 0.4)	4.7 ± 3.1	3.2 ± 1.2[e]
20:3ω9	---	(0.6, 0.2)	0.7 ± 0.5	---
20:3ω6	0.7 ± 0.1	(0.7, 0.3)	2.3 ± 1.0[e]	0.7 ± 0.4
20:4ω6	46.3 ± 2.6	(44.9,47.4)	39.0 ± 6.0	45.8 ± 1.6[f]
20:5ω3	0.5 ± 0.4	(1.9, 0.7)	0.8 ± 0.5	0.6 ± 0.1
22:4ω6	3.0 ± 2.2	(2.3, 3.3)	3.9 ± 1.5	1.5 ± 0.8
DMA[g]	3.3 ± 2.0	(3.9, 3.6)	3.0 ± 1.2	4.1 ± 1.5
(n)[h]	(4)	(2)	(4)	(3)

[a]See text for composition of diets; SFO, safflower oil.
[b]Dimethylacetal.
[c]Weight percent, reported as mean ± SD (n=3 or 4) or as the individual results (n=2).
[d]Significantly different from control group, $P < 0.01$.
[e]Significantly different from control group, $P < 0.05$.
[f]Significantly different from tallow-cholesterol group, $P < 0.05$.
[g]Sum of minor dimethylacetal components.
[h](n) = number of animals.

predominated, and this phospholipid contained the highest percentage of 22:4ω6 (5.5%). Plasmalogen PE (Table VIII) was high in 20:4ω6 (46.3%), as was the diacyl form. Dimethylacetals, formed by the action of MEOH-HCl on the α,β-unsaturated ether linkages of the plasmalogens, were composed primarily of 18:0 DMA (31.7%), 18:1 DMA (7.8%), and 16:0 DMA (4.0%) (Table VIII).

Effect of Supplementation with Tallow

Addition of tallow to the control diet caused a variable decrease in the percentage of 18:2ω6 in all of the phospholipids except PE plasmalogen (see Tables IV through VIII).

TABLE IX
Relative Ratio[a] of 20:4ω6 to 20:3ω6 in the Phospholipids from Platelets of Dogs on Experimental Diets

Diet[b]	Phospholipid[c] PC	PI	PS	PE	PPE	n[d]
Control	1.0	1.0	1.0	1.0	1.0	4
Tallow	1.3	1.3	1.4	1.7	1.4	2
Tallow Chol	0.3	0.3	0.6	0.6	0.3	4
Tallow, Chol, SFO	0.8	0.7	1.5	1.2	1.0	3

[a]Relative ratio is the ratio of 20:4ω6 to 20:3ω6 in the platelets of the experimental animals divided by that of the control animals.

[b]See text for composition of diets; SFO, safflower oil.

[c]PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PPE, plasmalogen phosphatidylethanolamine.

[d](n) = number of animals.

Effect of Cholesterol Supplementation

The addition of cholesterol to the tallow-containing diet resulted in several distinct alterations in the fatty acids of the platelet phospholipids. By comparison to control values, statistically significant increases occurred in 18:1ω9 (in PC, PI, PS, and diacyl PE) and 20:3ω6 and 20:3ω9 (in PC, PI, diacyl and alkenylacyl PE). Increases in 20:3ω6 and 18:1ω9 also occurred relative to the tallow-fed animals. Arachidonic acid was significantly decreased in the PC of cholesterol-fed animals. Although changes in 18:2ω6 and 20:4ω6 were not generally statistically significant because of the large variation among animals, a consistent effect of adding cholesterol to the diet was apparent. Tallow-cholesterol feeding caused an increase in 18:2ω6 and a decrease in 20:4ω6 relative to both control and tallow-fed animals in all the phospholipids except PI, in which the 20:4ω6 was decreased compared to control values but was the same as in tallow-fed animals.

Supplementation of the tallow-cholesterol diet with a polyunsaturated oil (safflower oil) caused a reduction in the percentages of 20:3ω9 and 20:3ω6 in all the phospholipids to the levels observed in the control animals, or below, thus reversing the effect of the cholesterol supplementation. The addition of safflower oil also elevated the arachidonic acid content above the level in the tallow-cholesterol-fed animals and, in some cases, to the control level in all the phospholipids except alkenylacyl PE.

A summary of the effects of the experimental diets on the ratio of 20:4ω6 to 20:3ω6 in the platelet phospholipids is presented in Table IX. Tallow feeding produced an increase in the ratio relative to control animals in all of the phospholipids. Supplementation of the tallow diet with cholesterol was associated with a reduction of the ratio of 20:4ω6 to 20:3ω6 to below the value in either the control or the tallow-fed group, reflecting the increased percentage of 20:3ω6 and decreased 20:4ω6 resulting from cholesterol supplementation of the tallow-containing diet. The addition of safflower oil to the tallow-cholesterol diet resulted in an increase in this ratio relative to the value in unsupplemented animals due to a partial reversal of the effect of cholesterol on the content of 20:3ω6 and 20:4ω6.

DISCUSSION

Human platelet lipids have been reported to contain 79% phospholipid (18). Results obtained for canine platelets from the control and tallow-fed animals (Table II) agreed with this value. Furthermore, the phospholipid composition of canine platelets (Table III) was similar to that of human platelets. Averaging literature values gave a composition for human platelets of PC, 38.3%; PE, 26.0%, Sph, 18.5%; PS, 9.5%; and PI, 4.5% (18-20). Lysolecithin ranged from 0.7 to 1.7% (18,19). The content of plasmalogen, 72.5 to 84.1% of the canine platelet PE (Table III), was higher than results reported for human platelets, for which values ranging from 50 to 60% have been reported (18,19). The absence of alkylacyl PE is consistent with the observation that the diacyl form and either the alkenylacyl (plasmalogen) or alkylacyl form, but not both, can exist in cells of the same species (21).

The fatty acid composition of canine platelet phospholipids was qualitatively similar to that of human platelets (22). The greatest difference in composition appeared to be in the percentages of 18:1 and 18:2ω6. PC from platelets of control animals contained 16.9% 18:1 and 11.5% 18:2ω6, while the percentage of 18:1 and 18:2ω6 in PC from human plate-

lets ranged from 27.5 to 31.6% for 18:1 and from 7.0 to 9.3% for 18:2ω6. PS, PI, and diacyl and alkenylacyl PE from canine platelets all had higher percentages of oleic and lower percentages of linoleic acid than human platelets.

Animals fed the tallow-cholesterol and tallow-cholesterol-safflower oil diets, in agreement with previous results (7), had severe atherosclerotic disease. Furthermore, platelets from animals fed tallow-cholesterol and tallow-cholesterol-SFO, but not from tallow-fed animals, showed a substantial increase in sensitivity of platelets to aggregation at low levels of ADP or collagen (R.M. Jaffe, unpublished observations). The cause of the alterations in platelet function noted upon cholesterol supplementation of the diets is unknown. However, platelet hypersensitivity to aggregation has been reported in patients with Type II hypercholesterolemia which is associated with accelerated atherosclerosis (23).

Addition of cholesterol to the diet was also associated with alterations in lipid composition. The most striking observation related to the phospholipid fatty acid composition of platelets from these animals was a consistent reduction in the ratio of 20:4ω6 and 20:3ω6 following the addition of cholesterol to the diet. The presence of safflower oil modulated, to a certain extent, the effect of the cholesterol on the content of 20:4ω6 and 20:3ω6.

Furthermore, we observed a decrease in the ratio of 20:4ω6 to 20:3ω6 in the PC of plasma phospholipids from animals on the cholesterol-supplemented diets and in the erythrocytes of these animals (unpublished observations). Other investigators have noted an increase in the percentage of 20:3ω6 and a decrease in 20:4ω6 in the liver phospholipids of animals fed diets supplemented with cholesterol (24,25). The reason for this decreased ratio is unknown, but it has been postulated to be the result of utilization of 20:4ω6 for cholesterol ester synthesis, with a resultant depletion of liver arachidonate and a build up of its metabolic precursors (24). Conversely, data suggesting a direct inhibition of conversion of 20:3ω6 to 20:4ω6 in the liver has also been reported (25).

The mechanism which leads to alterations in the fatty acid composition of platelets is complex, since platelet phospholipids are in a dynamic state. Their fatty acid compositions are the result of a balance between uptake and incorporation of serum lipids (26), de novo synthesis (27), and metabolism (28) of fatty acids. The reduction in the ratio of 20:4ω6 to 20:3ω6 associated with cholesterol supplementation of the diet may be the result of one or a combination of the above factors; the mechanism causing the alteration remains to be determined.

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Utilization of Various Sterols by Lecithin-Cholesterol Acyltransferase as Acyl Acceptors

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ABSTRACT

Highly purified lecithin-cholesterol acyltransferase of human plasma was used to study the utilization of various sterols as the acyl acceptor. The esterification of sterols was facilitated by the presence of a 3β-hydroxyl group and the *trans* configuration of the A/B rings, as was evident from the lack of acceptor activity of all 3α-hydroxy sterols tested and coprostanol. Cholesterol analogs in which the side chain is modified, such as campesterol, β-sitosterol, desmosterol and stigmasterol, were less effective than cholesterol as acyl acceptors. However, androstan-3β-ol, which completely lacks the side chain, was found to be more active than cholesterol. The transfer of the acyl group to all effective sterols required the presence of the cofactor peptide apolipoprotein A-I.

INTRODUCTION

Lecithin-cholesterol acyltransferase (EC 2.3.1.43) is known to catalyze the transfer of an acyl group from the carbon-2 position of phosphatidylcholine to cholesterol (1). The substrate specificity of the enzyme with respect to the acyl donor has been the subject of several studies which indicate a high specificity for phospholipids containing a basic nitrogen atom, and an increased reaction rate by N-methylation (2). Only the fatty acid esterified at the carbon-2 position of the phospholipid can be transferred (3); this fatty acid, however, can be of various chain lengths and degrees of saturation (4-7).

The specificity of the enzyme toward the acyl acceptor has been less thoroughly investigated. It has been shown that cholestanol, desmosterol, and β-sitosterol are esterified in human plasma (8,9), thus indicating that sterols other than cholesterol can also serve as the acyl acceptor. Water may also be considered as an acyl acceptor in view of a phospholipase A_2-like activity that was demonstrated with a partially purified enzyme (10) and confirmed with pure enzyme preparations (11,12). The present study was conducted to identify the structural features of sterols required for effective participation in the enzymatic transacylation.

MATERIAL AND METHODS

Experiments were performed in phosphate (KH_2PO_4-Na_2HPO_4) buffer, pH 7.4, ionic strength 0.1, containing 0.025% EDTA unless specified otherwise in the text. Lecithin-cholesterol acyltransferase was purified from human plasma according to the method recently developed in our laboratory (11). The enzyme showed a single band on both disc and sodium dodecyl sulfate electrophoreses (11). Apolipoprotein A-I and egg phosphatidylcholine were prepared as previously described (11). [2-(9,10)-^{3}H] Dipalmitoyl phosphatidylcholine (13) mCi/mmole) was purchased from Applied Science Laboratories, Inc., (State College, PA), and its specific labeling was confirmed by the hydrolysis with phospholipase A_2 from Naja naja (11). [7α-^{3}H] Cholesterol (9.3 Ci/mmole), and [22,23-^{3}H] β-sitosterol (47 Ci/mmole) were obtained from Amersham/Searle Corporation (Arlington Heights, IL). The radiochemical purity of these labeled compounds, given by the supplier, was in the range of 98-99%. Cholesterol (99+%) was obtained from Sigma Chemical Company (St. Louis, MO). Desmosterol (95%), β-sitosterol (98+%), stigmasterol (99.5%), campesterol (99%), cholestanol (95+%), and coprostanol (95+%) were purchased from Applied Science Laboratories, Inc. Epicholesterol (95+%), epicholestanol (95+%), lathosterol (95+%), androstan-3α-ol (95+%) and androstan-3α-ol (95+%) were purchased from Research Plus Steroid Laboratories, Inc. (Denville, NJ). The purity given in parentheses was supplied by the manufacturers. These materials were used without further purification.

The assay mixture for the transacylation consisted of the substrate vesicles (25 nmoles of sterols and 100 nmoles of phosphatidylcholine), 15 μg apolipoprotein A-I, 10 mM 2-mercaptoethanol, 0.7 mM EDTA, 2.5 mg bovine serum albumin, and 10 μl of the purified enzyme (40 ng as protein) in a final volume of 250 μl in phosphate buffer. For the determination of phospholipase A_2-like activity of lecithin-cholesterol acyltransferase in the absence of sterols, the substrate vesicles in the

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TABLE I

Comparison of Methods for Determination of Acyl Acceptor Activity

Acyl acceptor	Ester formed or phosphatidylcholine hydrolyzed (nmoles/hr/ml)		Ratio of Method I/Method II
	Method I[a]	Method II[b]	
Cholesterol	61.1 ± 1.4[c]	33.3 ± 1.0[c]	1.83
β-Sitosterol	48.1 ± 1.2	26.3 ± 0.9	1.83
—	15.3 ± 0.7	8.3 ± 2.6	1.84

[a]In Method I, vesicles of [2-(9,10)-^{3}H]dipalmitoyl phosphatidylcholine-egg phosphatidylcholine mixture (molar ratio, 1:4) containing 20 mole % of acyl acceptors, or vesicles of the phospholipid mixture alone were incubated for 30 min with purified enzyme. The rates of conversion of cholesterol and β-sitosterol to their corresponding esters were calculated from the percentage of the labeled palmitoyl group transferred from the dipalmitoyl phosphtidylcholine. The phospholipase A_2-like activity was calculated from the rate of the release of the labeled palmitic acid. All the rates were obtained assuming that the enzyme utilizes dipalmitoyl phosphatidylcholine and egg phosphatidylcholine equally, without any preference.

[b]In Method II, vesicles of egg phosphatidylcholine containing 20 mole % [7α-^{3}H]cholesterol, [22,23-^{3}H]β-sitosterol, or no sterol were incubated for 30 min with purified enzyme. The rate of ester formation was obtained from the amount of [7α-^{3}H]cholesterol and [22,23-^{3}H]β-sitosterol esterified. The rate of hydrolysis in the absence of acyl acceptor was determined by extracting the lipids from 1 ml incubation mixture and by analyzing the phosphorus content of the lysophosphatidylcholine and phosphatidylcholine fractions separated by thin layer chromatography on Silica Gel G using chloroform/methanol/acetic acid/water (75:45:12:6, v/v/v/v) as the developing solvent.

[c]The incubations were carried out in quadruplicate and the values represent means ± standard deviation.

assay mixture contained phosphatidylcholine alone. The substrate vesicles were prepared in phosphate buffer containing 10 mM 2-mercaptoethanol essentially as described by Batzri and Korn (13). The compositions of labeled substrate vesicles are described in the text. The vesicle solution and apolipoprotein A-I were preincubated for 30 min at 37 C under N_2 prior to addition into the assay mixture. The incubation mixtures in 8 ml screw-capped tubes were flushed with nitrogen, capped, and incubated for 30 min at 37 C with mechanical shaking. After the incubation, the lipids were extracted with chloroform/methanol (2:1, v/v), washed (14), and separated on silicic acid-impregnated, glass fiber sheets (Type ITLC-SA, Gelman Instrument Company, Ann Arbor, MI) using hexane/diethyl ether/acetic acid (70:30:1, v/v/v) as the developing solvent. The distribution of radioactivity on the chromatograms was measured by cutting the lipid spots and counting in a Beckman LS-250 liquid scintillation spectrometer. The scintillation mixture contained 5 g PPO, 300 mg POPOP, 130 ml methanol, 100 ml Beckman Biosolve BBS-3, and was diluted to 1 liter by toluene. The number of nmoles of ester and fatty acid produced was calculated from the distribution of radioactivity in the lipid spots and from the initial concentrations of labeled substrates. The results were corrected for the values obtained with control assays. All the reactions were linear during a 30 min incubation period under the assay conditions used.

The protein contents were determined by the method of Lowry et al. (15) with crystalline bovine plasma albumin as standard. Cholesterol and phospholipid phosphorus were determined by methods previously described (16).

RESULTS

Structural features of various sterols which facilitate their transacylation by lecithin-cholesterol acyltransferase can be determined by the use of labeled phosphatidylcholine and unlabeled sterols or by the use of unlabeled phosphatidylcholine and labeled sterols. It was observed that [2-(9,10)-^{3}H]dipalmitoyl phosphatidylcholine mixed with egg phosphatidylcholine at the molar ratio of 1:4 served as an efficient acyl donor. The rates of transesterification of cholesterol and β-sitosterol calculated from the transfer of the labeled palmitoyl group from the carbon-2 position of the dipalmitoyl phosphatidylcholine were ca. 80% higher than those computed from the esterification of the corresponding radioactive acyl acceptors (Table I). This effective and nearly equal degree of preferential utilization of dipalmitoyl phosphatidylcholine by the acyl acceptors provided a convenient means for the

TABLE II

Transesterification of Various Sterols by Purified Lecithin-Cholesterol Acyltransferase, and the Simultaneous Fatty Acid Release from Phosphatidylcholine[a]

Acyl acceptor	Palmitoyl ester formed (nmoles/hr/ml)	Palmitic acid released (nmoles/hr/ml)
Cholesterol (Cholest-5-ene-3β-ol)	12.2 ± 0.3[b]	1.8 ± 0.1[b]
Cholestanol (Cholestan-3β-ol)	11.7 ± 0.2	1.8 ± 0.1
Lathosterol (Cholest-7-ene-3β-ol)	10.1 ± 0.1	2.0 ± 0.1
Campesterol (24-isoergost-5-ene-3β-ol)	10.9 ± 0.1	1.7 ± 0.1
β-Sitosterol (Stigmast-5-ene-3β-ol)	9.6 ± 0.2	1.9 ± 0.1
Desmosterol (Cholest-5,24-diene-3β-ol)	5.6 ± 0.1	2.0 ± 0.1
Stigmasterol (Stigmast-5,22-diene-3β-ol)	4.2 ± 0.2	2.9 ± 0.3
Androsterol (Androstan-3β-ol)	15.6 ± 0.1	2.2 ± 0.1
Coprostanol (Coprostan-3β-ol)	< 0.1	3.2 ± 0.1
Epicholesterol (Cholest-5-ene-3α-ol)	< 0.1	3.2 ± 0.1
Epicholestanol (Cholestan-3α-ol)	< 0.1	3.1 ± 0.1
Epiandrosterol (Androstan-3α-ol)	< 0.1	3.6 ± 0.2
None	—	3.1 ± 0.1

[a]Vesicles of [2-(9,10)-^{3}H]dipalmitoyl phosphatidylcholine-egg phosphatidylcholine mixture (molar ratio, 1:4) containing 20 mole % of the various sterols were incubated in quadruplicate for 30 min with lecithin-cholesterol acyltransferase. The extent of labeled palmitoyl ester formation represents the acyl acceptor activity of the sterols, while the amount of labeled palmitic acid released shows the phospholipase A_2-like activity in the presence of sterols. When apolipoprotein A-I was omitted from the assay media, the results obtained did not differ significantly from those obtained with the control assay media.

[b]The values represent the means of the analysis of quadruplicate samples ± standard deviation.

determination of the effectiveness of the transacylation of various unlabeled sterols. The phospholipase A_2-like activity of lecithin-cholesterol acyltransferase was also determined by utilizing phosphatidylcholine alone as a substrate. The rate of the hydrolysis of phosphatidylcholine as determined from the liberation of labeled palmitic acid was also 80% higher than the rate obtained from the phosphorus analysis of lysolecithin produced (Table I).

The comparison of acyl acceptor activity of cholestanol and lathosterol with that of cholesterol revealed that the absence of the double bond in the steroid nucleus, or the presence of a double bond at carbon-7 instead of carbon-5, did not appreciably affect the acceptor activity (Table II). Only a small reduction in acceptor activity was observed with campesterol and β-sitosterol which possess methyl and ethyl groups, respectively, at carbon-24. The presence of a double bond at carbon-24 (desmosterol) or the presence of both a double bond and an ethyl group on the side chain (stigmasterol) reduced the acceptor activity to less than 50% of that of cholesterol. Interestingly, androstan-3β-ol, which lacks a side chain, showed a higher acceptor activity than cholesterol. Coprostanol was completely inactive, indicating the absolute requirement for the planar or *trans* configuration of the A/B rings. Epicholesterol, epicholestanol, and androstan-3α-ol were also not utilized by the enzyme, suggesting that the hydroxyl group at the position 3 must be in the β conformation. None of the sterols tested was esterified in the absence of apolipoprotein A-I. Various sterols differing in the side chain, sterol nucleus, and the configuration of the A/B rings and 3-hydroxyl group, were previously shown to be incorporated in egg phosphatidylcholine vesicles at concentrations considerably greater than that used in the present study (17).

When the substrate contained only phosphatidylcholine, the rate of palmitic acid release was about 3 nmoles/hr/ml (Table II). This hydrolytic activity of the enzyme, manifested by the release of free fatty acids, was reduced in the presence of 20 mole % of sterols. The degree of this reduction was generally greater with sterols exhibiting higher acyl acceptor activities.

DISCUSSION

In order for sterols to exhibit acyl acceptor activity, the following two structural features are absolutely required. (a) The hydroxyl group at carbon-3 must be in the β configuration, as evidenced by the complete lack of acyl acceptor activity shown by epicholesterol, epicholestanol, and androstan-3α-ol, in contrast to the high activity of the corresponding β-hydroxy sterols. (b) The A and B rings must be in the planar, *trans* configuration, since coprostanol, which is the A/B *cis* isomer of cholestanol, did not show acyl acceptor activity. Among sterols that satisfy the above requirements, androstan-

3β-ol, completely lacking a side chain, was found to be more effective than either β-cholestanol or cholesterol. This suggests that the hydrophobic side chain of sterols at the carbon-17 is not required for acyl acceptor activity. Moreover, sterols in which the side chain is more bulky and rigid due to the presence of an additional methyl or ethyl group were less active than cholesterol. It thus appeared that the presence of the side chain, especially when bulky or rigid, tends to interfere with the acceptor activity of sterols. On the other hand, the presence or absence and the location of the double bond in the B ring were not important for effective acyl acceptor activity.

Cholesterol and several other sterols are known to exhibit a condensing effect on phospholipids in the liquid expanded state of monolayers and to reduce the permeability of their bilayers as recently reviewed (18). These effects required not only the presence of a 3β-hydroxyl group and a *trans* A/B configuration but also a side chain at carbon-17 in the sterol molecule (17-19). Although the first two of these structural features were required for acyl acceptor activity, the side chain was not required, and it rather interfered with the transesterification by lecithin-cholesterol acyltransferase, possibly by reducing the mobility of sterols in the vesicles. Therefore, the sterol-phosphatidylcholine interaction which leads to the condensing effect and to the permeability changes, is not a prerequisite for the transesterification reaction. The β configuration of the hydroxyl group and *trans* A/B configuration may be important in providing an optimal orientation of the hydroxyl group for the transacylation at the active site of the enzyme bound to substrate vesicles.

We previously demonstrated the phospholipase A_2-like activity of lecithin-cholesterol acyltransferase (10,11). It is possible that the lecithin-cholesterol acyltransferase reaction proceeds via an acyl-enzyme intermediate. When an effective acyl acceptor, such as cholesterol, is present at the active site with the proper orientation of the hydroxyl group, the acyl group may be transferred mostly to the acyl acceptor. However, a small portion of the acyl enzyme intermediate may always be hydrolyzed, this hydrolysis being at a slower rate in the presence of effective acyl acceptors. The slow rate of fatty acid and lysophosphatidylcholine liberation even in the presence of phosphatidylcholine alone as the substrate suggests a limited accessibility of water molecules to the active site.

Although pure dipalmitoyl phosphatidylcholine as such is a poor substrate of lecithin-cholesterol acyltransferase (6) due to its highly ordered bilayer structure, the mixing with egg phosphatidylcholine at the molar ratio of 1:4 allowed the preferential utilization of the saturated phosphatidylcholine for the transacylation over egg phosphatidylcholine, which is primarily unsaturated at the carbon-2 position. This preferential utilization is in accordance with the results of Glomset et al. (20); their study showed a more effective transfer of the saturated acyl group of the endogenous phosphatidylcholine to cholesterol than of the unsaturated acyl group upon incubation of whole plasma. Apparently, the slow utilization of dipalmitoyl phosphatidylcholine in the absence of unsaturated phosphatidylcholine (6) is due to a strong intermolecular interaction between the saturated acyl groups (21).

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METHODS

Simultaneous Mass Spectrometric Measurement of Prostaglandins E_1 (PGE_1) and PGE_2 with a Deuterated Internal Standard[1]

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ABSTRACT

The use of (3,3,4,4-$^2H_4$)PGE_2 as internal standard for the quantitative measurement of PGE_1 by gas chromatography-mass spectrometry is reported. A method for the simultaneous analysis of PGE_1 and PGE_2 is also described. The prostaglandins were analyzed by selected ion monitoring as the methyl ester-trimethylsilyl ether (ME-TMS) derivatives of PGB_1 and PGB_2, respectively. In all cases, a linear response over the range 1 to 70 ng (2.8 to 198 pmoles) was demonstrated. The use of this procedure is exemplified by its application to the PG analysis of sheep seminal vesicles.

INTRODUCTION

Highly sensitive and specific analytical methods for the analysis of prostaglandins are essential to further the study of their physiological role in animal tissues. Radioimmunoassay (RIA) is sometimes the preferred method because of its sensitivity in the picogram range, the need for little or no preliminary purification of the extracts, and the simplicity of the equipment required. However, the problem of cross reactivity between PGE_1 and PGE_2 and their opposing antisera has not yet been entirely solved. In spite of recent developments in RIA technology (1,2) and its inherent advantages for analysis of large numbers of samples, combined gas chromatography-mass spectrometry (GC-MS) has emerged as the preferred method.

Samuelsson and collaborators (3) first introduced the stable isotope dilution and the selected ion monitoring (SIM) techniques in prostaglandin analysis. In their method, which was used to measure PGE_1, a ($^2H_3$)methoxime analog was the internal standard, and its addition after the derivatization stage was a disadvantage. Later Axen et al. (4) overcame this problem by using (3,3,4,4-$^2H_4$)PGE_2 and (3,3,4,4-$^2H_4$)$PGF_{2\alpha}$ for the analysis of PGE_2 and $PGF_{2\alpha}$, respectively. Because those deuterated molecules can be introduced before isolation and derivatization, they are nearly perfect standards.

In recent important developments in prostaglandin analysis, the use of GC-MS-SIM was extended to other prostaglandins, new derivatives and reagents were introduced, and the use of deuterated species was sometimes abandoned (5-10). Thus, Kelly (8) introduced the use of a 9,11-butylboronate derivative for the analysis of $PGF_{2\alpha}$. Middleditch and Desiderio (9) examined the use of ω-homo-PGE_1 and ω-nor-PGE_2, in place of deuterium-labeled analogs, as suitable internal standards for the analysis of prostaglandins of the E series. The method of Nicosia and Galli (10), who used trimethylsilylimidazole (TSIM) in piperidine, is applicable to all four primary PGs, and was a decisive improvement over existing ones. That method, which affords instant derivatization of PGE_1 and PGE_2 and their concomitant conversion to PGB_1 and PGB_2, respectively, eliminated the two major problems inherent in the oximation procedure, namely long reaction times, and the formation of *(syn-anti)* isomeric pairs of derivatives (4,9,11,12). It also eliminated delicate dehydration procedures sometimes used to convert PGE into PGB with caustic (7). Nicosia and Galli (10) proposed the use of the C_{20} fatty acid methyl ester as internal standard for the measurement of PGE_1. The drawbacks of such a procedure are: (a) methyl arachate cannot function as a carrier too; (b) the linearity range (to 17 ng) is shorter than might sometimes be desirable.

[1]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

[2]Author to whom correspondence should be addressed.

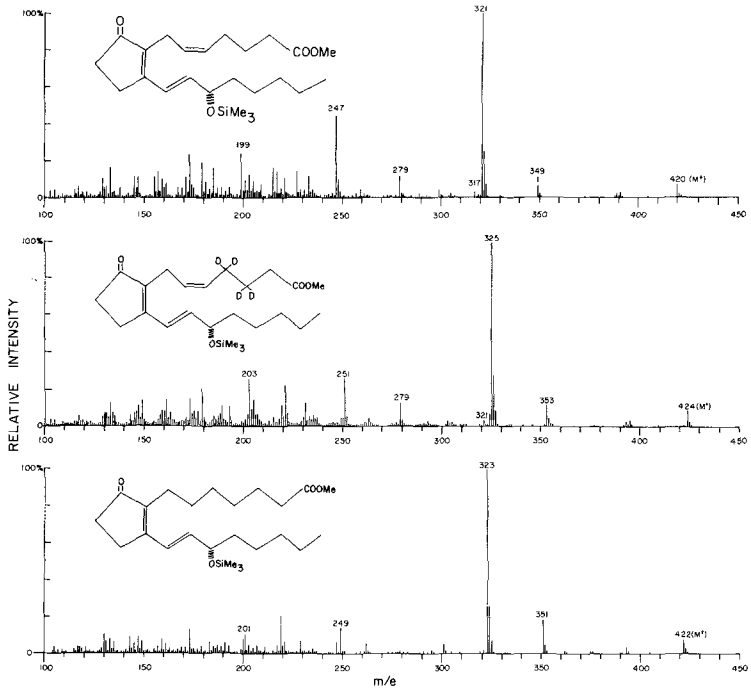

FIG. 1. Quadrupole mass spectra (40 eV) of PGB_2-ME-TMS (upper spectrum), (3,3,4,4-$^2H_4$)PGB_2-ME-TMS (middle spectrum), and PGB_1-ME-TMS (lower spectrum) obtained by derivatization of their PGE counterparts.

We now report the successful use of (3,3,4,4-$^2H_4$)PGE_2 as internal standard and carrier for the measurement of PGE_1. A procedure for the simultaneous analysis of PGE_1 and PGE_2 is also described. When this method is applied to a biological sample, the chromatographic system(s) used for the cleanup must be non-specific toward PGE_1 and PGE_2, i.e., the two species must elute together. We describe the application of the method to the analysis of sheep seminal vesicles.

Recently Perry and Desiderio (13) described the use of ($^2H_4$)PGE_2 as an internal standard to simultaneously quantify PGE_1, PGE_2, 19-hydroxy PGE_1, and 19-hydroxy PGE_2 in seminal fluid. However, their method of collecting and handling data is entirely different from the procedure we used. Some advantages related to accuracy and precision inherent in our procedure will be discussed below.

EXPERIMENTAL

Materials

PGE_1, PGE_2, and (3,3,4,4-$^2H_4$)PGE_2 were provided by J. Pike and U. Axen of the Upjohn Co. TSIM was purchased from Pierce Chemical Co., Rockford, IL. Piperidine was Fisher-certified (Fisher Scientific Co., Fair Lawn, NJ) and freshly distilled. Diazomethane was prepared by the action of KOH on N-methyl-N-nitroso-p-toluenesulfonamide (Aldrich Chemical Co., Milwaukee, WI) and collected in diethyl ether. We observed no adverse effect whatsoever when the reagent was used after storage at -28 C for up to 8 weeks. Lipidex-5000 was purchased from Packard Instrument Co., Downers Grove, IL.

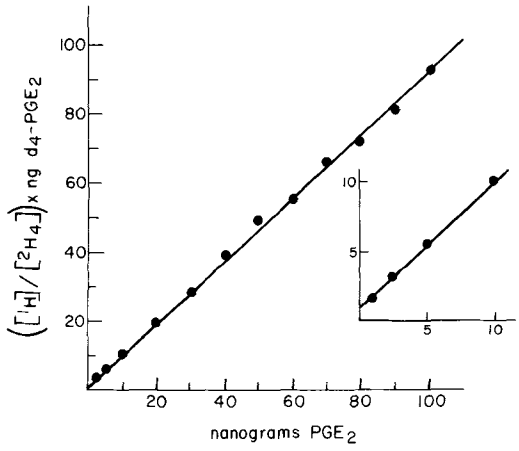

FIG. 2. Ratios of peak heights of (^1H)PGE_2 and 1 ng of ($^2H_4$)PGE_2 vs. amount of (^1H)PGE_2 injected.

TABLE I

Calibration of PGE_2 (See Fig. 2). Response Ratios

(^1H)PGE_2 injected (ng)	$\bar{y}$ ± SEM	n
100	93.4 ± 0.4	5
90	80.6 ± 0.3	4
80	71.2 ± 0.2	4
70	66.1 ± 0.1	4
60	57.6 ± 1.0	7
50	49.5 ± 0.1	4
40	39.5 ± 0.5	4
30	27.7 ± 0.1	4
20	18.30 ± 0.01	4
10	9.78 ± 0.07	5
5	5.62 ± 0.02	4
2.5	3.32 ± 0.03	4
1	1.25 ± 0.03	4

TABLE II

Blank Values for the Ion Triad m/e 321/323/325 from Pure Derivatized PGEs

	Ion pair (m/e)	Blank value[a]
PGB_2	323/321	0.0704 ± 0.0006 (n=8)
	325/321	<0.0025 (n=8)
PGB_1	325/323	0.0719 ± 0.0018 (n=5)
	321/323	0.0311 ± 0.0011 (n=5)
($^2H_4$)PGB_2	323/325	0.0151 ± 0.0007 (n=6)
	321/325	0.0295 ± 0.0004 (n=6)

[a]Means ± SEM.

Preparation of PG Derivatives

All analytical operations were performed in silanized glassware, and evaporations were conducted under a stream of N_2. Methylations were carried out by exposure of the dry residues to excess CH_2N_2 for 10 min. The methyl esters (ME) were converted to the ME-TMS derivatives of their PGB counterparts by action of TSIM in piperidine (1:1) according to a pub-

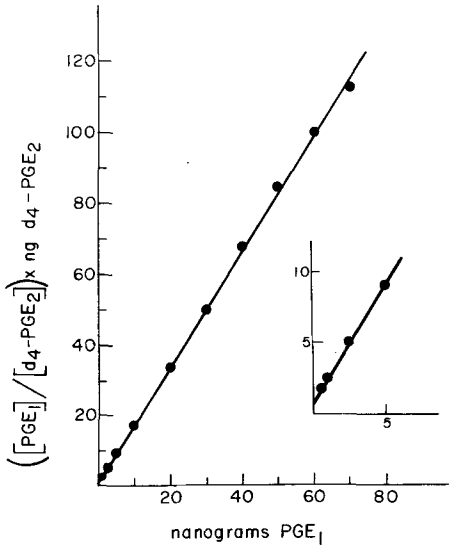

FIG. 3. Ratios of peak heights of PGE_1 and 1 ng of ($^2H_4$)PGE_2 vs. amount of PGE_1 injected.

TABLE III

Calibration of PGE_1 (See Fig. 3). Response Ratios

PGE_1 injected (ng)	$\bar{y}$ ± SEM	n
70	111.9 ± 0.7	4
60	99.4 ± 1.1	5
50	84.2 ± 0.6	4
40	66.8 ± 0.6	5
30	48.5 ± 0.3	4
20	32.7 ± 0.1	4
10	16.3 ± 0.1	4
5	8.97 ± 0.03	4
2.5	4.92 ± 0.01	4
1	2.56 ± 0.01	4
0.5	1.70 ± 0.02	4

lished procedure (10) and analyzed by gas chromatography-mass spectrometry. The derivatized PGs were generally used within a few min, but they remained viable for at least 48 hr when stored in the dark at 4 C.

Analysis of Sheep Seminal Vesicles

Sheep seminal vesicles (240 mg) were ground with a teflon pestle homogenizer in 24 ml of 0.05 M phosphate buffer (pH 7.5). The homogenate was centrifuged at 500 x g at 4 C for 10 min, and the supernatant was incubated at 37 C for 10 min. The pH was then brought to 3.5 by addition of 2 M citric acid, and 8 μg of ($^2H_4$)-PGE_2 was added immediately thereafter. Six 3-ml aliquots of fluid were placed in 6 centrifuge tubes. Exact and increasing amounts of PGE_1(200 to 1000 ng) and PGE_2 (50 to 500 ng) were added to 5 of the 6 tubes. The contents of each tube were then extracted twice with 5 ml of AcOEt. The combined extracts were evaporated and the residues were treated with CH_2N_2. The materials left after evaporation were dissolved in 0.1 ml of heptane/chloroform (7:3) and subjected to straight phase liquid-gel chromatography on a 10 x 200 mm Lipidex-5000 column with the same solvent system. The rate of elution was 15 ml/hr while the temperature was maintained at 25-26 C. The first 70 ml of the eluates were discarded. The following 30-ml fractions, which contained more than 90% of the PG under investigation, were evaporated, and the residues were treated with TSIM and analyzed with the GC-MS system. The amounts of PGE_2 and PGE_1 in each sample were determined from standard curves.

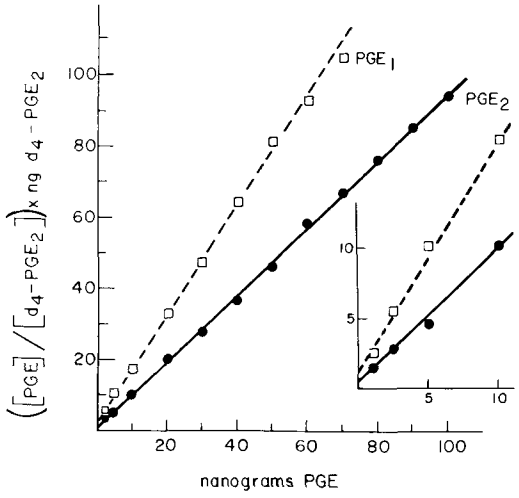

FIG. 4. Ratios of peak heights of PGE_1 and PGE_2 and 1 ng of ($^2H_4$) PGE_2 vs. amount of PGE_1 and PGE_2 injected in simultaneous measurements.

Instrumentation

The GC-MS system we used was a Finnigan 3200F with an all-glass jet molecular separator. The mass spectrometer was operated at 40 or 70 eV, and the continuous dynode electron multiplier was set at 2.3 kV. The U-shaped glass columns, 1.52 m long x 2 mm i.d., were treated with dimethylchlorosilane and packed with 1% OV-101 on 100/120 mesh Gas Chrom Q. Temperatures were: injector, 235 C; column, 225 C; separator, 230 C. The carrier gas (He)

TABLE IV

Simultaneous Calibration (See Fig. 4). Response Ratios

(^1H)PGE_2 and PGE_1 injected (ng each)	$\bar{y}$ ± SEM		n
	PGE_2	PGE_1	
100	94.4 ± 0.7	a	4
90	84.4 ± 0.7	a	4
80	76.4 ± 1.0	a	6
70	67.2 ± 0.4	104.6 ± 0.8	4
60	58.3 ± 0.1	92.6 ± 1.0	4
50	46.0 ± 0.4	81.6 ± 0.9	4
40	36.0 ± 0.2	63.8 ± 0.3	4
30	26.6 ± 0.3	46.6 ± 0.8	6
20	20.1 ± 0.1	33.3 ± 0.3	4
10	10.15 ± 0.22	17.8 ± 0.1	4
5	4.70 ± 0.08	10.05 ± 0.39	4
2.5	2.76 ± 0.08	5.65 ± 0.03	4
1	1.31 ± 0.03	2.37 ± 0.06	5

[a]Nonlinear area.

was supplied at a head pressure of 1.26 kg/cm^2 and the flow rate was about 20 ml/min. A manually operated effluent diverter was opened 75 sec after each injection. A standard PROMIM (Finnigan) modular electronic programmer was used for selected ion monitoring.

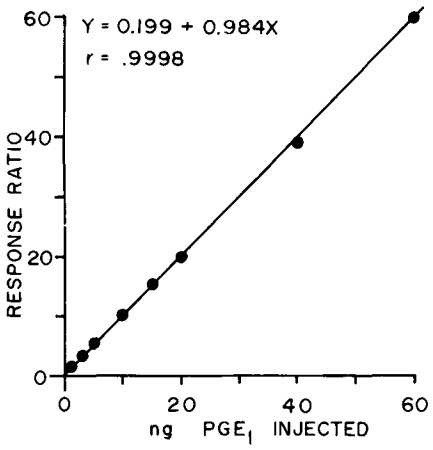

FIG. 5. Calibration line for PGE_1.

TABLE V

Effect of Reconditioning of the Ion Source. Calibration of PGE_1 (see Fig. 5). Response Ratios

PGE_1 injected (ng)	$\bar{y}$ ± SEM[a]
60	59.8 ± 0.4
40	38.8 ± 0.2
20	19.7 ± 0.1
15	15.1 ± 0.1
10	9.78 ± 0.06
5	5.21 ± 0.02
3	3.30 ± 0.03
1	1.45 ± 0.01

[a] n = 5.

The ions selected to monitor the prostaglandins were: m/e 321 for PGE_2, m/e 323 for PGE_1, and m/e 325 for $(^2H_4)PGE_2$. The signal from each selected m/e value was displayed on a Rikadenki multichannel pen recorder. Calibration and recovery curves were constructed from peak height measurements.

RESULTS

The Method

Figure 1 shows the quadrupole mass spectra of the ME-TMS derivatives of PGB_2, (3,3,4,4-$^2H_4$)PGB_2, and PGB_1 obtained by derivatization of their PGE counterparts as described under "Experimental."

In their excellent article, Sweetman et al. (7) reported that a "blank" can arise from the use of a large excess of the deuterated analog of PGE_2. Such a "carrier blank" would result from the presence of some protium form that would register in the protium (m/e 321) channel. Accordingly, those authors suggested use of the smallest possible amount of carrier. They also were first to call attention to a "fragmentation blank" at m/e 321, which occurred with $(^2H_4)PGB_2$-ME-TMS, in addition to the major ion at m/e 325. This m/e 321 ion is analogous to that at m/e 317 in the protium compound. Those two ions are clearly visible in the spectra of derivatized $(^2H_4)PGB_2$ (m/e 321) and PGB_2 (m/e 317) (Fig. 1), and in both they are about 3% of their respective base peaks. When we determined a calibration curve for PGE_2, with $(^2H_4)PGE_2$ as standard, we substracted the contribution of the labeled compound to the m/e 321 channel from the observed peak height at m/e 321. Figure 2 shows a typical calibration curve with a constant amount (50 ng) of $(^2H_4)PGE_2$. The resultant best fitting straight line has a linear regression coefficient of 0.914

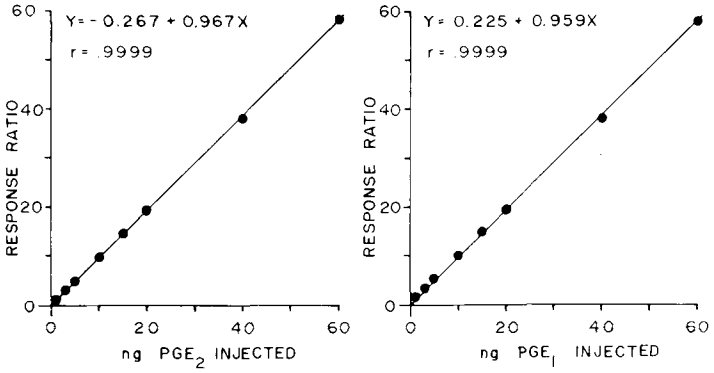

FIG. 6. Simultaneous calibration lines for PGE_1 and PGE_2 obtained when equal amounts were injected.

TABLE VI

Effect of Reconditioning of the Ion Source. Simultaneous Calibration (See Fig. 6). Response Ratios

(^{1}H)PGE_2 and PGE_1 Injected (ng each)	$\bar{y}$ ± SEM[a]	
	PGE_2	PGE_1
60	58.0 ± 0.1	58.1 ± 0.2
40	38.0 ± 0.1	38.1 ± 0.2
20	19.0 ± 0.1	19.2 ± 0.1
15	14.40 ± 0.03	14.76 ± 0.06
10	9.46 ± 0.02	9.71 ± 0.07
5	4.49 ± 0.03	4.98 ± 0.02
3	2.68 ± 0.01	3.16 ± 0.01
1	0.74 ± 0.01	1.40 ± 0.04

[a]n = 5.

and an intercept of 0.96. The mean values of the response ratios ($\bar{y}$) and the number of injections are listed in Table I.

When PGE_1 is measured with ($^2H_4$)PGE_2 as the internal standard, the situation is further complicated by the presence of the silicon satellites, specifically of a relatively intense ion (ca. 7% of the base peak) at m/e 325 in the spectrum of PGB_1-ME-TMS (Fig. 1). In SIM, this ion, of course, registers in the d_4 channel. Consequently, the gas chromatographic conditions must insure that the elution times of the two species either are sufficiently apart for SIM analysis without interference, or are coincident. In the former case, cross contributions can be ignored; in the latter, they must be fully taken into account. We found that coincidence is easier to attain than separation if the elution time is to be kept relatively short. The average elution times from several determinations at 225 C were as follows: 368 sec for ($^2H_4$)PGB_2-ME-TMS and PGB_2-ME-TMS, and 373 sec for PGB_1-ME-TMS. In general, cross contributions may result not only from the Si satellites but also from "fragmentation blanks" of the type described by Sweetman et al. (7). The elemental compositions and structures of such contributing ions can be determined only by high-resolution mass spectrometry which so far has not been applied to the PG derivatives under consideration. Here, however, we must be concerned with total peak heights (or areas) at nominal masses. For this reason, we decided to

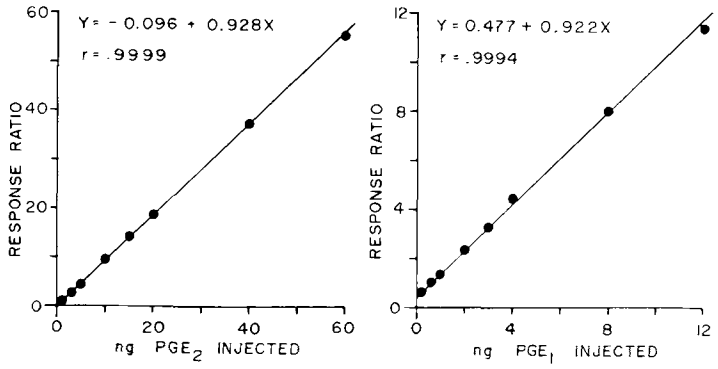

FIG. 7. Simultaneous calibration lines for PGE_1 and PGE_2 obtained when amounts of PGE_2 injected were systematically five times larger than those of PGE_1.

TABLE VII

Effect of Reconditioning of the Ion Source. Simultaneous Calibration (See Fig. 7). Response Ratios

(^{1}H)PGE_2		PGE_1	
ng Injected	$\bar{y}$ ± SEM[a]	ng Injected	$\bar{y}$ ± SEM[a]
60	55.3 ± 0.3	12	11.34 ± 0.05
40	37.3 ± 0.1	8	8.00 ± 0.05
20	18.6 ± 0.1	4	4.41 ± 0.03
15	14.02 ± 0.02	3	3.20 ± 0.01
10	9.36 ± 0.04	2	2.28 ± 0.02
5	4.26 ± 0.01	1	1.30 ± 0.01
3	2.54 ± 0.03	0.6	1.00 ± 0.01
1	0.75 ± 0.01	0.2	0.64 ± 0.01

[a]n = 5.

determine the blank values for the ion triad m/e 321/323/325 using pure PGB derivatives under closely controlled experimental conditions (see below). The results are shown in Table II.

In the present study, when PGE_1 was measured with $(^2H_4)PGE_2$ as standard, the blank value for the ion pair m/e 325/323 (Table II) was used to calculate the contribution of PGE_1 to the m/e 325 channel. This contribution was subtracted from the peak heights of the m/e 325 ion. The results are shown in Figure 3. The best fitting straight line has a linear regression coefficient of 1.623, and an intercept of 0.81. The constant amount of $(^2H_4)PGE_2$ injected was 40 ng. The linearity is limited to 70 ng, beyond which the curve bends. The mean values of the response ratios ($\bar{y}$) and the number of injections are shown in Table III.

Simultaneous determination of PGE_1 and PGE_2 by use of the set of ions at m/e 321, m/e 323 and m/e 325 in SIM is possible if the GC operating parameters allow simultaneous elution of the three species, and all relevant blanks are taken into consideration. We used the blank values of the ion pairs m/e 323/321, m/e 325/323, m/e 321/323, and m/e 321/325 (see Table II) in that sequence. Identical amounts of PGE_1 and PGE_2 were injected. The results are shown in Figure 4. Least-square analysis of the data points from this simultaneous calibration of PGE_2 and PGE_1 gave linear regression lines with practically the same slope coefficients as those from separate determinations. Table IV shows the mean values of the response ratios ($\bar{y}$) relative to this simultaneous calibration.

Relative ion abundances in a mass spectrum are functions of the residence times of the various ions, which in turn depend on instrumental parameters (14). Because the fidelity of the data in SIM work rests on the accurate and reproducible measurements of peak heights, the GC-MS conditions used to collect data for the calibration curve must be reproduced during the analysis of the biological materials. This important point is illustrated by Figure 5 and Table V which show calibration data for PGE_1 obtained after reconditioning of the ion source. Figure 6 and Table VI show results of a simultaneous calibration of PGE_2 and PGE_1 conducted under identical instrumental conditions. Equal amounts of the two species were injected in this experiment. Whereas the slopes in the PGE_2 plots did not change significantly (compare with Figs. 2 and 4), thanks to chemical identity between PGE_2 and the internal standard, the slopes in the PGE_1 plots decreased. We attribute this phenomenon to reconditioning of the ion source. Clearly, new standard curves must be obtained each time the ion source parameters are changed.

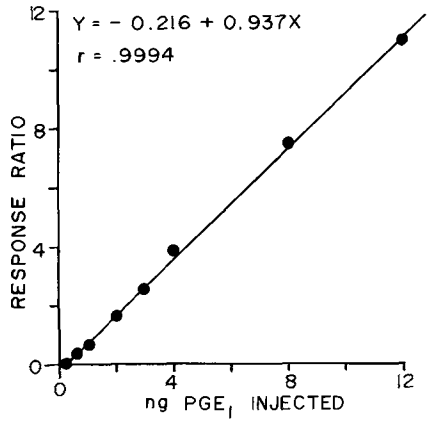

FIG. 8. Simultaneous calibration line for PGE_1 using response ratios corrected for the m/e 323/325 blank.

TABLE VIII

Effect of Reconditioning of the Ion Source. Simultaneous Calibration (See Fig. 8). Corrected Response Ratios

PGE_1 injected (ng)	$\bar{y}$ ± SEM[a]
12	10.95 ± 0.05
8	7.41 ± 0.05
4	3.80 ± 0.03
3	2.52 ± 0.01
2	1.63 ± 0.02
1	0.65 ± 0.01
0.6	0.32 ± 0.01
0.2	-0.04 ± 0.01

[a]n = 5.

Figure 7 and Table VII display data from a simultaneous calibration where the ratio of protium PGE_2 to PGE_1 injected was systematically 5 to 1. The high intercept in the PGE_1 plot is due to the m/e 323/325 blank which becomes important because of the relatively low levels of PGE_1 being measured. When the appropriate peak height correction was introduced, the "normal" curve of Figure 8 was obtained (see Table VIII).

Analysis of Sheep Seminal Vesicles

Straight phase liquid-gel chromatography with Lipidex-5000 afforded simultaneous elution of PGE_2 and PGE_1, and proved a satisfactory tool for cleanup of the biological extract as documented by the recovery curve shown in Figure 9. Table IX shows mean PG amounts calculated from five injections, and

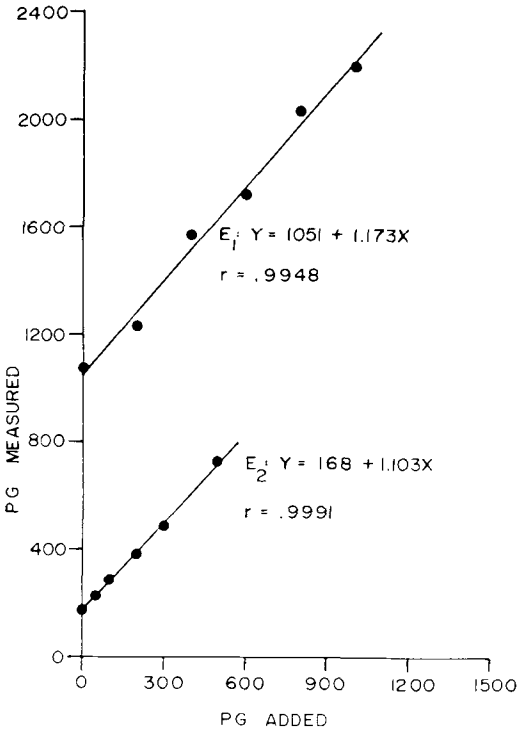

FIG. 9. Prostaglandin recovery from incubated sheep seminal vesicles.

TABLE IX

Recovery of PGE_2 and PGE_1 from Incubated Sheep Seminal Vesicles

ng PGE_2		ng PGE_1	
Added	Measured[a]	Added	Measured[a]
0	169.8 ± 1.2	0	1071.8 ± 13.3
50	226.0 ± 1.3	200	1228.4 ± 4.3
100	285.6 ± 1.7	400	1572.8 ± 10.7
200	378.2 ± 1.6	600	1720.1 ± 9.1
300	487.7 ± 1.4	800	2034.3 ± 19.4
500	728.3 ± 3.1	1000	2202.8 ± 19.2

[a]Means ± standard errors (n = 5).

the standard errors are relative to the GC-MS analysis.

DISCUSSION

Murphy et al. (15) showed that precision and accuracy are lost when the mass interval is large (several a.m.u.) between the ions used for SIM. In their work, with a 14 a.m.u. interval, the losses of accuracy and precision were severe. We therefore believe that, in this respect, our method of quantitative measurement of PGE_1 with $(^2H_4)PGE_2$ as the internal standard is superior to the method with ω-homo-PGE_1 as the internal standard for which the interval between the selected ions is 14 a.m.u. (9).

Some authors (9,13) implied that simultaneous elution is not necessary for SIM work, but we think that such chromatographic behavior is desirable in dealing with very small amounts of easily degradable materials like the PGs. Murphy et al. (15), on the basis of their studies, concluded that large differences in elution times between the standard and the species to be measured can also lead to loss of accuracy and precision, possibly due to time-dependent variations in ionization conditions or ion detection sensitivity. Furthermore, differences in elution times may result in different degrees of absorption of the biological compound and the internal standard. Our method, which is based on the simultaneous elution of PGE_1 and $(^2H_4)PGE_2$, is not subject to such potential sources of error. Thus, we extended the linearity range from that afforded by the use of methyl arachate as internal standard (10), 1 to 17 ng for PGE_1, to 1 to 70 ng. That extension might be attributed to the fact that PGB_1-ME-TMS and $(^2H_4)PGB_2$-ME-TMS have essentially identical elution times.

In conclusion, our study shows that both prostaglandins of the E series can be simultaneously quantified by use of $(^2H_4)PGE_2$ as the internal standard, if the GC conditions allow concurrent elution of the standard and the species to be measured, and if the cross contributions (blanks) are determined and properly subtracted. A nonspecific chromatographic system must be used for cleanup of biological extracts. This study demonstrates that Lipidex-5000 is a satisfactory tool to that effect. Chemical reactivity and partition coefficients of PGE_1 and $(^2H_4)PGE_2$ can safely be assumed to be identical. On this basis, $(^2H_4)PGE_2$ is a nearly ideal carrier/internal standard.

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A Rapid Sensitive Method for Determining Phospholipid Phosphorus Involving Digestion with Magnesium Nitrate

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ABSTRACT

A method is described for the rapid determination of phospholipid phosphorus in samples containing less than 0.5 μg phosphorus. Phospholipid phosphorus is first converted to inorganic phosphate by heating a dried lipid extract briefly over a Bunsen flame in the presence of magnesium nitrate, then dissolving the resulting residue in dilute hydrochloric acid at 95 C. The determination of the inorganic phosphate content of the sample then requires only the addition of Triton X-100 and an acidic ammonium molybdate-malachite green reagent. Absorbance is measured at 650 nm after 5 min at room temperature.

INTRODUCTION

For many years, phospholipid phosphorus has been determined in this laboratory by digesting lipid extracts with perchloric acid, then assaying inorganic phosphate (in the range 0-5 μg P) by a modification of the method of Chen et al. (1,2). The present method was developed to satisfy our requirement for a convenient, less time-consuming procedure with sensitivity in the range 0-0.5 μg P for a specific clinical application (2).

Phospholipid digestion can be achieved in a few seconds by ashing over a Bunsen flame in the presence of magnesium nitrate (3). This rapid digestion procedure has been coupled with a modification of a sensitive method for determining inorganic phosphate, based on the formation of a complex between phosphomolybdate and the basic dye, malachite green (4).

MATERIALS AND METHODS

All solutions were prepared from analytical grade reagents, unless otherwise stated, using glass distilled water. Because of the extreme sensitivity of the method, special care is required in the washing of glassware. All glassware was immersed overnight in conc. sulphuric acid containing ca. 5% conc. nitric acid, then rinsed thoroughly with tap water and glass distilled water.

Digestion of Phospholipid

A sample of washed lipid extract (up to 4 ml) containing $\leqslant$ 0.5 μg phosphorus was mixed with 30 μl of magnesium nitrate solution, 10% (w/v) $Mg(NO_3)_2.6H_2O$ in methanol, in a 17 mm test tube and evaporated to dryness by heating in a water bath at 85-100 C in a fume hood. Larger volumes were sometimes used, but care was necessary to avoid losses due to bumping of the solvent.

Digestion was achieved by heating the sample for 15 seconds in the top portion of a Bunsen flame, then lowering the tube so that its base was at the tip of the blue cone of the flame and heating steadily for a further 10 seconds. Because the toxic gas nitrogen dioxide is released during this procedure, both the digestion and subsequent cooling of the tubes should be carried out in a fume hood.

After brief cooling (to less than 100 C), 1 ml 1 M HCl was added. The tube was covered with a glass marble to minimize evaporation and heated together with suitable standards at 90-95 C for 15 min, then cooled to room temperature by immersion in tap water before proceeding with the determination of inorganic phosphate. Standards containing 0-0.5 μg inorganic phosphate-phosphorus (KH_2PO_4) in 1 ml M HCl were prepared in tubes in which 30 μl of magnesium nitrate solution had previously been evaporated to dryness and "digested" as described above.

Determination of Inorganic Phosphate

The following reagents were prepared.

A. 4.2% (w/v) ammonium molybdate in HCl: conc. HCl was diluted to 4.5 M, then used to dissolve the molybdate, $(NH_4)_6\,Mo_7O_{24}.4H_2O$.

B. 0.3% (w/v) malachite green solution: B.D.H. product No. 34045, color index 42000, was dissolved in distilled water just before use.

C. Dye-molybdate reagent: reagent A (1 vol.) was mixed with reagent B (3 vol.) and left at room temperature for 2-3 hr with occasional mixing, before filtering through Whatman No. 1 filter paper. The reagent is stored at room temperature and is stable for 3-5 weeks. A small amount of precipitate may form after 3 weeks. The

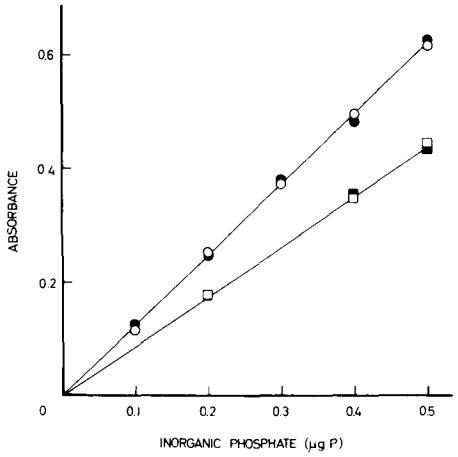

FIG. 1. Standard curves for inorganic phosphate prepared by the routine procedure (○,●) as described in the text, or by the method of Itaya and Ui (4) (□,■) in a final reaction volume of 3.1 ml. Mg^{2+} was included in some tubes (●,■), as described in the text. Absorbance was measured at 650 nm (○,●) or 660 nm (□,■).

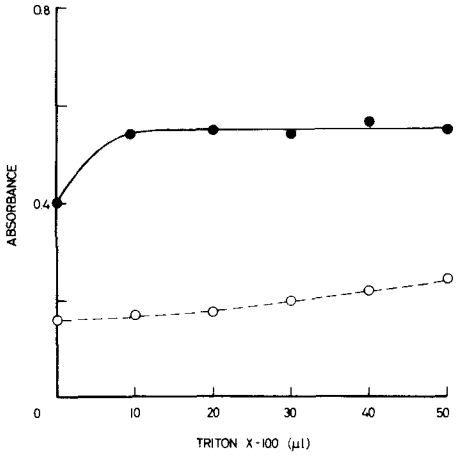

FIG. 2. The effect of Triton X-100 on absorbance in the determination of inorganic phosphate by the routine procedure; 0-50 μl of a 2% solution of Triton X-100 was used. The absorbance at 650 nm of both the reagent blank (○), and a 0.5 μg P standard (●) corrected for the reagent blank are shown. Mg^{2+} was included in all samples.

reagent is still usable provided that the precipitate is removed by filtration, but should be discarded when further precipitation occurs.

D. Triton X-100 (Rohm and Haas): a 1% (w/v) solution in distilled water. This reagent must be refrigerated. It should be replaced every 3 months or at the first sign of cloudiness.

In *the routine procedure,* 30 μl of reagent D was added to 1 ml of test solution or standard containing up to 0.5 μg P, as inorganic phosphate, in 1 ml 1 M HCl. Reagent C (2 ml) was added with immediate mixing and the color was allowed to develop at room temperature (15-25 C) without further mixing. The absorbance at 650 nm was measured 5-40 min after the addition of the color reagent using a Unicam SP800 or a Turner Model 350 spectrophotometer. In some experiments, 25 μl of 2% Triton X-100 was used, and the absorbance was measured 5-20 min after the addition of the color reagent. Because the dye-phosphomolybdate complex tends to absorb to glass (5,6), the use of disposable plastic cuvettes (Ratiolab) is recommended.

Preparation of Lipid Extracts

The liver of an adult female Wistar rat was homogenized in 0.9% NaCl (5 ml/g liver) and a portion was extracted with 20 vol chloroform/methanol (2:1, v/v). The extract was washed to remove nonlipid phosphorus (7). Washed lipid extracts were prepared in the same way from samples of human amniotic fluid, collected by amniocentesis during late pregnancy as part of routine clinical management. The extracts were made to a known volume with chloroform/methanol (1:1, v/v).

RESULTS

The Standard Curve and Absorption Spectrum

The routine procedure gave a linear standard curve up to 0.5 μg P (Fig. 1). Beyond 0.5 μg P, there was a gradual departure from linearity, the absorbance for 0.7 μg P being about 8% lower than that predicted by extrapolation of the standard curve.

Standard curves obtained by the recommended procedure and the original method of Itaya and Ui (4) are compared in Figure 1. For the recommended procedure, the molar extinction coefficient of the dye-phosphomolybdate complex was 115,000. In this laboratory, the molar extinction coefficient of 96,000-123,000 reported by Itaya and Ui (4) was not achieved using their method; the data in Figure 1 indicate a molar extinction coefficient of 84,000 for the Itaya-Ui method. In the recommended procedure, the formation of the dye-phosphomolybdate complex caused a shift in absorption maximum from 625 nm to 640-650 nm. The absorption spectrum of the complex, which was not affected by the inclusion of Mg^{2+}, was similar to that reported for the original method (4).

TABLE I

The Effect of Triton X-100 on the Stability of the Color[a]

Time	Triton X-100 (μl)		
	15[b]	25	50
min	%	%	%
5	98 ± 2	100	100
10	99 ± 1	98	97
20	100 ± 0	96	95
40	96 ± 0	92	89
60	95 ± 2	88	85
120	88 ± 1	86	71

[a]For each time point, a reagent blank and a standard (0.5 μg P) were prepared in tubes in which 30 μl of 10% magnesium nitrate solution had been ashed. After the addition of 2% Triton X-100 and color reagent, the tubes were incubated at 20 C for the time indicated before measuring absorbance at 650 nm. The absorbance is expressed as a percentage of maximal absorbance at each concentration of Triton X-100.

[b]Each result represents the mean of 2 independent experiments ± range.

In the present method, the final concentration of ammonium molybdate is lower than in the Itaya and Ui method (4). Increasing the concentration of molybdate in the color reagent to provide the same final concentration as in the original method did not affect the sensitivity of the method but reduced the stability of the color reagent.

Triton X-100 and the Stability of the Color

The inclusion of 10-50 μl of a 2% solution of the nonionic detergent Triton X-100 in the reaction mixture caused a 37-38% increase in sensitivity, with negligible increase in the reagent blank provided that less than 30 μl of Triton X-100 was used (Fig. 2). In the original method of Itaya and Ui (4), the detergent, Tween 20, ← added to the reaction mixture after the color reagent, served to stabilize the color; if added before the color reagent, it inhibited color formation. In the present method, the absorbance and stability were the same whether the Triton X-100 was added before or after the color reagent.

Increasing the concentration of Triton X-100 beyond the optimal level had a detrimental effect on the stability of the color (Table I). When 50 μl of 2% Triton X-100 was used, the dye-phosphomolybdate complex began to precipitate within 120 min. Stability was also reduced if the samples were mixed vigorously after color development was complete. Optimal sensitivity and stability were achieved with 15 μl of 2% Triton X-100 solution was used (Fig. 2, Table I). Under these conditions, the absorbance of both the reagent blank and standard were virtually constant for 5-40 min after the addition of the color reagent. To enhance pipetting accuracy, it is recommended that 30 μl of a 1% solution of Triton X-100 be used routinely.

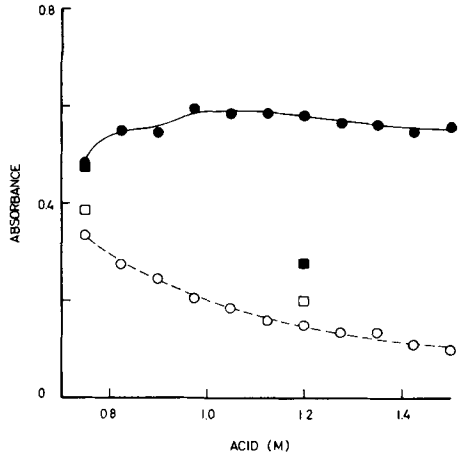

FIG. 3. The effect of acid on absorbance in the determination of inorganic phosphate by the routine procedure (○,●) and in the presence of Tween 20. The concentration of HCl in the sample was varied so that the acid concentration indicated was achieved in the final reaction mixture. In some samples (□,■), Triton X-100 was omitted and Tween 20 at the concentration recommended by Itaya and Ui (4) was added after the color reagent. The absorbance at 650 nm of both the reagent blank (○,□), and a 0.5 μg P standard (●,■) corrected for the reagent blank are shown. Mg^{2+} was included in all samples.

The Effect of Acid Concentration

In the routine procedure, the HCl in the dye-molybdate reagent itself produces a final acid concentration of 0.75 M. The effect on absorbance of varying the final acid concentration from 0.75-1.5 M by the addition of HCl to the 1 ml sample is shown in Figure 3. Maximal absorbance was achieved at a final acid concentration of 1.0-1.2 M; the final acid concentration in the routine procedure is ca. 1.1 M. Further tests were carried out at final concentrations of 1 or 1.2 M HCl. It was found that the final acid concentration could be varied within these limits without significantly affecting the sensitivity or linearity of the method or the stability of the color. However, as the absorbance of the reagent blank is dependent on acid concentration (Fig. 3), it is important that the final acid concentration be carefully controlled within a given batch of samples and standards.

When Tween 20, at the concentration suggested by Itaya and Ui (4), was used in the

TABLE II

The Effect of Digestion Time and Glassware on the Determination of Phospholipid Phosphorus[a]

Digestion procedure[b]		Apparent P content			
		Batch 1[c]		Batch 2[c]	
Step 1	Step 2	Mean	S.D.	Mean	S.D.
sec	sec	μg		μg	
10	5	0.437	± 0.017	0.404	± 0.009
15	10	0.432	± 0.006	0.405	± 0.010
15	30	0.426	± 0.010	0.404	± 0.005

[a]Replicate samples of a washed lipid extract of amniotic fluid were digested under various conditions. Each result represents the mean of three determinations.

[b]Digestion was carried out as described in the Materials and Methods section. Step 1 represents the first stage of digestion at the top of the Bunsen flame; step 2 represents the second stage of digestion at the top of the blue cone of the flame.

[c]Each batch was set up in test tubes supplied by a different manufacturer. Standards and the reagent blank were prepared in test tubes of the type used for batch 1.

present method instead of Triton X-100, comparable results were obtained in the absence of added HCl, but there was a marked loss of sensitivity when the final acid concentration was increased to 1.2 M (Fig. 3).

The Effect of Temperature

The rate of formation of the color, its stability and the maximum absorbance for 0.5 μg P were virtually identical at 15, 20 or 25 C. The reaction can, therefore, be safely carried out at room temperature in most laboratories. Since brief warming at 37 C was shown to cause instability of color, all samples and standards must be at room temperature when the color reagent is added.

The Choice of Dye

It is recommended that malachite green be purchased from the source indicated. When malachite green from another manufacturer was used, there was a 28% loss of sensitivity in the present method, while the Itaya-Ui method became unusable because of loss of both linearity and sensitivity.

The Digestion Procedure

An early report of this procedure indicated that digestion takes only a few seconds and that subsequent acid hydrolysis is necessary to convert any pyrophosphate formed during digestion into inorganic phosphate, but provided no supporting data (3). The procedure was, therefore, tested to determine optimal conditions for the present purpose.

Digestion time. Three digestion procedures were compared, with special attention being given to the position of the tube in the Bunsen flame (Table II). Although digestion was complete within 15 sec, the 25 sec procedure is recommended, since carbon sometimes remained in samples containing a large amount of organic material if a shorter digestion time was used.

Glassware. The data in Table II emphasize the need for caution in the choice of glassware, as pointed out in the original report of the magnesium nitrate digestion procedure (3). Interfering substances arising from the glassware may contribute significantly to the apparent P content of the sample. This problem, which persits even after repeated washing and reusing of the tubes, is adequately corrected for by standardizing on one brand of glassware and by preparing the reagent blank and standard curve in tubes in which magnesium nitrate has been "digested."

Acid treatment. The data for method A in Table III indicate that the P content of some phospholipid samples would be variably underestimated if the acid treatment of the digested residue were omitted, a finding consistent with the suggestion that some inorganic pyrophosphate is formed during digestion (3). The mean difference between all treated and untreated samples was only 4%, but for individual samples a maximum difference of 9% was observed for both liver and amniotic fluid extracts. The acid treatment also ensures that the digested residue is completely solubilized before the addition of the color reagent.

A Comparison of the Present Method with Another Method for Determining Phospholipid Phosphorus

The present method for determining phospholipid phosphorus was compared with an alternative method which had been used routinely in this laboratory for many years. The latter procedure involves digestion of the phospholipid by heating for 1½ hr with perchloric acid before determining inorganic

TABLE III

A Comparison of Two Methods for the Determination of Phospholipid Phosphorus

Source of lipid extract[a]	Expt. No.	Method A[b] Not heated mean ± S.D.	Method A[b] Heated 95 C mean ± S.D.	Method B[c] mean ± S.D.
		μg P/100 ml	μg P/100ml	μg P/100ml
Liver	1	13.2 ± 1.2	12.7 ± 0.7	13.1 ± 0.4
	2	21.4 ± 1.5	23.4 ± 0.3	23.3 ± 1.1
	3	31.3 ± 0.2	31.9 ± 0.6	33.4 ± 0.5
	4	39.1 ± 0.8	37.7 ± 1.4	36.1 ± 0.6
	5	42.9 ± 0.5	45.4 ± 0.1	45.0 ± 0.8
Amniotic fluid	2	9.7 ± 0.3	9.9 ± 1.1	11.3 ± 0.6
	3	19.3 ± 0.6	21.0 ± 0.5	19.9 ± 0.5
	4	20.7 ± 0.2	20.4 ± 0.5	20.3 ± 0.7
	5	45.5 ± 0.5	49.1 ± 0.9	47.2 ± 0.5
	6	48.0 ± 2.0	51.5 ± 1.0	49.4 ± 0.8

[a]Extracts from rat liver were assayed at different dilutions corresponding to 12-36 mg liver/100 ml extract. Five different samples of amniotic fluid were extracted; 100 ml extract was derived from 6.25 ml amniotic fluid in each case.

[b]For each determination, six 0.8 ml samples were assayed by the routine procedure, as described in the text. For 3 of the samples, the incubation at 95 C after the addition of HCl was omitted.

[c]For each determination, three 4 ml samples were evaporated to dryness and digested with 70% perchloric acid at 180 C for 1½ hr. Inorganic phosphate was determined by a modification of the method of Chen et al. (1,2).

phosphate by a method based on the reduction of phosphomolybdate by ascorbic acid (1,2). Washed lipid extracts from rat liver or human amniotic fluid were assayed in triplicate by both procedures, the sample size being adjusted to allow for the ca. 5-fold difference in sensitivity of the two methods (Table III). Comparison of data in column 2 (Method A, heated 95 C) and column 3 (Method B) of Table III indicates that there is satisfactory agreement between the two methods.

DISCUSSION

Several methods for the determination of phospholipid phosphorus making use of malachite green for the determination of inorganic phosphate have already been described (5, 8-11). In every case, perchloric acid or a mixture of sulphuric and perchloric acids was used for digesting the phospholipid. Digestion with 70% perchloric acid at 180 C may take 60 min or longer (C.G. Duck-Chong, unpublished data). The addition of sulphuric acid accelerates the process, but complete digestion may still take up to 50 min (11). (A shorter digestion procedure involving heating with perchloric and sulphuric acid over a Bunsen flame (9) is considered undesirable because of the risk of explosion). The digestion procedure of Ames (3) used in the present method takes no more than 20 min, including the 10-15 min acid treatment at 95 C, and therefore offers a considerable saving of time.

For the determination of inorganic phosphate, the use of Triton X-100 in the present method, instead of Tween 20 as in the original method (4), increases the sensitivity of the method (Fig. 1) and provides an optimal acid concentration in a region which readily accommodates the additional acid required for the hydrolysis of pyrophosphate formed during digestion (Fig. 3). An additional advantage is the rapid rate at which a plateau of absorbance is reached in both samples and reagent blanks on addition of the color reagent (Table I). In this laboratory, the absorbance of both reagent blanks and samples processed by the original method (4) continued to increase for more than 20 min, whether Tween 20 was added immediately after the color reagent or after a delay of several minutes.

Other modifications of the original method have been described for the determination of inorganic phosphate. However, for the purposes of this laboratory, they are considered to be less satisfactory than the present method for one or more of the following reasons: (a) they are less sensitive (12-14), (b) the rate of color development is slower (12,13,15,16), or (c) the color reagent is less stable (11,13). The method described in this paper is recommended for any application where the speed of processing is an important consideration. Since the digestion procedure of Ames (3) is suitable for the determination of phosphorus in a wide variety

of biological materials, the present method may have a more general application in the determination of organic phosphorus in compounds other than phospholipids.

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COMMUNICATIONS

Desaturation of Eicosa-11,14-Dienoic Acid in Human Testes

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ABSTRACT

The metabolism of [1-^{14}C]eicosa-11,14-dienoic acid was investigated in human testes using whole tissue minces and microsomal preparations. Both types of preparations catalyzed the desaturation of the labeled diene to eicosa-8,11,14-trienoic as well as eicosa-5,11,14-trienoic acid. The reported results, therefore, indicate that human testicular tissue, as well as rat testicular tissue (reported previously), is capable of utilizing eicosa-11,14-dienoic acid as a precursor of arachidonic acid. Since it is known that there is no Δ^8 desaturase activity in rat liver and brain, these studies support the concept that there is a tissue variation in this enzymatic pathway.

INTRODUCTION

The lipids of human testes contain a large proportion of polyunsaturated fatty acids, including arachidonic acid (1). Although it has been demonstrated that human testes can synthesize polyunsaturated fatty acids from labeled precursors (2), the particular pathways used for the biosynthesis of polyunsaturated fatty acids such as arachidonate have yet to be established in this tissue. Biosynthesis of arachidonate is generally thought to proceed through the intermediates octadeca-6,9,12-trienoic and eicosa-8,11,14-trienoic acids (3), although an alternative pathway in which linoleic is first elongated to eicosa-11,14-dienoic acid before desaturation to eicosa-8,11,14-trienoic and finally to arachidonic acid has been proposed (4). The latter pathway is inoperative in rat liver (5) and rat brain (6) but may be functional in rat testis (7) as well as human normal and cancerous colonic, bladder, and ovarian tissue (8). Thus, there is apparently a variation among tissues in Δ^8 desaturase activity, and there may also be a variation among species. Since evidence had already been obtained of Δ^8 desaturase activity in rat testis, it appeared that Δ^8 desaturase activity variation was probably due to tissue differences. In view of these previous findings and since the fatty acid spectrum of testicular lipids of rat differs from that of the human (9), we thought it important to establish whether or not human testicular tissue can desaturate eicosa-11,14-dienoic acid and utilize it for synthesis of arachidonic acid.

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METHODS

Testes were placed directly into ice after removal at orchidectomy from patients with prostatic cancer. The testes themselves were not affected by the disease. The tissue was freed of extraneous material and the tunica albuginea was removed. Approximately two grams of the tissue was either thoroughly minced with a razor blade for tissue mince incubations or homogenized for preparation of the microsomal fraction as described by Peluffo et al. (10). Incubations were in 3 ml of the medium described by Ayala et al. (11), containing the following: 10-20 mg protein, tissue mince or microsomal fraction, 0.45 mmol KCl, 0.75 mmol sucrose, 4 μmol ATP, 2.5 μmol NADH, 15 μmol $MgCl_2$, 4.5 μmol glutathione, 1 μmol nicotinamide, 125 μmol NaF, 125 μmol phosphate buffer (pH 7.0), 0.2 μmol CoA, and 1 μCi of [1-^{14}C]eicosa-11,14-dienoic acid (specific activity 59 μCi/μmol; DHOM Products, Hollywood, CA) dissolved in 10 μl propylene glycol. Duplicate incubations were done for 3 hr at 37 C under oxygen and terminated by addition of potassium hydroxide. Procedures for the hydrolysis, extraction and methylation of total fatty acids from incubation mixtures were previously described (7). Total fatty acid methyl esters were separated by degree of unsaturation on layers of silica gel impregnated with 9% silver nitrate and the triene fraction subjected to ozonolysis followed by reduction with triphenylphosphine. Gas radiochromatography of methyl esters and of aldehydo esters produced from the ozonolysis and reduction of methyl esters was done as described previously (7).

TABLE I

Distribution of Radioactivity in Products Formed during Incubation of Tissue Minces and of Microsomes from Human Testes with [1-^{14}C]eicosa-11,14-dienoic Acid

Tissue preparation	% of Desaturation product formed[a]		% of [^{14}C] of Eicosatrienoic Acid fraction[b]	
	Eicosatrienoic acid	Arachidonic acid	5,11,14 Isomer	8,11,14 Isomer
Mince	87.7 (3.74)	12.3 (3.74)	19.8 (10.1)	80.2 (10.1)
Microsomes			32.5 (15.0)	67.5 (15.0)

[a]Results are means of triplicate analyses of two different tissues with standard deviation in parentheses.

[b]Results are means of duplicate analyses of two different tissues with standard deviation in parentheses.

RESULTS AND DISCUSSION

Human testicular tissue was shown to metabolize eicosa-11,14-dienoic acid by desaturation. After incubation of minced tissue with the labeled substrate, ca. 5% of the recovered radioactivity was found in unsaturated fatty acids (namely eicosatrienoic and eicosatetraenoic acids) other than the initial substrate. A substantial proportion of this amount was found in arachidonate (Table I) indicating that eicosa-11,14-dienoic acid can be metabolized by the testis to an intermediate such as eicosa-8,11,14-trienoic acid which is suitable for desaturation to arachidonate.

That human testis is in fact capable of forming eicosa-8,11,14-trienoic acid as an intermediate was confirmed by analysis of the labeled isomers in the eicosatrienoic acid fraction obtained in incubations with [1-^{14}C]-eicosa-11,14-dienoic acid. Although most of the label was present in the 8,11,14-isomer, a substantial proportion was found in the 5,11,14 isomer (Table I). The latter isomer is believed to be a "dead-end" product unsuitable for further desaturation (12), whereas the former can be readily desaturated to arachidonate (3). Microsomal preparations also synthesized labeled eicosa-8,11,14-trienoic acid upon incubation with [1-^{14}C]eicosa-11,14-dienoic acid, thus indicating that the labeled eicosa-8,11,14-trienoic acid was not derived from mitochondrial elongation of octadeca-6,9,12-trienoic acid (Table I).

It is not yet clear whether the desaturation of eicosa-11,14-dienoic acid in testis results from a specific Δ^8 desaturase or from a Δ^9 desaturase with less specificity than the ones found in liver and in other tissue with no Δ^8 desaturase activity. The latter possibility was proposed by Gurr et al. (13) to explain the ability of some animal species (e.g., chickens, goat, pig) but not others (rat and hamster) to desaturate 12-octadecenoic to octadeca-9,12-dienoic acid. They proposed that the tissues active in this desaturation contain, in addition to stearoyl CoA desaturase, a second desaturase with more activity toward short chain saturated acids but capable of desaturating longer chain monoenes because of their geometrical configuration (13,14). If that were so, it might not be unreasonable to suggest that the twenty-carbon diene might also serve as a substrate for the same short chain desaturase. However, no evidence is available for or against this possibility.

The importance of this pathway to the overall rate of biosynthesis of arachidonate in testis and in the organs in which it has been reported is not known. Even if it is a minor pathway, it might presumably become important in the event of a decrease in activity of the regular pathway.

The results of the present investigation and those previously reported by us (2) and by others (8) indicate that there is a variation in tissue Δ^8 desaturase activity rather than a variation in different species.

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β-Oxidation of the Coenzyme A Esters of Vaccenic, Elaidic, and Petroselaidic Acids by Rat Heart Mitochondria

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ABSTRACT

Rates of β-oxidation of the coenzyme A esters of vaccenic, elaidic, and petroselaidic acids as well as their respective *cis* isomers by rat heart mitochondria were measured and compared. At all concentrations studied, vaccenoyl-CoA was oxidized more rapidly than elaidoyl-CoA, but more slowly than oleoyl-CoA except at high substrate concentrations. All *trans* octadecenoyl-CoA esters were oxidized at a slower rate than their respective *cis* or saturated isomers. Oxidation rates decreased as the double bond approached the carboxyl-end.

INTRODUCTION

Vaccenic acid (*trans*-11-octadecenoic acid) is the major *trans* fatty acid found in the milk fat (1,2) and the adipose tissue (3,4) of ruminants and is the only significant natural *trans* fatty acid consumed in the Western diet. Vaccenic acid is a product of rumen bacterial fermentation and constitutes ca. 1-6% of the total bovine milk fat, varying somewhat with seasonal changes (1,2). Ruminant adipose tissue generally contains ca. 5% vaccenic acid (3,4). Partially hydrogenated plant oils are also a major source of vaccenic acid and contain 4-8% of the acid, representing ca. one-fourth of the total *trans* fatty acid content (5).

In light of recent concern about dietary *trans* fatty acids and with evidence mounting that elaidic acid (*trans*-9-octadecenoic acid) is catabolized at a significantly slower rate than is its *cis* isomer, oleic acid, by both rat heart and liver mitochondria (6,7), it was considered important to compare the rate of β-oxidation of vaccenic acid relative to elaidic acid as well as to oleic, stearic, and palmitic acids. To facilitate the comparison of oxidation rates with respect to double bond position, the β-oxidation rate of petroselaidic acid (*trans*-6-octadecenoic acid), which is present in small amounts (0-1.5%) in partially hydrogenated plant oils (5), was also examined and compared to its *cis* isomer, petroselinic acid.

MATERIALS AND METHODS

Chemicals

Oleic acid (99%), *cis*-vaccenic acid (99%), *trans*-vaccenic acid (99%), coenzyme A/lithium salt (84%), adenosine 5'-diphosphate (ADP)/sodium salt (97%), and fatty acid free bovine serum albumin were purchased from Sigma, St. Louis, MO. Petroselaidic (99%) acid and petroselinic acid (99%) were purchased from NuChek Prep, Elysian, MN. Elaidic acid (99+%) was purchased from Aldrich, Milwaukee, WI. (-)-Carnitine chloride was purchased from Koch-Light, Colnbrook, Bucks, England.

Synthesis of Acyl-CoA Esters

The CoA esters of the fatty acids were prepared by the method of Goldman and Vagelos (8). Acyl-CoA esters were assayed on the basis of molar absorbance at 260 nm. The molar extinction coefficient of oleoyl-CoA (Sigma) of known purity was used as a standard; it was found to be 13.7 $mM^{-1}cm^{-1}$ at pH 7.0. Purities for the acyl-CoA esters were 90 to 99%. The absorbance ratio of 232/260 nm was also determined and ranged from 0.49 to 0.57, a range similar to previous reports for acyl-CoA esters (9).

Mitochondrial Oxidations

Hearts were removed from 450 ± 40 g male Holtzman rats after short ether exposure and decapitation. The mitochondria from single hearts were immediately isolated by a minor modification of the procedure of Pande and Blanchaer (10). The use of a 50 mM, rather than 10 mM, Tris-Cl buffer (pH 7.4) during the Nagarse incubation was found to improve mitochondrial respiratory control ratios. Approximately 0.25 mg of mitochondrial protein was used per assay. Protein was determined by the method of Lowry et al. (11), with bovine serum albumin as the protein standard. The mitochondria maintained high respiratory control ratios (5.5-11 for oleoyl-CoA) and high ADP/O ratios (2.8-3.1 for oleoyl-CoA) for 6 to 9 hr after isolation.

Oxygen uptake rates were measured polarographically using a Yellow Springs Instrument Model 53 (Yellow Springs, OH) equipped with

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TABLE I

Mitochondrial Oxidation of the Geometric Isomers of 6-, 9-, and 11-Octadecenoyl-CoA, Stearoyl-CoA, and Palmitoyl-CoA

Acyl-CoA[a]	Optimal substrate to albumin ratio (mole/mole)	Oxygen uptake (natoms/min per mg protein)	Relative rate[b]
18:1 Δ^{11}-*trans*	2.5	350 ± 10 (4)[c,d]	0.83
18:1 Δ^{9}-*trans*	2.5	250 ± 5 (4)	0.60
18:1 Δ^{6}-*trans*	2.5	95 ± 5 (3)	0.23
18:1 Δ^{11}-*cis*	1.9	445 ± 5 (4)	1.06
18:1 Δ^{9}-*cis*	2.2	420 ± 5 (4)	1.00
18:1 Δ^{6}-*cis*	2.5	195 ± 10 (3)	0.46
18:0	2.7	430 ± 10 (4)	1.02
16:0	1.8	430 ± 15 (4)	1.02

[a]All substrates were used at a concentration of 20 μM.
[b]Oxygen uptake rate relative to oleoyl-CoA.
[c]Mean ± S.E. for the indicated number of experiments.
[d]All *cis-trans*, *trans-trans* and saturated-*trans* differences are significant ($P < 0.005$).

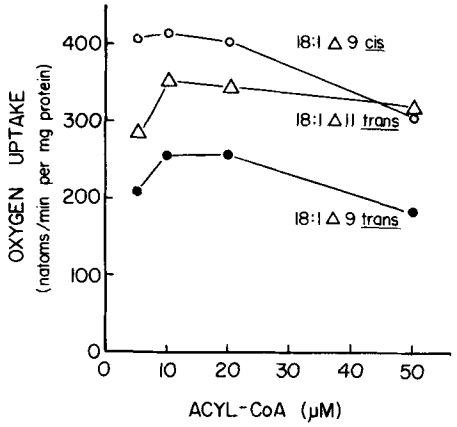

FIG. 1. The effect of substrate concentration on the β-oxidation rates of oleoyl-CoA (○), vaccenoyl-CoA (△), and elaidoyl-CoA (●), at optimum substrate/albumin ratios.

a Clark oxygen electrode. The incubation medium used by Swarttouw (12) for oxidation of acylcarnitine esters was used except that addition of cytochrome C was found to be unnecessary and, therefore, was omitted. To 2.90 ml of air-saturated incubation medium maintained at 37 C were added 2 mM (-)-carnitine chloride, albumin, and substrate. One minute after adding the mitochondria, 300 nmoles of ADP were added, bringing the total reaction cell volume to 3 ml. The oxygen uptake rate, respiratory control ratio, and ADP/O ratio were determined as described by Estabrook (13).

Mitochondrial oxidation rates decreased slightly, but proportionally to the length of time after isolation. Consequently, substrate comparisons were made by alternating the substrate order. After applying the F-test for determining equal variances, data were compared for significant differences by the Student t-distribution test.

RESULTS AND DISCUSSION

Rates of fatty acid oxidation by isolated mitochondria are dependent on the substrate/albumin ratio (12,14). Consequently, the optimal ratio was determined for each substrate for each mitochondrial preparation. Given oxidation rates are those observed at the optimal substrate/albumin ratio.

As depicted in Table I, vaccenoyl-CoA was oxidized at a significantly faster rate than was elaidoyl-CoA, but more slowly than was oleoyl-CoA or *cis*-vaccenoyl-CoA. With increasing substrate concentrations, as can be seen from Figure 1, the oxidation rate of vaccenoyl-CoA remained intermediate between that of oleoyl-CoA and of elaidoyl-CoA, and at higher concentrations (50 μM), the oxidation rates for oleoyl-CoA and vaccenoyl-CoA became nearly equal. The physiological concentration of long chain fatty acyl-CoA esters in rat heart has been determined to be 43 μM, although the true concentration is probably lower due to intracellular binding (15). Therefore, at physiological concentrations the oxidation rate of vaccenoyl-CoA is probably lower than that of oleoyl-CoA.

From Table I it can be seen that the oxidation rates of both the *trans* and the *cis* acyl-CoA esters decreased as the double bond approached the carboxyl-end. Rate differences between the Δ^9 and Δ^6 isomers decreased markedly. Such differences were expected for the *cis* isomers because β-oxidation of *cis* unsaturated fatty acids with even-positioned

double bonds requires an auxiliary enzyme, 3-hydroxy fatty acyl-CoA epimerase (16). However, β-oxidation of *trans* unsaturated fatty acids with even-positioned double bonds does not require additional transformation and, in fact, bypasses the first step of β-oxidation, fatty acyl-CoA dehydrogenase. From these considerations, petroselaidoyl-CoA would be expected to be oxidized at a faster rate than would be its *cis* isomer. On the contrary, however, petroselaidoyl-CoA was oxidized at only half the rate of its *cis* isomer. Evidently, transport of petroselaidoyl-CoA across the mitochondrial membrane was inhibited.

These results demonstrate that vaccenic acid may be a better substrate for heart mitochondrial oxidation in vitro than elaidic acid. Indeed, fatty acids which are slowly oxidized by heart mitochondria, such as elaidic and erucic acids, have been shown to accumulate in the cardiac tissue (6,17).

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Evidence for the Lipoprotein Heterogeneity of Human Plasma High Density Lipoproteins Isolated by Three Different Procedures[1]

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ABSTRACT

Quantitative electroimmunoassays of apolipoproteins in ultracentrifugally isolated high density lipoproteins (HDL) of normolipidemic subjects showed that A-I and A-II are the major (80-85% of total HDL protein) and B, C-III, E, D and F are the minor protein constituents of this density class. A comparison between the apolipoprotein composition of ultracentrifugally isolated HDL and heparin-Mn^{++} supernates showed no significant difference in the levels of A-I and C-III. However, the concentration of ApoE in the heparin-Mn^{++} supernates was almost twice as high as that in the ultracentrifugally isolated HDL; ApoB was only detectable in trace amounts in the heparin-Mn^{++} supernates. To establish whether these apolipoproteins are parts of a single macromolecular complex or form separate, discrete lipoprotein particles, the high density lipoproteins were isolated by three different procedures including ultracentrifugation, heparin-Mn^{++} precipitation and agarose column chromatography. The double diffusion analyses of each of these HDL preparations with antisera to A-I, A-II, ApoB, C-III, ApoD, ApoE, and ApoF showed nonidentity reactions between each possible combination of these antisera. The only exception was a reaction of partial identity between antisera to A-I and A-II polypeptides indicating the occurrence of two types of lipoprotein particles, a major one (LP-A) containing both polypeptides and a minor one (LP-A-I) containing A-I as the sole protein constituent. These findings indicate that high density lipoproteins, regardless of the manner of isolation, do not consist of a single macromolecular complex, but represent a mixture of several, discrete lipoprotein families.

INTRODUCTION

Gofman and his coworkers demonstrated that the human serum lipoproteins represent a wide spectrum of particle sizes and hydrated densities characterized by regions of maximal and minimal concentrations of 0.92-1.20 g/ml (1-3). On the basis of this discontinuous distribution, lipoproteins were classified into five major density classes including chylomicrons, very low density (VLDL), low density (LDL), high density (HDL) and very high density (VHDL) lipoproteins. The availability of ultracentrifugal techniques for the preparative isolation of lipoproteins (3,4) in conjunction with numerous clinical studies which related a particular density class to a lipid transport derangement (5) has led to the prevailing conceptual view that density classes represent the fundamental physical-chemical and metabolic entities of the plasma lipoprotein system. However, more recent studies on the characterization and distribution of apolipoproteins have shown that the major density classes are heterogeneous not only with respect to particle size and hydrated density but also with respect to apolipoprotein composition (6-8). To account for the heterogeneity of proteins in density classes, we postulated (6,9) that the serum lipoprotein system consists of discrete lipoprotein families or particles each of which is characterized by the presence of a single apolipoprotein or its constitutive polypeptides. Studies on the isolation of lipoprotein X (LP-X), an abnormal low density lipoprotein of obstructive jaundice, provided the first experimental evidence for the existence of lipoprotein families (10). By employing a procedure which combined ultracentrifugation, heparin precipitation and ethanol fractionation, the LDL were separated into lipoprotein A (LPA), lipoprotein B (LP-B) and LP-X. More recently, several procedures have been developed in this and other laboratories for the isolation of lipoprotein families from LDL (11-15) and HDL (16-23). By demonstrating that both of these two major density classes represent mixtures of discrete, separable lipoprotein families, these studies have disclosed an unexpected complexity of lipoproteins and cast serious doubts on the

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validity of concepts which consider operationally defined lipoproteins as the fundamental physical-chemical entities of the system. Recent epidemiological studies (24) substantiating earlier reports (25,26) on the inverse relationship between the levels of HDL and the incidence of coronary heart disease have created, if not necessitated, a renewed interest in studies on the lipoprotein composition of density classes, in general, and HDL, in particular.

The purpose of this study was to determine the apolipoprotein composition of normolipidemic human HDL and to establish whether these apolipoproteins are parts of a single macromolecular complex or form discrete lipoprotein families. Since ultracentrifugation may cause compositional and structural alterations in major lipoproteins (27-30), experiments described in this report were carried out with HDL isolated by three different procedures including ultracentrifugation, heparin-Mn^{++} precipitation and agarose column chromatography.

MATERIALS AND METHODS

Blood Donors

Blood was drawn into tubes containing solid Na_2EDTA by venipuncture from healthy, normolipidemic, young men and women who had fasted overnight. The plasma was separated from formed cells by low-speed centrifugation at 4 C. Plasma samples for ultracentrifugal isolation of lipoproteins were obtained from the same donors by plasmapheresis. All plasma samples contained sodium azide (1 mg/ml) and Thiomerosal (0.1 mg/ml) as preservatives.

Isolation of HDL by Ultracentrifugation

Plasma samples were adjusted to a solution density of 1.073 g/ml by adding solid KBr (this corresponds to a small molecule solvent density of 1.062 g/ml which after salt redistribution yields an average density of 1.053 g/ml), and the mixtures were centrifuged in the Ti60 rotors of the Spinco Model L-265B ultracentrifuges for 22 hr at 105,000 x g at 5 C. The top lipoprotein layer was removed by a tube-slicing technique, and the solution density of the bottom portion was adjusted to 1.23 g/ml by adding solid KBr. After centrifuging for 22 hr at 105,000 x g, the HDL present in the top layer were removed and recentrifuged three times under identical conditions until free of albumin, as demonstrated by immunodiffusion tests (9).

Isolation of HDL by Heparin-Mn^{++} Precipitation

Plasma samples were treated with 5% heparin solution (Heparin sodium, 100 U/mg, ICN Pharmaceuticals, Inc., Cleveland, OH) and 1 M $MnCl_2$ according to the procedure described by Burstein et al. (31); final concentrations in plasma were 0.2% heparin and 0.05 M $MnCl_2$. After addition of reagents, samples were kept for 30 min at 4 C and the precipitated ApoB-containing lipoproteins were sedimented by centrifugation at 10,000 x g for 10 min at 6 C. The clear supernatant solutions were removed by aspiration. Aliquots of some of the supernatant solutions were adjusted to a solution density of 1.23 g/ml, and the mixtures were centrifuged for 22 hr at 105,000 x g to obtain concentrated HDL preparations for immunologic characterization. The floating HDL were removed by a tube-slicing technique and dialyzed for 48 hr against three changes of 0.15 M NaCl containing 0.05% Na_2EDTA.

Isolation of HDL by Agarose Column Chromatography

The isolation of HDL by agarose column chromatography was carried out according to a modified procedure of Margolis (32). Plasma samples (5 ml) were chromatographed on a Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.5 x 90 cm) employing 0.05 M potassium phosphate, pH 8, as equilibrating and eluting buffer. The column was run at 4 C. The second major peak eluting between 390-510 ml was considered to be analogous to HDL because it was the only segment of the elution profile that reacted with antibodies to A-I and A-II as demonstrated by immunodiffusion tests.

Immunological Methods

The double diffusion analyses of HDL preparations were performed in 1% agarose (Seakem Agarose, Bausch and Lomb, Inc., Rochester, NY) employing Veronal buffer, pH 8.6, ionic strength 0.1 (33). The immunodiffusion reactions of identity, partial identity and nonidentity between various antigens were evaluated according to the recommendations of Ouchterlony (34).

Apolipoproteins A-I, A-II, C-I, C-II, C-III, D, E and F and lipoprotein B, and their corresponding monospecific antisera were prepared and characterized according to the previously published procedures from this laboratory (9,11,17,20,22). Rabbit antiserum to human albumin was purchased from Behring Diagnostics, Inc., Woodburry, NY.

Electroimmunoassay of HDL Apolipoproteins

The quantitative determination of apolipoproteins in HDL preparations isolated either by

TABLE I

Concentrations of Apolipoproteins in Ultracentrifugally Isolated HDL from Normolipidemic Subjects[a]

A-I	A-II	ApoB	C-I	C-II	C-III	ApoD	ApoE	ApoF
97 ± 10[b] (52.9)[c]	50 ± 5 (27.3)	12 ± 5 (6.5)	4.8 ± 1.7 (2.6)	2.2 ± 0.8 (1.2)	7.0 ± 2.5 (3.8)	5.5 ± 1.5 (3.0)	3.4 ± 1.7 (1.9)	1-2 (0.8)

[a]Results represent five separate HDL preparations obtained from male subjects.
[b]Values are means ± SD.
[c]Results expressed as a percentage of the sum of individually determined apolipoprotein values.

TABLE II

Concentrations of Apolipoproteins in the Heparin-Mn^{++} Supernates of Normolipidemic Men and Women

Subjects	Number	A-I	ApoB	C-III	ApoE
Men	6	104.0 ± 20.2[a]	< 1.0	6.74 ± 1.91	5.98 ± 3.55
Women	4	103.48 ± 17.6	< 1.0	7.92 ± 2.75	7.81 ± 3.34

[a]Values are means ± SD.

ultracentrifugation or the heparin-Mn^{++} precipitation method was carried out by previously described electroimmunoassays including A-I and A-II (35), ApoB (36), C-I, C-II and C-III (37), ApoD (38) and ApoE (39). The ApoF was also determined by electroimmunoassay (unpublished experiments).

RESULTS

Qualitative double diffusion analyses showed that all known apolipoproteins (A-I, A-II, B, C-I, C-II, C-III, D, E and F) were present both in the ultracentrifugally isolated HDL and heparin-Mn^{++} supernates. A detailed quantitative analysis of ultracentrifugally isolated HDL (Table I) revealed that A-I and A-II polypeptides account for 80-85% of the total apolipoprotein content of this density class. The ApoC polypeptides and ApoB may comprise up to 15% and apolipoproteins D, E and F account for the remainder of the HDL proteins. The levels of apolipoproteins A-I and C-III in heparin-Mn^{++} supernates (Table II) were similar to those in ultracentrifugally isolated HDL. On the other hand, the levels of ApoE in the heparin-Mn^{++} supernates were almost twice as high as those in the ultracentrifugally isolated HDL. The ApoB was only detectable in trace amounts (< 1 mg/dl) in the heparin-Mn^{++} supernates. The concentrations of C-III and ApoE were slightly higher in women than men (Table II).

Immunological characterization of ultracentrifugally isolated HDL confirmed results of several previous studies indicating that apolipoproteins A (A-I and A-II), B, C (C-I, C-II and C-III), D, E and F reside on discrete lipoprotein particles (11,14,15,17-23,39). To establish the possible effect of preparative ultracentrifugation on the structural integrity of lipoproteins, double diffusion analyses were also carried out with HDL preparations isolated by heparin-Mn^{++} precipitation method and agarose column chromatography. The heparin-Mn^{++}supernates showed nonidentity reactions with every tested combination of monospecific antisera to known apolipoproteins. Thus, the precipitin lines of even the negligible amounts of ApoB-containing lipoprotein particles crossed those of A-I, A-II, ApoD, ApoE, ApoF (Fig. 1) and C-III (Fig. 2). The immunodiffusion pattern in Figure 1 also shows a nonidentity reaction between the ApoD- and ApoE-containing lipoproteins. The immunological behavior of ApoC-containing lipoproteins was studied with antibodies to C-III, the major polypeptide of ApoC. As shown in Figure 2, the precipitin lines of C-III displayed the typical nonidentity reactions with those of A-I, ApoD and ApoE. Finally, the testing of heparin-Mn^{++} supernates with antisera to ApoE and ApoA polypeptides showed nonidentity reactions between ApoE and both A-I and A-II (Fig. 3). There was no difference in the immunological behavior between the heparin-Mn^{++} supernates and those supernates washed by ultracentrifugation at 1.23 g/ml.

The immunologic behavior of lipoproteins isolated by agarose column chromatography of whole plasma was only studied with the second major peak (390-510 ml) which, due to its positive reactions with antibodies to A-I and A-II, was considered to be analogous to HDL.

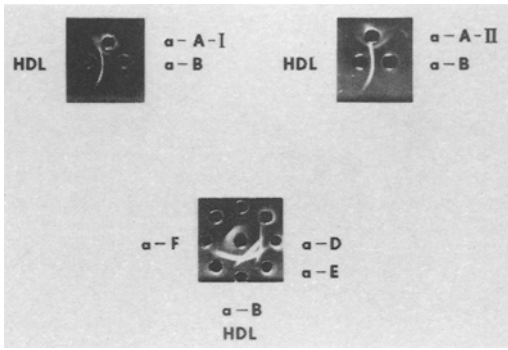

FIG. 1. Double diffusion analyses of HDL isolated by heparin-Mn^{++} procedure. In the upper patterns, HDL sample is placed in the lower left wells. In the bottom pattern, HDL sample is placed in the center well. The placement of antisera is indicated in each pattern; a = anti.

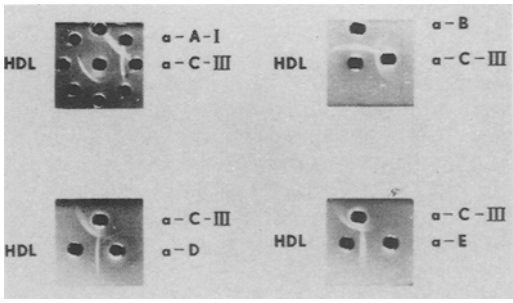

FIG. 2. Double diffusion analyses of HDL isolated by heparin-Mn^{++} procedure. In the upper left pattern, HDL sample is placed in the center well. In all other patterns, HDL sample is placed in the lower left well. The placement of antisera is indicated in each pattern; a = anti.

Double diffusion analysis of this fraction with antisera to A-II and ApoD showed a non-identity reaction between these two apolipoproteins (Figure 4). The nonidentity reactions were also observed between antisera to A-II and ApoE, A-I and ApoD, and A-I and ApoE. However, ApoC polypeptides were not detectable in this fraction, because the major part of this lipoprotein family is eluted prior to the elution of the major HDL peak examined in this study.

In analogy with the previously published results on the ultracentrifugally isolated HDL (16, 17,19,20), the heparin-Mn^{++} supernates and the major HDL peak obtained by the agarose column chromatography gave reactions of partial identity between A-I and A-II when tested by double diffusion (Fig. 5).

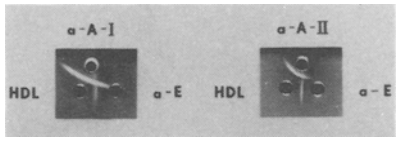

FIG. 3. Double diffusion analyses of HDL isolated by heparin-Mn^{++} procedure. The HDL samples are placed in the lower left wells. The placement of antisera is indicated in each pattern; a = anti.

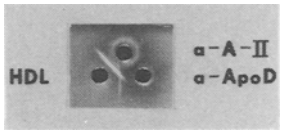

FIG. 4. Double diffusion analysis of HDL isolated by agarose column chromatography. The HDL sample is placed in the lower left well and the antisera to A-II and ApoD as indicated in the pattern; a = anti.

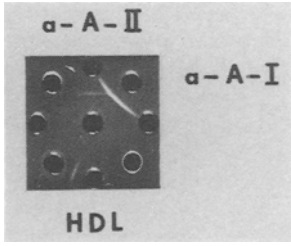

FIG 5. Double diffusion analysis of HDL isolated by heparin-Mn^{++} procedure. The HDL sample is placed in the center well, and the antisera to A-I and A-II as indicated in the pattern; a = anti.

DISCUSSION

Results of this study confirm the already established apolipoprotein heterogeneity of HDL isolated by preparative ultracentrifugation (6-9) and provide for the first time a complete set of data on the concentration of all nine well characterized polypeptides of the apolipoproteins. Although A-I and A-II account for the major part of the HDL protein, the contribution of apolipoproteins B, C (C-I, C-II, C-III), D, E and F may be as high as 20% of the total protein. Significant differences in the molar concentrations between the major (A-I and A-II) and minor (B, C-III, D, E and F) apolipoproteins argue against the concept of a single HDL complex with all apolipoproteins present in each lipoprotein particle.

The most significant difference between the compositions of HDL isolated by ultracentrifugation and the heparin-Mn^{++} procedure was the very low concentration, if not absence, of

ApoB-containing lipoproteins in the latter preparation. The ApoB in ultracentrifugally isolated HDL occurs as LP-B of high hydrated density (12,17,21,40) and as part of the protein moiety of Lp(a) (41). The presence of small amounts of ApoB in heparin-Mn^{++} supernates is due to its incomplete precipitation by 0.05 M $MnCl_2$ (42). The difference in the concentration of ApoE between HDL isolated by ultracentrifugation and the heparin-Mn^{++} procedure most probably reflects the polydisperse character of its lipoprotein family; heparin-Mn^{++} supernates contain LP-E particles of both low and high hydrated densities, whereas ultracentrifugally isolated HDL only contain particles of densities greater than 1.063 g/ml. The similarity in the concentration of A-I and C-III in HDL preparations isolated by ultracentrifugation and the heparin-Mn^{++} procedure indicates that the major part of their polydisperse lipoprotein families have high hydrated densities.

Immunologic analyses of HDL preparations isolated by three different procedures showed clearly that apolipoproteins A (A-I + A-II), B, C-III, D, E and F reside on separate, discrete lipoprotein particles. Identical immunologic behavior of all three HDL preparations also shows that the already recognized occurrence of discrete lipoprotein particles in ultracentrifugally isolated HDL (11,14,15,17-23,39) is a result of normal metabolic processes rather than dissociation of a macromolecular lipoprotein complex during ultracentrifugation of serum in salt solutions of high ionic strength. The reported enrichment of VHDL with ApoA- and ApoE-containing lipoproteins (28,30) during ultracentrifugation of HDL is most probably due either to a partial loss of lipids (27,28) or apolipoprotein (29,30) from these lipoprotein particles accompanied by a concomitant increase in their hydrated densities. However, there is also evidence (unpublished experiments) for an opposite effect, i.e., formation of lipoprotein associations in a manner similar to that described for VLDL in the S_f 100-400 range (29). Although each of the isolation procedures used in this study yields HDL preparations characterized by the presence of several discrete lipoprotein families or particles, there are some differences in the apolipoprotein composition between HDL preparations commensurate with the nature of their isolation. For this reason, it is advisable to conduct studies on the composition and interaction of lipoprotein families within the HDL range with preparations isolated by at least two different procedures. This applies especially to HDL from patients with various types of dyslipoproteinemia.

The reaction of partial identity between the A-I and A-II precipitin lines indicates the existence of two types of lipoprotein particles: the major one, referred to as LP-A, is characterized by the presence of both polypeptides, whereas the minor LP-A-I contains only A-I as its protein moiety. Both of these lipoproteins have already been identified and described (16,17,19). It still remains to be established, however, whether ApoC polypeptides comprise the protein moiety of a lipoprotein family (LP-C) and/or separate subfamilies (LP-C-I, LP-C-II, LP-C-III, etc.) in the HDL range.

The present findings of lipoprotein heterogeneity of HDL preparations, regardless of the manner of isolation, are incompatible with the view that operationally defined lipoproteins represent the fundamental physical-chemical entities of the system. These experimental findings provide, however, strong evidence for the lipoprotein family concept which views serum lipoproteins as a system of polydisperse lipoprotein families each of which is characterized by the presence of a single apolipoprotein (6,9,43). According to this concept, the individual lipoprotein families represent the fundamental physical-chemical entities of the system and exist as discrete, free forms at higher densities and as various association complexes at lower densities of the spectrum. In a recent review (8), Osborne and Brewer have suggested that the free forms of lipoprotein families be referred to as "primary" lipoproteins and the association complexes as "secondary" lipoproteins. Some lipoprotein families or "primary" lipoproteins can reversibly interact with other lipoprotein families to form the association complexes. For example, lipoproteins C and E form association complexes or "secondary" lipoproteins with the triglyceride-rich lipoprotein B (8,9,39,44-47). In turn, such association complexes of very low hydrated densities are dissociated during lipolysis into free forms of lipoprotein families B, C and E of higher hydrated densities (8,48,49). Some free forms of lipoprotein families such as LP-A, LP-A-I, LP-C and LP-E, seem to be secreted from liver and/or intestine as the so called nascent lipoproteins (50-53).

In conclusion, this study presents data on the concentration of apolipoproteins in high density lipoproteins from normolipidemic subjects and provides evidence that this density class consists of a mixture of free forms of lipoprotein families or "primary" lipoproteins.

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The Composition and Metabolism of High Density Lipoprotein Subfractions

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ABSTRACT

The composition and metabolism of high density lipoprotein (HDL) subfractions were investigated in seven normal individuals. Mean HDL_2 (d, 1.063-1.125 g/ml) composition (by weight) was 43% protein, 28% phospholipid, 23% cholesterol, and 6% triglyceride, and mean HDL_3 (d, 1.125-1.21 g/ml) composition was 58% protein, 22% phospholipid, 14% cholesterol, and 5% triglyceride. The mean apoA-I; apoA-II weight ratio was 4.75 for HDL_2 and 3.65 for HDL_3. HDL_2 protein was proportionally slightly richer in C apolipoproteins and higher molecular weight constituents (including apoE) than HDL_3. Kinetic studies utilizing radiolabeled HDL_A (d, 1.09-1.21 g/ml), HDL_2, and HDL_3 demonstrated rapid exchange of apoA-I and apoA-II radioactivity among HDL subfractions, similar fractional rates of catabolism of apoA-I and apoA-II within HDL, and similar radioactivity decay within HDL subfractions. Mean plasma residence time was 5.74 days for radiolabeled HDL_2 and 5.70 days for radiolabeled HDL_3. Differences in HDL protein mass among individuals were largely due to alterations in catabolism, and in general both HDL_2 and HDL_3 were catabolized via a plasma and a nonplasma pathway. Data from simultaneous radiolabeled very low density lipoprotein and HDL studies in 2 individuals are consistent with the concept that apoC-II and apoC-III are catabolized at a different rate than are apoA-I and apoA-II within the HDL density range.

INTRODUCTION

The composition and metabolism of high density lipoproteins (HDL) have recently become a subject of increasing interest, largely due to the finding that the cholesterol constituent of these lipoproteins is inversely correlated with the incidence of coronary artery disease (1-4). Human HDL, isolated by ultracentrifugation in the density range 1.063-1.21 g/ml from plasma, are composed (weight percent) of ca. 50% protein, 25% phospholipid, 20% cholesterol, and 5% triglyceride (5,6). Apolipoproteins (apo) A-I, A-II, B, C-I, C-II, C-III, D, E, and F are all protein constituents of HDL (7-10). ApoA-I and apoA-II comprise ca. 90% of HDL protein, with an apoA-I:apoA-II weight ratio of ca. 3:1 (5-10). ApoA-I has a molecular weight of ca. 28,000 and has been reported to activate the enzyme lecithin cholesterol acyl transferase (11-13). ApoA-II consists of two identical peptides linked by a single disulfide bond (14,15). Research on the lipid-binding properties of apoA-I and apoA-II have recently been reviewed (6,16). These proteins also self associate in aqueous solutions (17,18). In plasma from normal human subjects, almost all apoA-I and apoA-II is found within HDL (19-21).

Total HDL levels in normal human plasma are around 270 mg% (22). Classically, HDL has been divided into two density classes: HDL_2 (d, 1.063-1.125 g/ml) and HDL_3 (d, 1.125-1.21 g/ml) (23), largely due to the demonstration of a bimodal distribution of particle flotation within HDL (24). HDL_2 is composed of ca. 55% lipid and 45% protein, while 55% of HDL_3 mass is due to protein (6,25). Recently HDL has been separated into three subfractions, and these have been designated HDL_{2b} (d, 1.063-1.100 g/ml), HDL_{2a} (d, 1.100-1.125 g/ml), and HDL_3 (d, 1.125-1.21 g/ml) (26). Fluctuations in HDL levels are largely due to variations in HDL_2 levels (22,27). In the present study, we have investigated the composition and metabolism of several HDL subfractions.

METHODS

Subjects Studies

The sex, age, height, weight, plasma lipid and lipoprotein cholesterol values for the subjects studied are given in Table I. All subjects had normal hepatic, renal, and thyroid function, and were studied while on an isocaloric 20% protein, 40% carbohydrate, 40% fat (polyunsaturated/saturated ratio 0.2) 300 mg cholesterol diet. The diet was begun at least two weeks prior to the start of the metabolic study and continued throughout the course of the study. Normal activity was permitted, and the subjects were requested to adhere to the same amount of activity daily. For subject six (Table I), normal physical activity including running 8 miles per day in the evening, which

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TABLE I

Subjects Studied[a]

Subject	Age (yrs)	Sex	Height cm	Weight KG	Plasma cholesterol mg/dl	Plasma triglyceride mg/dl	Cholesterol VLDL mg/dl	Cholesterol LDL mg/dl	Cholesterol HDL mg/dl
1	23	F	160	57.1	177	84	15	121	41
2	21	F	172	50.2	159	40	8	98	53
3	22	F	160	59.6	136	58	11	86	39
4	23	F	172	53.5	177	117	22	115	40
5	21	F	165	53.6	249	76	15	152	82
6	23	M	184	72.3	134	42	5	60	69
7	22	M	177	64.2	181	79	16	116	49

[a]Represents the mean of 14 plasma cholesterol and triglyceride determinations and 3 lipoprotein cholesterol determinations; the standard deviation for all values was $\leq 8\%$.

he continued daily during the baseline and study period. Subjects were given supersaturated potassium iodide (1 g/day) to block thyroid uptake of radioactivity, and ferrous sulfate (900 mg/day) before and during the metabolic study. No other medications were given during the study. During all studies, the body weight of subjects fluctuated by less than 1 kg. Informed consent was obtained from all subjects.

Isolation, Characterization and Labeling of Lipoproteins

Plasma and lipoprotein cholesterol and triglyceride concentrations were measured by the Lipid Research Clinic protocol utilizing Autoanalyzer II methodology (28). The collection of plasma, the isolation, radioiodination, and characterization of HDL, as well as immunoelectrophoresis techniques, were carried out as previously described (29). All HDL preparations utilized for radiolabeling were isolated in Beckman 60 Ti rotors, (Beckman Instruments, Inc., Fullerton, CA) at 59,000 rpm and were subjected to equal amounts of centrifugation time (29). Aliquots of each preparation were delipidated and chromatographed on Sephadex G-200 (superfine) (29). The apolipoprotein composition of the peaks eluting from the column was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (29,30), and tetramethylurea (TMU) PAGE (31).

The following HDL preparations were used for kinetic studies: Subjects 1 and 2, HDL_A (d, 1.09-1.21 g/ml); subject 3, HDL_3 (d, 1.125-1.21 g/ml); subject 4, HDL_2 (d, 1.063-1.125 g/ml), and subjects 5, 6, and 7, HDL_2 and HDL_3. In subjects 3 and 4, very low density lipoproteins (VLDL) were also isolated, characterized, and radiolabeled as previously described (32), for simultaneous VLDL and HDL studies. Autologous, radiolabeled, sterile, and pyrogen-free lipoproteins were given intravenously in all subjects (29). The following tracers were used: Subjects 1 and 2, [^{125}I]-HDL_A; subject 3, [^{125}I]-VLDL and [^{131}I]-HDL_3; subject 4, [^{125}I]-VLDL and [^{131}I]-HDL_2; subjects 5, 6, and 7 [^{125}I]-HDL_2 and [^{131}I]-HDL_3. The characterization of radiolabeled lipoproteins was carried out as previously described (29).

Metabolic Studies

Blood samples in all studies were obtained just prior to injection, at 10 min, 6, 12, and 24 hr, and then daily for 14 days following injection. In subjects 3 and 4, samples were also obtained at 3 and 9 hr after injection. All samples were collected in 0.1% EDTA, and immediately placed on ice. Plasma was separated from blood by a brief centrifugation. All urine output was collected, and collections were ended and restarted at the time blood samples were obtained. Radioactivity in urine, plasma, and plasma lipoprotein fractions was determined by a Packard Model 3375 gamma counter (Packard Instrument Company, Downers Grove, IL). In subjects 1 and 2, HDL_B (d, 1.063-1.09 g/ml) and HDL_A (1.09-1.21 g/ml) were isolated from plasma obtained prior to injection, and at 10 min, 6, 12, and 24 hr, and day 4, 7, 10, 12, and 14 following injection. In subjects 3, 4, 5, 6, and 7 HDL_2 and HDL_3 were isolated from plasma obtained at these same time points. In subjects 3 and 4, VLDL ($d < 1.006$ g/ml), intermediate density lipoproteins (IDL, d, 1.006-1.019 g/ml), and low density lipoproteins (LDL, d, 1.019-1.063 g/ml) were also isolated at these same time points, as well as at 3 and 9 hr following injection.

All lipoprotein density fractions from these time points were isolated in Beckman 40.3 rotors at 39.000 rpm, and their composition (protein, phospholipid, cholesterol, and triglyceride) was quantified as described previously

TABLE II

High Density Lipoprotein Composition[a]

Subject		Protein mg%	Phospholipid mg%	Cholesterol mg%	Triglyceride mg%	Total mg%
1	HDL_B	18 (.41)	11 (.26)	10 (.24)	4 (.10)	43
	HDL_A	137 (.66)	32 (.15)	30 (.15)	8 (.04)	207
	HDL	155 (.62)	43 (.17)	40 (.16)	12 (.05)	250
2	HDL_B	30 (.43)	19 (.28)	17 (.25)	3 (.04)	69
	HDL_A	126 (.64)	30 (.15)	35 (.18)	6 (.03)	197
	HDL	156 (.58)	49 (.18)	52 (.19)	9 (.03)	267
3	HDL_2	47 (.53)	12 (.14)	24 (.27)	5 (.05)	88
	HDL_3	72 (.63)	19 (.17)	16 (.15)	7 (.05)	114
	HDL	119 (.59)	31 (.15)	40 (.20)	12 (.05)	202
4	HDL_2	67 (.50)	35 (.26)	25 (.19)	8 (.06)	135
	HDL_3	89 (.65)	29 (.22)	15 (.11)	4 (.03)	137
	HDL	156 (.51)	64 (.24)	40 (.15)	12 (.04)	272
5	HDL_2	84 (.39)	67 (.31)	49 (.23)	16 (.07)	216
	HDL_3	111 (.54)	48 (.23)	34 (.17)	13 (.06)	206
	HDL	195 (.46)	115 (.27)	83 (.20)	29 (.07)	422
6	HDL_2	80 (.40)	58 (.29)	51 (.26)	8 (.04)	198
	HDL_3	72 (.59)	29 (.24)	18 (.15)	4 (.03)	123
	HDL	152 (.47)	87 (.27)	70 (.22)	12 (.04)	321
7	HDL_2	61 (.42)	48 (.33)	29 (.20)	6 (.04)	144
	HDL_3	87 (.58)	38 (.26)	20 (.14)	6 (.04)	151
	HDL	148 (.50)	86 (.29)	49 (.17)	12 (.04)	295
Mean	HDL_B	24 (.42)	15 (.27)	14 (.24)	4 (.07)	56
	HDL_A	143 (.65)	31 (.15)	33 (.16)	7 (.03)	203
	HDL_2	78 (.43)	44 (.28)	36 (.23)	9 (.06)	157
	HDL_3	86 (.58)	33 (.22)	21 (.14)	7 (.05)	147
	HDL	154 (.53)	67 (.23)	53 (.18)	14 (.05)	288

[a]HDL_B = (1.063-1.09 g/ml), HDL_A = (1.09-1.121 g/ml), HDL_2 = (1.063-1.125 g/ml), HDL_3 = (1.125-1.21 g/ml), HDL = (1.063-1.21 g/ml). Numbers in parenthese indicate fraction of total. The values given for HDL protein, phospholipid, cholesterol, and triglyceride represent the sum of the HDL subfraction values.

(29). All HDL fractions were isolated by sequential ultracentrifugation, and were subjected to the same amount of ultracentrifugation. The distribution of radioactivity within lipoproteins apoproteins was assessed by SDS and TMU PAGE (30,31). In subjects 1 and 2, apoA-I and apoA-II specific activity were determined within HDL (1.063-1.21 g/ml) isolated from plasma obtained at 10 min, and day 1, 4, 7, 10, 12, and 14 following injection as previously described (29). In subjects 3 and 4, the distribution of radioactivity in all lipoprotein fraction was assessed before and after TMU precipitation and by TMU PAGE in order to determine the radioactivity associated with apoC-II and apoC-III (31). In subjects 6 and 7, HDL_2 and HDL_3 were isolated from plasma, incubated for 30 min at 37 C with $[^{125}I]$-HDL_2 and $[^{131}I]$-HDL_3, and from plasma, obtained 24 hr following injection. These fractions were subjected to Sephadex G-200 column chromatography, in order to determine the apolipoprotein radioactivity distribution among HDL subfractions. Plasma and urine radioactivity data from HDL tracer studies were analyzed utilizing the metabolic model given previously (29). Plasma obtained just prior to injection in all subjects was shipped on ice to the Donner Laboratory, Berkeley, CA for analytical ultracentrifugation (22,23,26).

RESULTS

Plasma lipid and lipoprotein cholesterol values for the subjects studied are shown in Table I. Subjects 5 and 6 had HDL cholesterol levels which were significantly higher than the normal range in our laboratory of 50 ± 14 mg% (± S.D.). Subject 6 was running 8 miles per day during the study. High density lipoprotein compositional data on fasting subjects are given in Table II. The protein values represent the mean of seven determinations, and the phospholipid, cholesterol, and triglyceride values are the mean of three determinations. Standard deviations for all determinations were within ± 10%, and little fluctuation in these values occurred during the course of kinetic

TABLE III

Results of Analytical Ultracentrifugation[a]

Subject	VLDL (Sf >20)	IDL (Sf 12-20)	LDL (Sf 0-12)	HDL_2 (F 3.5-9)	HDL_3 (F 0-3.5)	Total HDL
1	81.8	.4	240.1	76.9	183.1	260.0
2	28.0	14.0	213.0	119.4	201.2	320.6
3	54.1	3.6	235.4	75.3	147.7	223.0
4	38.0	27.2	236.4	74.6	138.6	213.2
5	51.0	17.1	444.7	188.7	233.5	422.2
6	.8	2.9	164.6	169.5	145.1	314.6
7	61.6	47.7	302.8	107.7	189.6	297.3

[a]All numbers are in mg/dl.

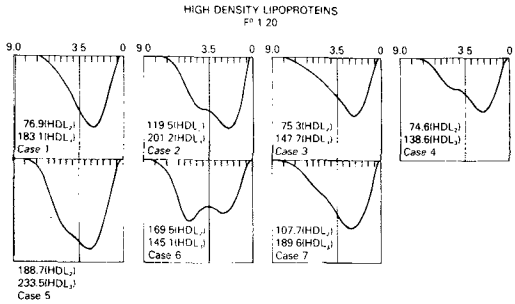

FIG. 1. Analytical ultracentrifugation patterns of high density lipoproteins.

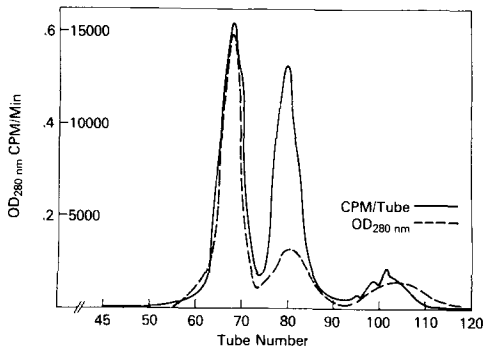

FIG. 2. Sephadex G-200 column chromatography of HDL_3 (subject 6), indicating optical density (280 nm) and radioactivity profile.

studies. As expected, HDL_2 was richer in lipid than HDL_3. The mean HDL_2 composition was: 43% protein, 28% phospholipid, 23% cholesterol, and 6% triglyceride, while the mean HDL_3 composition was 58% protein, 22% phospholipid, 14% cholesterol, and 5% triglyceride. HDL_B was slightly richer in lipid content than HDL_2.

The results of analytical ultracentrifugation for each subject's plasma are shown in Table III. Individual HDL patterns for each subject are shown in Figure 1. F° is the HDL flotation rate in Svedbergs. The F° 3.5-9.0 and F° 0-3.5 subclasses correspond roughly to HDL_2 and HDL_3 (21). Total HDL levels are comparable to those obtained by compositional analysis. The HDL_2 mass predicted by the F° 3.5-9 interval as obtained by analytical ultracentrifugation was consistently lower than that obtained by compositional studies, and the converse was true for HDL_3 mass. This HDL flotation interval (F° 3.5-9) therefore does not precisely correspond to HDL_2 mass, as determined by conventional methods. Subjects 5 and 6 had elevated total HDL and HDL_2 levels.

The apoprotein content of the HDL preparations utilized for radioiodination and kinetic studies was determined by Sephadex G-200 column chromatography, and a typical profile is shown in Figure 2. The recovery of protein following chromatography averaged 92.6%. The initial large peak contained apoA-I, the second peak contained apoA-II with small amounts of the C apolipoproteins, and the third small peak contained the C apolipoproteins contaminated by some apoA-II. Column fractions eluting prior to the apoA-I peak were pooled and designated higher molecular weight constituents. Column fractions were analyzed by SDS PAGE for apolipoprotein content (Fig. 3). Higher molecular weight constituents (Fraction 1) probably represent apoE and ApoA-I, while the other fractions represent proteins as indicated in Table IV and Figure 2. The amount of protein in each of the four fractions is shown in Table IV. The proportion of total protein due to higher molecular weight constituents (Fraction 1) and C apolipoproteins (Fraction 4) appeared to be slightly greater in HDL_2 than in HDL_3. In subjects 5 and 6, the apoA-I/apoA-II ratio was greater in HDL_2 than in HDL_3 while in subject 7 these ratios were similar. Subject 5 had the highest HDL levels, and the highest

apoA-I/apoA-II ratio in both HDL_2 and HDL_3.

The HDL preparations isolated for kinetic studies were free of albumin as determined by immunoelectrophoresis. All HDL_3 fractions were free of apoB, while traces of apoB were found in all HDL_2 preparations. Immunoelectrophoresis of radioiodinated HDL with antisera to HDL demonstrated precipition lines identical to those obtained with unlabeled lipoprotein. Efficiency of labeling averaged 59% (range 47-73%), and the mean lipid labeling of radiolabeled HDL was 2.1% (range of 0.6 to 3.4%). Lipid labeling was slightly greater for HDL_2 than for HDL_3. Mean specific activity was 256 microcuries of [^{125}I] or [^{131}I] per mg for HDL protein. A mean of 0.6 moles (range 0.4-0.9) of iodine per mole of HDL protein were incorporated into HDL during the labeling procedure, assuming a molecular weight of 30,000 for HDL protein. SDS PAGE of radiolabeled HDL revealed a mean (± standard deviation of 51.2 ± 2.4% of radioactivity associated with apoA-I, 33.1 ± 1.9% associated with apoA-II, 10.4 ± 1.4% associated with higher molecular weight constituents, and 4.6 ± .8% associated with the C apo-lipoproteins. The percent of radioactivity associated with higher molecular weight constituents and the C apolipoproteins was consistently slightly higher for HDL_2 than for HDL_3.

Following injection of radioactivity into plasma, a mean (± standard deviation) of 1.9 ± .8% of radioactivity was associated with the 1.063 g/ml supernatant, 93.7 ± 4.1% with HDL, and 4.4 ± 0.7% was associated with the 1.21 g/ml infranatant. This distribution of radioactivity was virtually identical regardless of which HDL subfraction was labeled. In subjects 6 and 7, HDL_2 and HDL_3 were isolated from each subject's plasma incubated with both tracers and from plasma obtained 24 hr following injection, and subjected to column chromatography. These studies demonstrated that the [^{131}I]HDL_3 radioactivity isolated in HDL_2, and the [^{125}I]HDL_2 radioactivity isolated in HDL_3 following injection were due to the exchange of both apoA-I and apoA-II radioactivity, and that this exchange occurred with in vitro incubation as well. The apoA-I and apoA-II specific radioactivity for each HDL subfraction are given in Table I. These data are consistent with the concept that apoA-I and apoA-II can exchange among HDL subfractions but that their specific activities are not identical within these subfractions.

In subjects 1 and 2, apoA-I and apoA-II specific radioactivity decay was determined in HDL (1.063-1.21 g/ml) following the injection of radiolabeled HDL, and is shown in Figure 4.

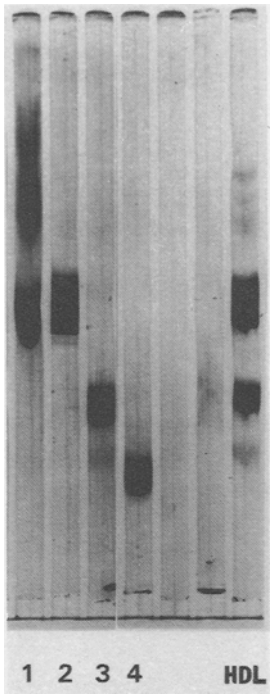

FIG. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of column fraction 1 (1), column fraction 2 (2), column fraction 3 (3), column fraction 4 (4) and apo-HDL (HDL) for subject 6 for column elution profile shown in Figure 2.

These data are consistent with the concept that apoA-I and apoA-II are catabolized at similar rates within HDL. The decay of radioactivity within HDL subfractions following the injection of HDL_A, HDL_2 and HDL_3 is shown in Figure 5. In Figure 6, plasma radioactivity decay is shown in three subjects who simultaneously received [^{125}I]HDL_2 and [^{131}I]-HDL_3. These data are interpreted to indicate that the major protein constituents of HDL subfractions are catabolized at similar rates.

The plasma and urine radioactivity data in all studies were analyzed with the SAAM computer program (33), utilizing a model generated from previous radiolabeled HDL kinetic studies (29). This model, shown in Figure 7, consists of a plasma and a nonplasma compartment, with catabolism occurring from both compartments. This multicompartmental model provides an excellent fit for the radiolabeled HDL kinetic data generated in this investigation. Mean uncertainties for metabolic parameters were as follows (± fractional standard deviation): synthesis rate (mg/kg/day) ± .012; plasma residence time (days) ± .021; whole body residence time (days) ± .075;

TABLE IV

Percent Apoprotein Composition of HDL[a]

Study		FX 1	FX 2 (apoA-I)	FX 3 (apoA-II)	FX 4	FX 2/FX 3	FX 2 + FX 3
1	HDL_A	5.1	64.1	25.2	5.6	2.55	89.2
2	HDL_A	9.1	63.1	23.0	4.7	2.75	86.1
3	HDL_3	4.2	62.2	20.5	13.2	3.03	82.7
4	HDL_2	8.9	63.7	11.1	16.3	5.74	74.8
5	HDL_2	11.4	57.8	8.2	22.6	7.05	66.0
	HDL_3	7.4	69.9	12.4	10.3	5.64	82.3
6	HDL_2	8.7	66.8	12.4	12.1	5.39	79.2
	HDL_2	4.8	69.3	14.9	11.0	4.65	84.2
	HDL_3	1.4	66.9	23.3	8.5	2.87	90.8
7	HDL_2	5.8	61.4	20.7	12.2	2.97	82.1
	HDL_2	5.9	61.7	22.7	9.7	2.72	84.4
	HDL_3	4.0	67.3	21.9	6.8	3.07	89.2
Mean	HDL_2	7.6	63.5	15.0	14.0	4.75	78.4
	HDL_3	4.3	66.6	19.5	9.7	3.65	86.3

[a]Fraction 1 (FX 1) contains high molecular weight constituents; Fraction 2 (FX 2) contains apoA-I; Fraction 3 (FX 3) contains apoA-II, with small amounts of C peptides; Fraction 4 (FX 4) contains C peptides but is contaminated with apoA-II. HDL_A, HDL_2, and HDL_3 are HDL fractions having the densities as designated in the text. For subjects 6 and 7, HDL_2 preparations were analyzed in duplicate. Other analyses represent one column run.

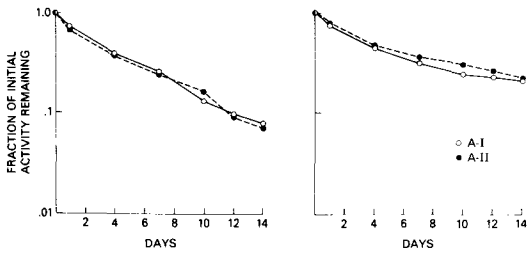

FIG. 4. Normalized specific activity decay curves for spoA-I and apoA-II within HDL following the injection of radiolabeled HDL_A in subject 1 (left panel) and subject 2 (right panel).

plasma volume (ml) ± .040; plasma flux (mg/kg/day) ± .086; nonplasma flux (mg/kg/day) ± .151; plasma catabolic rate constant ($days^{-1}$) ± .009; nonplasma catabolic rate constant ($days^{-1}$) ± .020; exchange catabolic rate constant (L(2,1), $days^{-1}$) ± .116 and exchange catabolic rate constant (L(1,2), $days^{-1}$) ± .150. Results of these analyses are presented in Table VI. In order to calculate synthesis rates, the protein mass of HDL (1.063-1.21 g/ml) was used in all studies. The plasma residence time for each tracer, calculated from the area under the radioactivity decay curve, represents the average life span in plasma for the tracer, and equals the reciprocal of the fractional catabolic rate (FCR). In subjects 5, 6, and 7, the synthesis rates and residence times were very similar regardless of whether HDL_2 or HDL_3 was used as a tracer, consistent with the concept that the major protein moiety of both subfractions of HDL are catabolized at similar fractional rates. In those studies where radiolabeled HDL_2 and HDL_3 were simultaneously injected, the radioactivity curves were fitted individually. Since no difference in the exchange rate constants (L (2,1) and L (1,2) was noted, the data were refitted with the exchange rates being the same for both tracers. Only small differences in the rates of catabolism (L (3,1) and L (3,2) were noted. Studies in subjects 3, 4, 6, and 7 indicate that both HDL_2 and HDL_3 protein are catabolized by a plasma and a nonplasma route. In subject 5, HDL_3 was catabolized via both routes, while HDL_2 was catabolized solely from the plasma pool. The reason for this latter observation remains unclear, but may reflect increased labeling of lipid, higher molecular weight constituents, and C apolipoproteins in this HDL_2 preparation. These constituents are probably catabolized at a greater fractional catabolic rate than apoA-I and apoA-II, resulting in a decreased residence time, and a more rapid appearance of radioactivity in the urine. The metabolic data presented are consistent with the view that the major protein constituents of HDL_2 and HDL_3 are metabolized at similar rates in plasma, and that in general these constituents are catabolized via both a plasma and a nonplasma route.

In order to simultaneously compare the metabolism of apoC-II and apoC-III within

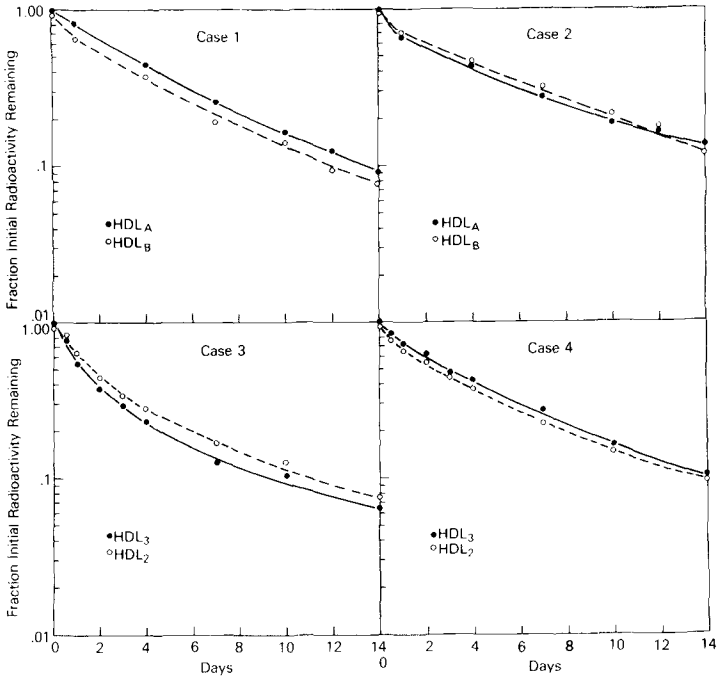

FIG. 5. Radioactivity decay curves in HDL subfractions (as a fraction of initial radioactivity per ml). Following the injection of [^{125}I] HDL_A (d, 1.09-1.21 g/ml in cases 1 and 2, [^{131}I] HDL_3 (d, 1.125-1.21 g/ml) in case 3 and [^{131}I] HDL_2 (d, 1.063-1.125 g/ml) in case 4. HDL_B has a density of 1.063-1.09 g/ml.

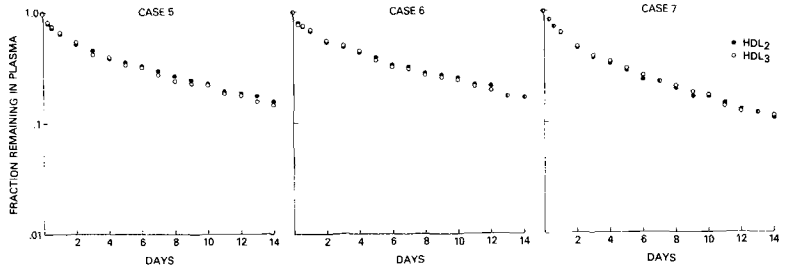

FIG. 6. Plasma radioactivity decay curves (as a fraction of initial radioactivity per ml) in cases 5, 6, and 7 following the simultaneous injection of [^{125}I] HDL_2 and [^{131}I] HDL_3.

HDL with that of apoA-I and apoA-II, we also studied [^{125}I] VLDL kinetics in subjects 3 and 4. The mean efficiency of iodination for these preparations was 15.5% and the lipid labeling was 9.7%. A mean of 0.7 moles of iodine per mole of VLDL protein were incorporated into VLDL during labeling assuming a molecular weight of 250,000 for VLDL protein. The mean percent of radioactivity associated with apoB was 31.2%, with the remainder being primarily associated with apoC-II and apoC-III. ApoC-I was not labeled in these experiments since this protein contains no tyrosine. Following the injection of radioactivity (10 min), a mean of 39.2% of radioactivity was associated with HDL. The radioactivity associated with apoC-II and apoC-II was greater than 90% of the radioactivity remaining in all lipoprotein fractions following this precipitation, as assessed by TMU PAGE. In Figure 8, the radioactivity decay of apoC-II and apoC-III in all lipoprotein fractions is shown for subject 4. In these studies we combined apoA-II and apoC-III radioactivity even though it is possible that these proteins may be catabolized differently. The terminal slope of the radioactivity decay curve was similar in all fractions indicating similar rates of catabolism in this subject, as

TABLE V

Specific Radioactivity in HDL Subfractions[a]

	[^{125}I]-HDL_2		[^{131}I]-HDL_3	
	apoA-I	apoA-II	apoA-I	apoA-II
	dpm/mg protein		dpm/mg protein	
Subject 6				
Incubation				
HDL_2	124344	61131	48108	57804
HDL_3	39104	43050	15930	21330
24 Hour Point				
HDL_2	6589	3384	976	1358
HDL_3	2112	2510	313	526
Subject 7				
Incubation				
HDL_2	75057	46707	19994	25980
HDL_3	30156	33603	14184	14376
24 Hour Point				
HDL_2	7370	5203	1427	1649
HDL_3	3187	3571	504	912

[a]Represents the mean of four determinations from pure protein column fractions on single column chromatography runs; the fractional standard deviation was less than ± .10 for all determinations. The incubation was carried out at 37 C for 30 min with each subject's plasma.

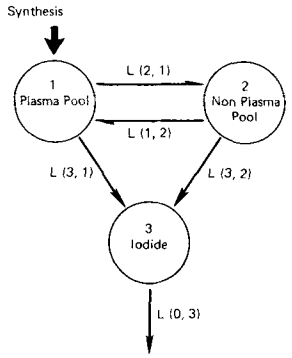

FIG. 7. Multicompartmental model utilized for the analysis of radiolabeled HDL plasma and urine radioactivity data.

well as in subject 3. For subject 4, the half life based on the terminal slope of apoC-II and apoC-III radioactivity within HDL (HDL_2 and HDL_3) was 1.56 days, while for subject 3 it was 1.29 days. These values are significantly different than the half lives (based on the terminal slope of the plasma decay curve) of HDL radioactivity (largely apoA-I and apoA-II radioactivity) in these same subjects, which were 5.54 days and 4.83 days, respectively. These data are consistent with the view that apoC-II and apoC-III radioactivity decay occurs at a significantly greater fractional rate within HDL than does apoA-I and apoA-II radioactivity in these subjects.

DISCUSSION

The data presented on the composition of HDL subfractions are consistent with previous reports indicating that HDL_2 is higher in cholesterol and phospholipid content than HDL_3 (6,24,25). With regard to protein composition, it has been reported that the apoA-I/apoA-II ratio is lower(34), identical (35) or higher (7,19,21,25) in HDL_2 than in HDL_3. Our data would tend to support the latter observations, and also indicate that HDL_2 is proportionally richer in higher molecular weight constituents (including apoE) and the C apolipoproteins than is HDL_3.

Following the injection of radiolabeled HDL subfractions, there was rapid exchange of both apoA-I and apoA-II among these subfractions. Similar data have previously been reported from both in vitro (36) and in vivo experiments (37). In the in vivo studies, HDL subfractions were labeled by in vitro incubation with radio-labeled apoA-I and the exchange of radioactivity among HDL subfractions, as well as plasma kinetics were examined in one individual. We have investigated the metabolism of HDL subfractions utilizing radiolabeled lipoproteins. Our data are consistent with the concept that apoA-I and apoA-II can exchange among HDL subfractions, and the specific radioactivity of apoA-I and apoA-II within these subfractions is not identical. Because of this exchange, the catabolism of apolipoproteins within HDL subfractions is difficult to evaluate.

TABLE VI

Metabolic Parameters[a]

Subject	Study	Synthesis rate	Residence times		Plasma volume	Plasma flux	Nonplasma flux	Plasma CRC	Nonplasma CRC	Exchange CRC	Exchange CRC
			plasma	whole body		R(3,1)	R(3,2)	L (3,1)	L (3,2)	L (2,1)	L (1,2)
		mg/kg/day	days	days	ml	mg/kg/day	mg/kg/day	$days^{-1}$	$days^{-1}$	$days^{-1}$	$days^{-1}$
1	^{125}I HDL_A	12.97	5.17	6.79	2156	8.49	2.54	.144	.087	.426	.262
2	^{125}I HDL_A	9.44	6.63	12.00	2307	5.80	4.94	.082	.116	.350	.471
3	^{131}I HDL_3	11.49	4.34	7.26	2514	9.51	1.98	.191	.059	.589	.430
4	^{131}I HDL_2	11.98	4.98	8.60	2044	6.11	5.53	.101	.129	.885	.731
5	^{125}I HDL_2	11.31	6.32	6.40	1957	11.31	0	.156	0	.470	.835
	^{131}I HDL_3	10.40	6.81	10.17	1957	6.36	4.03	.090	.115	.470	.835
6	^{125}I HDL_2	11.75	6.71	9.53	3757	7.91	3.84	.100	.117	.849	1.909
	^{131}I HDL_3	11.76	6.70	9.52	3757	7.91	3.84	.100	.117	.849	1.909
7	^{125}I HDL_2	11.52	4.96	7.75	2779	6.34	5.17	.111	.161	.600	.908
	^{131}I HDL_3	11.53	4.95	7.75	2779	6.36	5.17	.111	.161	.600	.908

[a]CRC is catabolic rate constant. Residence time in plasma was calcualted from the area under the radioactivity decay curve, and represents the average life span in plasma for the tracer. Residence time in the whole body was calculated by dividing the HDL protein mass in the plasma and nonplasma space by the synthesis rate (mg/day).

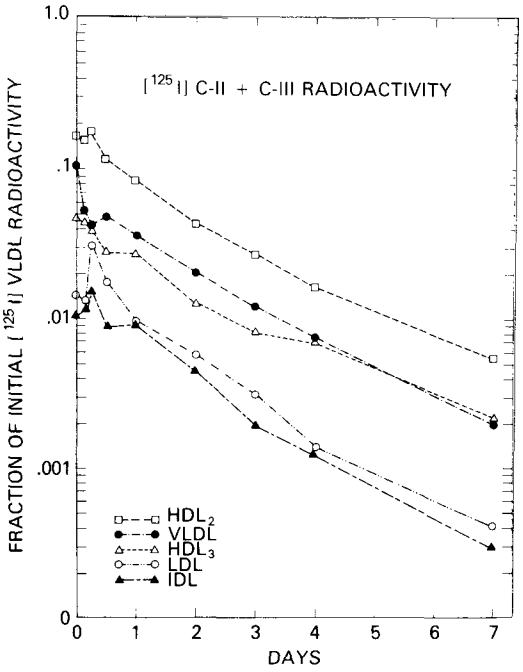

FIG. 8. ApoC-II and apoC-III radioactivity decay (as a fraction of initial radioactivity per ml of plasma) in lipoprotein density classes following the injection of [^{125}I]VLDL.

The plasma kinetics of radioiodinated HDL in man have been previously studies, and recently reviewed (38). Plasma half lives, based on the terminal slope of the radioactivity decay curve, have ranged from 3.5 to 5.8 days (29,39-42). Radioactivity decay was similar if injected as labeled HDL or as delipidated HDL (40). It has been demonstrated that the decay of specific radioactivity of apoA-I and apoA-II within HDL is similar following the injection of labeled HDL (29). The catabolism of HDL proteins is enhanced in hephrotic patients (39), in hypertriglyceridemic subjects (40), in normal individuals on high carbohydrate diets (29), and is markedly increased in patients with familial HDL deficiency (Tangier Disease) (42). In these latter patients, apoA-I is catabolized at a faster fractional rate than is apoA-II, and a large fraction of both apoA-I and apoA-II apolipoproteins is found in the 1.063 g/ml supernatant in plasma (42). In all these subjects, HDL protein levels were decreased, largely due to enhanced catabolism. In this study (Table V), all subjects had similar synthesis rates, and alterations in catabolism also appear to be largely responsible for variability of HDL protein levels among individuals. Changes in catabolism, therefore, appear to play a major role in the regulation of HDL levels. The factors affecting catabolism via the plasma and non-plasma route are not clear; however, it does appear that, in general, radiolabeled HDL_2 and HDL_3 protein constituents are catabolized via both pathways.

The presence of distinct lipoprotein species within HDL, characterized not by their hydrated density, but rather by their apolipoprotein content, has been reported (7-10). Two types of HDL particles containing apoA-I and apoA-II have been isolated: one in HDL_2 with 40% protein content, and the other in HDL_3 with a 56% protein content (7). In addition, a species containing only apoA-I has been noted within HDL_2 (LP-A-I) (25). Particles containing both apoA-I and apoA-II (LP-A-I, A-II) appear to comprise the major species within HDL. Other HDL species that have been reported include LP-C-I, C-II, C-III, LP-D, LP-E, and LP-F (7-10). LP-C-I, C-II, and C-III reportedly contains apoC-I, apoC-II, and apoC-III, and consists of 51% protein (7), while LP-E contains only apoE, and has been isolated within HDL_2 (9). The data presented in this report are consistent with the concept that apoA-I and apoA-II radioactivity decay is markedly different than that observed for apoC-II and apoC-III within the HDL density range. Our data could be explained by the concept that different lipoprotein species exist within HDL (7). An alternative possibility would be that apoC-II and apoC-III are on the same HDL particles as apoA-I and apoA-II, but are removed at a faster rate than apoA-I and apoA-I from HDL particles during their catabolism. Further work is needed to elucidate the sites of synthesis, composition, and catabolism of the various lipoprotein species which appear to exist within HDL.

Intestinal and liver perfusion studies in the rat indicate that both these organs can synthesize apoA-I, while the liver appears to be the major source of the C apoliporoteins and apoE (43-47). Both apoA-I and apoA-II have been localized in human jejunal epithelial cells by immunochemical techniques (48,49). It has been reported that human lymph chylomicron apoA-I and apoA-II can serve as precursors for these constitutents within plasma HDL (50). The relative contribution of the intestine and the liver to apoA-I and apoA-II synthesis remains to be established, as do the forms in which lipoproteins containing these proteins enter the plasma. In vitro experiments indicate that when VLDL particles are incubated with lipase and albumin, particles in the HDL density range containing the C apolipoproteins can be isolated (51). Similarly, when radio

iodinated VLDL is injected into plasma, apoC-II and apoC-III radioactivity can be rapidly detected within HDL, and may subsequently recirculate back to newly formed VLDL (32,52). Apolipoproteins A-I, A-II, C-II and C-III appear to exchange among plasma lipoproteins (32,36,37,42,53). The data cited above are consistent with the view that constituents within HDL arise following the catabolism of both intestinal and hepatic triglyceride-rich lipoproteins. HDL levels are decreased in hypertriglyceridemic patients (1-5,22,40,54,56), and HDL cholesterol concentration is inversely correlated with adipose tissue lipase activity (55). In subjects who engage in strenuous physical activity, plasma triglyceride levels, are low while HDL cholesterol values are elevated (55,56). Synthetic pathways and the state of the lipolytic system may regulate the levels and the catabolism of the lipoprotein species within HDL.

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Incorporation of Label from [9,10-methylene-^{14}C] Sterculic Acid in Rainbow Trout, *Salmo gairdneri*[1]

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ABSTRACT

The distribution of radioactivity from sterculic acid, labeled on the 9,10-methylene carbon of the cyclopropene ring, was investigated in trout, *Salmo gairdneri*. Fifty percent of the administered dose was excreted in feces and urine by 168 hr, but less than 1% of the dose was expired as carbon dioxide during the same time period. Incorporation of radioactivity into most organs peaked at 119 hr, and the majority of the label in the liver was in the fatty acid portion of the lipid fraction. Total lipid radioactivity in liver was higher in trout conditioned to cyclopropene lipids, and a substantial amount of label was found in phosphatidylcholine and ethanolamine phospholipids as well as neutral lipid. The data demonstrate that rainbow trout readily absorb, transport and incorporate sterculic acid into tissue lipid, including membrane lipid, but cannot oxidize the methylene carbon of the cyclopropene ring to carbon dioxide.

INTRODUCTION

Sterculic acid is a naturally occurring cyclopropenoid fatty acid (CPFA) containing a highly strained and reactive unsaturated three membered ring in the center of an eighteen carbon chain. Two food oils, cottonseed oil and kapok oil, contain CPFA (1). Many physiological disorders in animals, including altered egg production and fertility in chickens, delayed sexual maturity and retarded growth in rats, and neonatal and postanatal mortality in rats are attributed to the cyclopropene ring (2,3). CPFA-fed rats show fatty infiltration and degeneration of the livers along with renal tubule degeneration (3), altered lipid metabolism from inhibition of fatty acyl desaturase (4,5), and partial loss of membrane integrity as indicated by changes in erythrocyte hemolysis rate, mitochondrial swelling, and microsomal codeine demethylase activity (6). Scarpelli et al. (7) reported that hepatocytes from sterculic acid-fed rainbow trout and rats exhibited morphologic alterations in membranes of endoplasmic reticulum. In trout, Sinnhuber et al. (8) demonstrated that dietary CPFA was a cocarcinogen with Aflatoxin B_1 and M_1 (9), and recently reported dietary CPFA to be a carcinogen (10).

The metabolism and distrituion of [9,10-methylene-^{14}C] sterculic acid in the rat was partially described by Yoss et al. (11) and Nixon et al. (12). They demonstrated that the rat could not oxidize [9,10-methylene-^{14}C] sterculic acid to $^{14}CO_2$, that the main excretory pathway was via the urine, and that the principal urinary metabolite was *cis*-3,4-methylene adipic acid, a cyclopropane dicarboxylic acid. This metabolite was verified by Eisele et al. (13) along with the identity of 7 additional rat urinary metabolites. The formation of these metabolites required β- and ω-oxidation of sterculate (13).

Trout have been shown to be more sensitive to the physiological effects of dietary CPFA than rats (14). However, little information has been published to explain this phenomenon, and sterculic acid metabolism in the rainbow trout has not been elucidated. The intent of this study was to provide basic knowledge of the biological pathway of this unique fatty acid in rainbow trout. This paper reports the distribution of label from [9,10-methylene-^{14}C] sterculic acid as a function of time after administration in rainbow trout tissue, liver subcellular fractions, liver fractions (acid soluble, protein, lipid) and liver lipid components. This information should prove useful for explaining the difference in toxic response of trout and rat to dietary CPFA and may provide insight into the mechanism of carcinogenic activity of CPFA in trout and to cancer in general. This information also has economic significance because these fatty acids are included in commercial trout rations in cottonseed products.

MATERIALS AND METHODS

Source and Purity of Label

Methyl sterculate, labeled in the 9,10-methylene bridge of the cyclopropene ring (110 μCi/mmol), was synthesized in our laboratory (15) and shown to be 96.8 ± 2.5% or 99.0

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TABLE I

Recovery of Radioactivity from Control-Fed Trout Tissues after Administration of [9,10-Methylene-^{14}C] Sterculic Acid by Stomach Intubation[a]

Hours	2	4	18	74[b]	119	168
Stomach	98.8 ± 10.4	49.8 ± 5.2	10.9 ± 5.9	1.4 ± 1.6	2.3 ± 2.5	0.6 ± 0.3
Pyloric caecum	3.7 ± 3.6	1.0 ± 1.6	10.9 ± 7.8	30.2 ± 14.1	21.7 ± 5.5	8.2 ± 3.0
Lower intestine	0.1 ± 0.1	0.1 ± 0.2	2.9 ± 2.8	7.4 ± 0.8	7.0 ± 0.7	5.3 ± 3.4
Bile	0.0	0.0	0.3 ± 0.3	4.6 ± 3.3	10.0 ± 4.1	10.6 ± 2.0
Liver	0.0	0.0	0.4 ± 0.2	2.7 ± 0.1	2.8 ± 0.6	1.3 ± 0.6
Blood[c]	0.0	0.0	1.3 ± 1.3	24.4 ± 0.3	50.1 ± 18	13.0 ± 3.4

[a]Data are percent of administered radioactivity expressed as the mean ± standard deviation from 3 trout.

[b]Data from 2 trout.

[c]Radioactivity in blood expressed as dpm per mg serum protein divided by the dpm administered per kg body weight.

± 2.0% cyclopropene by the Halphen Test (16) and nuclear magnetic resonance (NMR) (17), respectively. The methyl ester was converted to the free acid by saponification with 0.5 N 95% ethanolic KOH at 45-50 C for 30 min and again shown to be 93.2 + 1.1% or 96.7 + 2.0% cyclopropene by these assays. A second preparation of sterculic acid (598 μCi/mmol) was used in the label distribution of liver lipid fractions and components. The radiopurity of both preparations was checked by thin layer chromatography (TLC) in a hexane/ethyl ether/benzene (70:30:1) system, and a single radioactive spot (99 + %) for the methyl ester was found at an Rf of 0.60. The second preparation was also shown to be 97 + % radiochemically pure by high pressure liquid chromatography (HPLC) on a μBondapak C^{18} column (Waters Associates, Milford, MA) with acetonitrile/water (9:1) as solvent.

Animals and Diet

Rainbow trout, *Salmo gairdneri*, of the Mt. Shasta strain were spawned and hatched at the Food Technology and Nutrition Laboratory at Oregon State University. The fry were fed a basal semipurified diet described previously (18) and were designated control-fed trout. At one year of age, control-fed trout were fed a 300 ppm cyclopropene diet for 3 or more months by adding CPFA to the basal diet as *Sterculia foetida* oil. The purpose was to condition the trout with CPFA with the intent of increasing the incorporation of label into various liver fractions and lipid components. All trout were fasted from 3 days prior to administration of labeled sterculic acid until termination of the experiment. Number 5 gelatin capsules (Eli Lilly, Indianapolis, IN) containing 3.84 ± 1.41 μCi/kg body wt. of labeled sterculic acid (110μCi/mmol) were inserted with a flexible trocar into the stomachs of control-fed trout weighing 356 ± 96 g. Trout used in the excretion studies were individually placed in 3.5 x 12 in. glass cylinder metabolism chambers in which water was replaced at 5 to 12 hr intervals. Air was bubbled through the chambers and then through two 100 ml IN KOH traps to collect $^{14}CO_2$. Trout used in deposition studies were placed in 2 x 2 x 2 ft. fiberglass tanks with a water flow rate of 2 gal/min and held for specific time periods. Each tank held 3 trout and all trout in each tank were dosed and terminated at the same time.

Fractionation and Radioactivity Counting Procedures

Organs and tissue. Blood was collected by heart puncture and centrifuged to prepare serum. Gall bladder contents were removed with a disposable syringe and stored frozen at -10 C. Organs and tissue were removed, weighed, wrapped in aluminum foil and stored at -10 C, along with the carcass and metabolism chamber water contents.

Organs and tissue were homogenized in 3 or 4 volumes (w/v) distilled water with a Tissumizer SDT 100 N (Tekmar Co., Cincinnati, OH). Aliquots of the homogenates were digested in NCS tissue solubilizer (Amersham/Searle, Arlington Heights, IL), counted in Aquasol (New England Nuclear, Boston, MA), and corrected for quenching. Aliquots of the chamber water and KOH traps were also counted in Aquasol.

Liver subcellular fractions. A measured portion of the liver from each trout from above was used to prepare subcellular fractions (12). The liver tissue was minced in 4 volumes (w/v) 0.25 M ice-cold sucrose and homogenized in a Potter-Elvehjem homogenizer. A model L-2 ultracentrifuge (Beckman, Inc., Palo Alto, CA)

TABLE II

Recovery of Radioactivity in Excreta and Carbon Dioxide from Control-Fed Trout after Intubation of [9,10-methylene-^{14}C] Sterculic Acid[a]

Hours	12	24	36	48	106[b]	120[b]
Excreta	20 ± 18	23 ± 19	36 ± 6	37 ± 6	51	52
CO_2	0	0	0	<1	<1	<1

[a]Data are percent of administered radioactivity expressed as the mean ± standard deviation from 3 trout.

[b]Data from one trout.

was used to isolate the following fractions: cell debris, mitochondrial, microsomal, and supernatant by spinning at 800 x g for 10 min, 12,000 x g for 10 min, and 105,000 x g for 60 min, respectively. Each sediment was washed once with 0.25 M sucrose and the wash was combined with the previous supernatant. Aliquots of the subcellular fractions were digested in NCS tissue solubilizer and counted for radioactivity in Aquasol.

Liver fractions. Four control and 3 CPFA-fed trout weighing 104 ± 10 g were injected intraperitoneally (IP) with 5.0 μCi sterculic acid (598 μCi/mmol) in 0.2 ml corn oil. After 42 hr, the livers were removed and fractionated according to the method of Shibko and coworkers (19) into 3 fractions: acid soluble compounds, lipid, and protein. The lipids were saponified and fractionated into 3 more fractions (20): sterols and unsaponifiable material (nonpolar, hexane soluble), fatty acids (ethyl ether soluble), and glycerol (water/ ethanol soluble). Aqueous samples were counted in Aquasol and samples in nonpolar solvents were counted in toluene containing 6 g PPO (2,5-diphenyloxazole, Sigma Chemical Co., St. Louis, MO) plus 50 mg POPOP (1,5-bis-[2,5-phenyloxazole]benzene, Nuclear; Chicago, Des Plaines, IL) per liter.

Liver lipid components. Four CPFA-fed trout weighing 177 ± 59 g were injected IP with 5.0 μCi sterculic acid (598 μCi/mmol) in 0.2 ml corn oil. After 24 or 72 hr, livers were removed from 2 trout, perfused with cold 0.9% NaCl, and the lipids extracted by the method of Folch (21). A neutral lipid fraction was obtained by one-dimensional TLC on Silica Gel H developed in chloroform/methanol/ammonium hydroxide (65:35:5). Neutral lipids were separated into monoacylglycerols, diacylglycerols, triacylglycerols, and cholesteryl esters by one-dimensional TLC on Silica Gel G developed in hexane/ethyl ether/glacial acetic acid (80:20:1). Separation of polar lipids was performed using two-dimensional TLC as reported previously (20). Spots were visualized by brief exposure to iodine vapor, scraped into scintillation vials, and counted in toluene-POPOP fluor. Known lipid standards were run on duplicate plates for two-dimensional TLC and on the same plate for one-dimensional TLC.

RESULTS AND DISCUSSION

Distribution of Radioactivity in Organs and Tissue with Time

After 2 hr the gelatin capsules that were inserted into the stomachs of the control-fed rainbow trout were only partially dissolved. Most of the label (98.5%) was found in the upper intestine (pyloric caecum) and stomach and contents (Table I). After 18 hr, the amount of label in the stomach dropped to 10.9% of the administered dose. As the radioactivity decreased in the stomach and intestinal tract, the label in the liver increased to a peak radioactivity level of 2.7-2.8% of the administered dose between 27 and 119 hr. The radioactivity in the blood serum reached a peak at 119 hr while the label in bile began to plateau at 119 hr and maintained a level of 10.0-10.6% of the administered dose to 168 hr. The relatively high percentages of [^{14}C] in the intestinal tract, blood, liver and bile during this 3 to 5 day period indicated that a substantial amount of enterohepatic circulation had occurred. In contrast, we found that the rat exhibited minimal enterohepatic circulation of label from sterculate (12). The relatively high, sustained level of label in the bile also suggests bile conjugation of sterculic acid and/or metabolites. This was supported by the finding that an additional 4% and 23% of the label in bile could be extracted into ethyl ether at pH 1 following β-glucuronidase and ethanolic KOH treatment. Retention of label in bile could also be affected by disruption of normal gall bladder emptying patterns from fasting during the test periods.

The excreta (urine and feces) contained 50% of the administered dose between 48 and 106 hr (Table II). Flux of label through control-fed trout was 3 to 6 times slower than through

TABLE III

Recovery of Radioactivity in Liver Subcellular Fractions from Control-Fed Trout after Intubation of [9,10-Methylene-^{14}C] Sterculic Acid[a]

Subcellular fraction	Hours				
	4	18	74[b]	119	168
800 x g cell cebri	0	0.14 ± 0.06	0.54 ± 0.16	0.41 ± 0.03	0.45 ± 0.26
12,000 x g mitrochondrial	0	0.02 ± 0.02	0.16 ± 0.02	0.35 ± 0.14	0.21 ± 0.04
105,000 x g microsomal	0	0.05 ± 0.05	1.32 ± 0.71	0.91 ± 0.26	0.57 ± 0.36
Supernatant	0	0.17 ± 0.10	0.70 ± 0.01	1.06 ± 0.21	0.43 ± 0.14

[a]Data are percent of administered radioactivity expressed as the mean ± standard deviation for 3 trout.

[b]Data from 2 trout.

TABLE IV

Radioactivity in Liver Fractions of Control and Cyclopropene-Conditioned Rainbow Trout at 42 hr Expressed as Percent of Administered Dose of [9,10-Methylene-^{14}-C] Sterculic Acid[a]

Fraction	%Administered Dose	
	Control livers	Cyclopropene-conditioned livers
Acid soluble	0.22 ± 0.03	0.20 ± 0.04
Protein	0.23 ± 0.17	0.35 ± 0.10
Total lipids	3.26 ± 0.40	14.70 ± 4.22[b]
nonsaponified	0.20 ± 0.02	0.55 ± 0.03[b]
fatty acids	3.00 ± 0.38	13.97 ± 4.10[b]
glycerol	0.06 ± 0.01	0.18 ± 0.15

[a]Values are mean ± standard deviation for livers from 4 controls and 3 cyclopropene-conditioned trout.

[b]Cyclopropene-fed trout significantly different ($P<0.05$) from controls in these fractions using Student "t" test.

control-fed rats, which excreted 59% of the administered dose in 16 hr (12).

Trout expired less than 1% of the administered radioactivity as carbon dioxide during test periods as long as 5 days (Table II). The low amount of label in carbon dioxide suggests that trout cannot oxidize the methylene carbon of the cyclopropene ring. The same observation was made with rats (12). The slow rate of oxidation of CPFA is not typical for oxidation of long chain fatty acids by salmonoids. In other work in our laboratory, salmon oxidized up to 40% of IP-injected ^{14}C-linolenic acid to labeled carbon dioxide in 24 hr (22).

Distribution of Radioactivity in Liver Subcellular Fractions

It is assumed that rat liver mitochondria and microsomes (endoplasmic reticulum) are involved in the metabolism of sterculic acid to its primary urinary metabolite, *cis*-3,4-methylene adipic acid (11,13). Thus, the purpose of this experiment was to measure the amount of label from [9,10-methylene-^{14}C] sterculic acid bound to trout liver subcellular fractions as a function of time (Table III) to obtain an indication of the sequence and involvement of subcellular fractions in the catalysis of various reactions in the oxidation of sterculic acid. The microsomal fraction peaked at 74 hr compared to 119 hr in the mitochondria. The high activity in the supernatant was probably due to the transport of sterculic acid and/or metabolites between organelles in the cytosol. The variations in the relative concentration of radioactivity in the different fractions as a function of time suggest that various phases of sterculic acid metabolism may be catalyzed by different subcellular fractions. Since radioactivity peaked in the microsomal fraction prior to the mitochondrial fractions, it is possible the endoplasmic reticulum catalyzed the initial steps of sterculate oxidation followed by catalysis of subsequent steps by the mitochondria. Different rates of incorporation and turnover of label from sterculate and/or metabolites into lipid components of the subcellular fractions would influence this interpretation.

TABLE V

Radioactivity Incorporated into Liver Lipid Components of Cyclopropene-Conditioned Rainbow Trout after IP Injection with [9,10-Methylene-^{14}C] Sterculic Acid[a]

Time (hr)	24	72
Total lipid[b]	4.32 ± 0.75	4.60 ± 0.00
Neutral lipid[b]	3.17 ± 0.94	3.70 ± 0.14
Triacylglycerols[c]	57.67 ± 25.84	75.85 ± 1.88
Diacylglycerols	6.51 ± 6.12	2.40 ± 0.62
Monoacylglycerols	7.85 ± 7.85	0.45 ± 0.16
Cholesteryl esters	1.17 ± 0.34	0.40 ± 0.00
Free fatty acids	0.36 ± 0.04	0.41 ± 0.01
Phospholipids[b]	1.15 ± 0.18	0.90 ± 0.14
Phosphatidylcholine	14.18 ± 3.77	12.01 ± 1.40
Phosphatidylethanolamine	9.13 ± 4.95	4.48 ± 0.21
Phosphatidylinositol	2.18 ± 1.88	1.11 ± 0.68
Phosphatidylserine	0.76 ± 0.54	0.78 ± 0.54
Diphosphatidylglycerol	0.41 ± 0.41	0.13 ± 0.13
Lysophosphatidylcholine	0.29 ± 0.20	tr[d]

[a]Data are expressed as the mean ± deviation of two determinations.

[b]Values for total lipid, neutral lipid and phospholipid are percent of total administered radioactivity.

[c]Values for individual lipid components are percent of radioactivity recovered in liver lipid.

[d]The amount of radioactivity in this component, and sphingomyelin and phosphatidic acid was less than 0.01% of the total organ lipid radioactivity.

Distribution of Radioactivity in Liver Fractions

Control-fed trout were compared to CPFA-fed trout to determine if conditioning to CPFA would cause a change in label distribution. Table IV shows the distribution of label in the various liver fractions at 42 hr after injection with sterculic acid. Little radioactivity was incorporated into substituents other than fatty acids. The aqueous acid soluble fraction probably contained metabolites which had not been transported from the liver. Only a small amount of label, 0.23% and 0.35% of the administered dose and 6% and 2% of the total liver radioactivity for the control and CPFA trout, respectively, was bound to proteins. In the lipid fraction, most of the label was in the fatty acid fraction with the CPFA-fed trout exhibiting a 4-fold increase in fatty acid radioactivity over the controls (Table IV). This difference was probably due to conditioning of the trout to CPFA. Rats fed CPFA also exhibited an increase in metabolism of sterculic acid compared to controls (21).

Distribution of Radioactivity in Liver Lipid Classes

CPFA-fed trout incorporated more label into the total lipid fraction (Table IV) than control trout; therefore, CPFA-fed trout were used exclusively for this study. Total lipid radioactivity (Table V) in liver did not change from 24 to 72 hr. An increase in neutral lipids was balanced by a decrease in phospholipid.

Table V shows that the percentage of label in liver triacylglycerols increased from 57% to 75% in the 72 hr period while label in the combined mono- and diacylglycerols decreased from 14% to 3%. Label was found in cholesteryl esters and free fatty acids at ca. 1% or less. The free fatty acids may be free sterculic acid and/or carboxylic acid metabolites.

A substantial amount of label was incorporated into phospholipids. Choline and ethanolamine phosphoglycerides contained the largest amount of radioactivity, and considerable amounts remained after 72 hr. The data show that label from sterculic acid is readily incorporated into phospholipids, an occurrence which is of particular importance since phospholipids comprise a large proportion of cellular membranes. The physical and biochemical consequences of altered membrane lipid geometry have been noted by several authors (6,23,24). Possible alterations in the molecular geometry of membranes resulting from inclusion of ring structures could account for some of the biological activity of the cyclopropenoid fatty acids.

The data from this study demonstrate that rainbow trout readily absorb and transport sterculic acid but cannot appreciably oxidize the methylene carbon of the cyclopropene ring to carbon dioxide. Radioactivity was excreted via the urine and feces. Conditioning of rainbow trout to CPFA appeared to increase the

incorporation of radioactivity into various liver fractions. The phospholipids of the livers of conditioned trout contained ca. 27% of the liver lipid radioactivity at 24 hr. The isolation and identification of the excretory metabolites is in progress.

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The Formation of Phosphatidylinositol by Acylation of 2-Acyl-*sn*-Glycero-3-Phosphorylinositol in Rat Liver Microsomes

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ABSTRACT

The conversion of 2-acyl-*sn*-glycero-3-phosphorylinositol into phosphatidylinositol via acyl-CoA: 2-acyl-*sn*-glycero-3-phosphorylinositol acyltransferase activity was found to occur in rat liver microsomes. Over a wide range of conditions, stearic acid was preferred over palmitate by the acyltransferase when these acids were presented in mixtures as acyl-CoA derivatives. The potential importance of this enzyme activity for the entry of stearic acid into the 1-position of hepatic phosphatidylinositol is further supported by its greater preference for stearate relative to the acyl-CoA:2-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase under certain assay conditions.

INTRODUCTION

The various phospholipids from liver and most other tissues contain mainly saturated (palmitate plus stearate) and unsaturated fatty acids in the 1- and 2-positions, respectively (1). Phosphatidylinositol (PI) from rat liver is known to be markedly enriched in stearic acid at the 1-position and arachidonic acid at the 2-position relative to phosphatidylcholine (PC) such that the 1-stearoyl 2-arachidonoyl molecular species greatly predominates in the former phospholipid (1). The biochemical basis for the characteristic fatty acid composition of PI is not clearly understood. It is known that arachidonate can enter PI via CDP-diacylglycerol: inositol phosphatidyltransferase activity (2) and by the acylation of 1-acyl-*sn*-glycero-3-phosphorylinositol (1-acyl GPI) via acyl-CoA:1-acyl GPI acyltransferase activity as found in microsomal preparations from liver (3) and brain (4). Liver microsomal preparations from various animals have acyltransferases which can acylate both 1- and 2-acyl-*sn*-glycero-3-phosphorylcholine (2-acyl GPC) to form PC (5).

The purpose of the work described herein was to document the acylation of 2-acyl-*sn*-glycero-3-phosphorylinositol (2-acyl GPI) via acyl-CoA:2-acyl GPI acyltransferase activity in rat liver microsomes which results in the formation of PI. The relative rates of entry of palmitate and stearate into PI by the latter reaction were determined and compared to those for the corresponding enzymatic reaction in PC synthesis.

MATERIALS AND METHODS

The unlabeled acyl-CoA esters were obtained and characterized as described previously (3). The [1-^{14}C]palmitoyl-CoA, [9,10-^{3}H(N)]-stearoyl-CoA, and [1-^{14}C]oleoyl-CoA were purchased from the New England Nuclear Corp. (Boston, MA) and diluted with nonlabeled acyl-CoA derivatives to the desired specific radioactivities. PI and PC were isolated from rat liver by thin layer chromatography (TLC) as described elsewhere (6).

For the preparation of 2-acyl GPI, hepatic PI (1.7 μmole) was incubated at 22 C in 1 ml of 100 mM borate buffer (pH 7.0) containing 12.5 mM $CaCl_2$ to which was added 500 μl of diethyl ether and 200 μl of a lipase Type IX suspension from Rhizopus arrhizus (Sigma Chemical Co., St. Louis, MO). The latter preparation contained 0.4 mg of protein in 3.2 M $(NH_4)_2SO_4$ solution (pH 7.0). After an incubation period of 60 min, 1 ml of ethanol was added, and the mixture was extracted four times with 2 ml of petroleum ether (30-60 B.P.)-diethyl ether (1:1, v/v). The 2-acyl GPI was extracted from the residual aqueous phase using 2 ml of chloroform, and aliquots were taken for quantitation by gas liquid chromatography (6). The 2-acyl GPI was used within 2-3 hr as a substrate for the acyltransferase. The preparation of 2-acyl GPC from PC utilized identical conditions to the above except that digestion times were 30 min in duration. The yields of 2-acyl GPI and 2-acyl GPC were 78 ± 8% (mean ± S.E., n = 5) and 72 ± 5%, respectively, of theoretical values.

Liver microsomes were prepared from male Wistar rats exactly as described (7). Microsomal protein was measured by the method of Lowry et al. (8).

Unless indicated otherwise in the text, the standard incubation mixture for monitoring acyl-CoA:2-acyl GPI acyltransferase activity contained 32 μM 2-acyl GPI, 16 μM mixed acyl-CoA (8 μM [^{14}C]palmitoyl-CoA plus 8 μM [^{3}H]stearoyl-CoA) containing 5,000-9,000 cpm/nmole, and 20 μg of microsomal protein in 500 μl Tris-HCl buffer (pH 7.4). Incubations were conducted at 37 C in a shaking water bath for 1 or 2 min after which the reaction was terminated and PI formation was determined

TABLE I

Fatty Acid Composition of Phosphatidylinositol and 2-Acyl-*sn*-Glycero-3-Phosphorylinositol

Fatty acids	Phosphatidylinositol[a]	2-Acyl-*sn*-Glycero-3-Phosphorylinositol[a]
16:0	8.1	2.6 ± 0.5
18:0	45.2	4.3 ± 0.6
18:1	2.2	1.6 ± 0.2
18:2	2.4	5.4 ± 0.1
20:2	1.8	3.5 ± 0.1
20:3	3.5	5.3 ± 0.2
20:4	31.7	64.4 ± 1.2
22:5	1.4	4.0 ± 0.2
22:6	2.4	6.3 ± 0.4

[a]Values are given as mole % of the total. Any fatty acid contributing <1% has been omitted from the table. Data for the 2-acyl GPI are means ± S.E. (n=5).

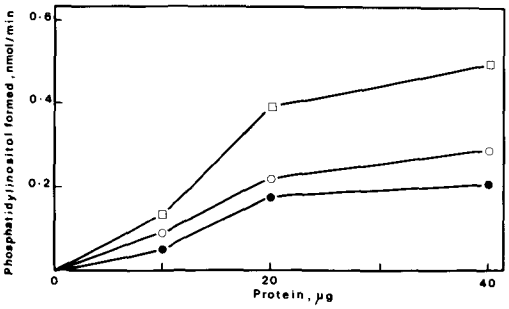

FIG. 1. The formation of PI in relation to the amount of microsomal protein in the incubation medium. □, total (1-palmitoyl plus 1-stearoyl species); ●, 1-palmitoyl species; ○, 1-stearoyl species.

by liquid scintillation counting following TLC (6). Substitution of 2-acyl GPC for 2-acyl GPI in the assay mixture was employed when measuring acyl-CoA:2-acyl GPC acyltransferase activity.

To confirm the positional location of the radioactive fatty acids in the product, 0.8 μmole of carrier was added, and the PI was hydrolyzed with phospholipase A_2 from *Crotalus adamanteus* (Sigma Chemical Co., St. Louis, MO). The phospholipid was dispersed in 3 ml of diethyl ether containing 1% by volume of 50 mM Tris-HCl buffer (pH 7.4), 5 mM $CaCl_2$, and lipase (0.4 mg of lyophilized powder). After a digestion period of 8 hr at 22 C, the distribution of radioactivity among the reaction products (1-acyl GPI and free fatty acids, was assessed following TLC (6). In the case of PC hydrolysis, 0.25 mg of the lipase preparation and a digestion period of 10 min were employed.

RESULTS

Table I shows that the saturated fatty acids (palmitate plus stearate) represented 53% of the total acids in hepatic PI, but only 7% of those in the 2-acyl GPI. The major unsaturated acid, arachidonate, represented 32% and 64% of the acids in the total PI and in the 2-position, respectively, in good agreement with previous findings (1,9). The saturated fatty acids contributed 42% to the total PC but only 7% to the 2-acyl GPC; the latter contained oleate (8%), linoleate (32%), arachidonate (32%), and docosahexaenoate (12%) as the major unsaturated acids.

Figure 1 gives the relationship between microsomal protein concentration and PI formation from 2-acyl GPI and a mixture of [^{14}C]palmitoyl-CoA plus [^{3}H]stearoyl-CoA under standard incubation conditions (see Materials and Methods). The amount of palmitate and stearate utilized for PI synthesis increased when the protein was increased from 10 μg to 40 μg per incubation. The amount of PI formed in the absence of added 2-acyl GPI was 5% or less of that formed under standard assay conditions. Hydrolysis of the newly formed PI with phospholipase A_2 revealed that at least 90% of the labeled palmitate and stearate were incorporated into the 1-position. Control experiments with 2-acyl GPC revealed that 97% of the radioactivity fatty acids were esterified to the 1-position of the newly synthesized PC. When [^{14}C]oleoyl-CoA was the acyl donor, 92% of this labeled unsaturated acid was found associated with the 1-position of the PI and PC when 2-acyl GPI and 2-acyl GPC, respectively, served as acyl acceptors.

An increase in acyl-CoA:2-acyl GPI acyltransferase activity was observed with an increase in 2-acyl GPI concentration (Fig. 2a) from 8 to 64 μM. Regardless of 2-acyl GPI or acyl-CoA concentration (8 to 64 μM), stearate was preferred by 1.6 to 2.3-fold over palmitate

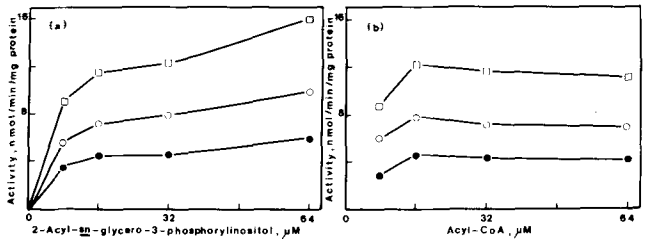

FIG. 2. The effect of (a) 2-acyl GPI and (b) acylCoA concentration on the formation of PI. Explanation of symbols as in Figure 1.

TABLE II

Utilization of Palmitate and Stearate from Acyl-CoA Mixtures for Acylation of 2-Acyl-*sn*-Glycero-3-Phosphorylinositol or 2-Acyl-*sn*-Glycero-3-Phosphorylcholine

	Phospholipid formed	Synthetic rate[a], nmole/min/mg protein			
		1-Palmitoyl species	1-Stearoyl species	Total	Selectivity index
Expt. 1	PI	6.33 ± 1.04	8.95 ± 1.21	15.28 ± 2.19	0.70 ± 0.07[b]
	PC	17.01 ± 1.52	13.74 ± 1.17	30.75 ± 2.68	1.24 ± 0.01
Expt. 2	PI	5.32 ± 0.95	6.64 ± 0.75	11.96 ± 1.66	0.76 ± 0.08[c]
	PC	7.11 ± 0.66	10.01 ± 0.73	17.12 ± 1.38	0.70 ± 0.02

[a]Values for Expt. 1 (n=5) and Expt. 2 (n-6) are means ± S.E. The total acyl-CoA (4 μM palmitoyl plus 4 μM stearoyl) was 8 μM in Expt. 1 and Expt. 2; the acyl acceptor was 32 μM and 8 μM in Expt. 1 and Expt. 2, respectively. The selectivity index equals (rate of palmitate entry/rate of stearate entry); an index greater or less than unity indicates enzyme selectivity for palmitate or stearate, respecitvely. All means in the vertical column under selectivity index are significantly different ($P < 0.01$) from 1.00 when analyzed statistically by Student's t-test (10).

[b]Significantly different from PC in Expt. 1 ($P < 0.01$).

[c]Not significantly different from PC in Expt. 2 ($P > 0.01$).

by the acyltransferase when these two acids were presented as equi-molar mixtures of their thiolester derivatives (Figs. 2a,2b).

Multiple experiments were conducted with different microsomal preparations to compare the selectivities of the 2-acyl GPI and 2-acyl GPC acyltransferases for palmitate versus stearate (Table II). With acyl acceptor and donor maintained at 32 μM and 8 μM, respectively, stearate was preferred in PI formation and palmitate in PC synthesis. Stearate was selectively utilized over palmitate for the biosynthesis of both PC and PI when both acceptor and donor were fixed at 8 μM.

DISCUSSION

The present results provide direct evidence for the acylation of 2-acyl GPI in rat liver microsomes via acyl-CoA:2-acyl GPI acyltransferase activity. The preferential association (> 90%) of newly incorporated saturated and unsaturated fatty acids with the 1-position of both PI and PC indicates that the 2-acyl GPI and 2-acyl GPC preparations used as enzyme substrates were not subject to significant acyl migration. The moderately selective utilization of stearate over palmitate by the acyl-CoA:2-acyl GPI acyltransferase (Table II) suggests that it may partly contribute to the preponderance of stearic acid over palmitic acid in the 1-position of PI isolated from rat liver (1,9). The 1-stearoyl species of PI can also originate from CDP-diacylglycerol, which contains a significant amount of this acid (11), via CDP-diacylglycerol:inositol phosphatidyltransferase activity (2). However, the much higher ratio of stearate:palmitate in hepatic PI relative to CDP-diacylglycerol (11) supports the potential importance of deacylation-reacylation reactions at the level of PI for stearate entry into this latter phospholipid. The conversion of PI to its monoacyl derivative has been demonstrated in mammalian tissues (12). The greater selectivity exhibited by the acyl-CoA:2-acyl GPI acyltransferase for stearate over palmitate relative to the acyl-CoA:2-acyl GPC acyltransferase under certain conditions (Table II) may contribute to the preponderance of stearic acid

in the 1-position of PI relative to other phospholipids.

ACKNOWLEDGMENT

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The Influence of Phthalate Esters on Human Plasma Lecithin/Cholesterol Acyltransferase

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ABSTRACT

The effect of various phthalate esters on the lecithin/cholesterol acyltransferase activity in man was studied in vitro. The enzymatic activity was strongly reduced with all phthalates except for the dimethyl phthalate. The inhibition rate depends on the phthalate concentration and also on the carbon number of the alkyl groups of phthalates.

INTRODUCTION

The intensive use in medicine as well as in foodstuff (1) of polyvinyl chloride materials plastified with phthalic acid esters results in the human body being contaminated by these phthalates. In fact, recent research works have shown that phthalates were present in blood stored in plastic bags (2) and in blood of patients with extracorporal circulations particularly during hemodialysis (3). Several authors (4) have found that rats fed phthalate esters had decreased cholesterol concentration in the blood.

In order to define more accurately the influence of phthalate esters on the cholesterol metabolism in man, we have studied the action of various phthalates in vitro on the activity of the lecithin/cholesterol acyltransferase (LCAT).

MATERIAL AND METHODS

The blood came from voluntary donors whose ages ranged from 20 to 40 whose lipidic profiles were normal.

The phthalates used were as follows: dimethyl phthalate (DMP), diethyl phthalate (DEP), diallyl phthalate (DAP), di-n-butyl phthalate (DBP), diethyl-2 hexyl phthalate (DEHP) and di-n-nonyl phthalate (DNP). Their chemical purity was checked by gas liquid chromatography.

The plasma LCAT activity was determined in the presence of each phthalate by the method of Stokke and Norum (5), and the results obtained were controlled by the method of Alcindor et al. (6).

In the method of Stokke and Norum (5), the radioactive y substrate was prepared as follows: plasma heated at 56 C for 30 min was incubated for 12 hr with 4 μCi of [4-^{14}C] cholesterol in a stabilized emulsion of albumin. The incubation mixture in each experiment consisted of 0.2 ml of plasma, 0.1 ml of radioactive substrate and 0.7 ml of buffer:Tris (0.01 M) EDTA (5 mM) NaCl (0.15 M) at pH 7.40. The incubation was performed in a shaking water bath at 37 C for 4 hr and for 6 hr. Lipids were extracted according to FOLCH et al. (7). The lipid extract was separated by thin layer silica gel chromatography. The spots identified by autoradiography or more simply by iodine vapors were scraped and the esterification rate calculated by measuring the radioactivity of each in a Packard-Tri-Carb 2425 instrument. The phthalates were used with increasing concentrations from 1 to 25 μM and, for each concentration, 3 tests and 3 control samples without any phthalate were run in parallel. After evaporation of the solvent (heptane), the incubation mixture was added to the phthalate residue.

The method of Alcindor et al. (6) used [1-^{3}H] cholesterol in plasma free of its light lipoproteins (LDL and VLDL) by precipitation with dextrane sulfate in presence of $CaCl_2$. The incubation was stopped after 1 hr and after 2 hr by propanol-2, and the esterification rate was obtained as in the previous method.

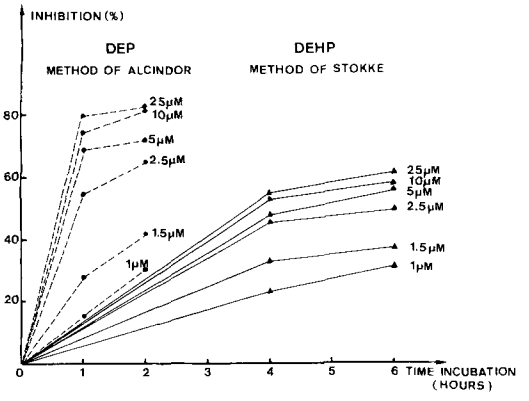

FIG. 1. Influence of incubation time on inhibition of LCAT activity. The DEP was studied by the method of Alcindor et al. (●——●) (6) and the DEHP by the method of Stokke and Norum (▲——▲) (5).

TABLE I

Influence on LCAT Activity for 5 μMoles of Phthalate Esters in the Incubation Mixture

	DMP		DEP		DAP		DBP		DEHP		DNP	
	S[a]	A[b]	S	A	S	A	S	A	S	A	S	A
LCAT activity % inhibition	24.2	70.2	74.4	69.1	59.1	39.6	64.4	50.6	48.2	54.2	40.5	38.2

[a]Method of Stokke and Norum (5).
[b]Method of Alcindor et al. (6).

RESULTS AND DISCUSSION

All the phthalates, except for DMP with low concentrations, reduced the LCAT activity. Such results were confirmed by the method of Alcindor et al. (6) (Table I). There was a relationship between the phthalate concentration and the inhibition rate. Indeed, the inhibition quickly increased when the phthalate concentrations were low but reached a steady state with high concentrations. This explained, for all phthalates, the existence of a very good logarithmic type correlation between the inhibition rate and the phthalate concentration – according to the methods and the phthalate ester types, the correlation coefficients were fluctuating between 0.82 and 0.99. The incubation time influenced the inhibition rate of the enzyme activity only for the low phthalate concentrations as shown in Figure 1. For the high phthalate concentrations, the maximum inhibition was nearly reached in 4 hr using the method of Stokke and Norum (5) and in 1 hr with the method of Alcindor et al. (6).

Taking into account such incontestable enzyme inhibition, several problems can be discussed. The phthalate impact upon the LCAT mechanism of action must be first considered. Our first results showed that the inhibition was competitive. Apparently there is some competition for the enzyme between lecithin, which is the LCAT natural substrate, and the phthalate molecule interfering through one of its two ester bonds.

The inhibition action is important for all the phthalates tested except for the DMP with small concentrations, which was also observed by Bell et al. (4). The inhibition rate varies with phthalate concentrations until it makes a steady level for high concentrations, but it also seems to depend on the carbon number of the alkyl groups of the inhibitor since, for instance, a 5 μM phthalate concentration of DEP gives 74.4% inhibition and 40.5% only with DNP.

The pathological aspect of such inhibition of an important enzyme such as LCAT in the cholesterol metabolism is still to be defined more precisely. Phthalate rates have been found (3) in patients with periodical hemodialysis for whom precocious atherosclerosis can be observed. Such experiments in vitro will have to be compared with the results obtained in vivo on frequently phthalate-contaminated men because other pathological effects have been discovered such as the decrease of the squalene synthesis (8), the teratogenic action in rats (9) or the ever increasing abortion frequency in women (10).

CONCLUSION

The phthalate-inhibiting action upon the enzymatic activity seems to have been demonstrated that it depends on the phthalate concentration and also on the carbon number of the alkyl groups of phthalate esters. The widespread distribution of these pollutants and the frequency and the seriousness of vascular diseases with lipoprotein perturbations justify the study and the development of their relationships.

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Isoflavones and Hypercholesterolemia in Rats

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ABSTRACT

Isoflavones isolated from three commonly used pulses such as Bengalgram (*Cicer arietinum*), greengram (*Phaseolus aureus*) and blackgram (*Phaseolus mungo*) and p-coumaric acid were supplemented to hypercholesterolemia-inducing diet of rats. Among isoflavones, Biochanin A and Formononetin showed hypolipidemic activity but diadzein did not; p-coumaric acid also produced a significant reduction in serum cholesterol levels.

INTRODUCTION

Although enough experimental data has accumulated to show that Bengalgram (1-5) (*Cicer arietinum*) can lower serum cholesterol, the mechanism of action is, as yet, not fully clear. Excretion of fecal bile acids and neutral sterols has been reported to increase after its ingestion indicating that there may be increased catabolism of cholesterol (3). It has also been suggested that Bengalgram may contain Biochanin A and Formononetin, both of which have estrogenic property, and that these are the active compounds, since rats fed these do show lowered levels of serum cholesterol (5). Dormant seeds of Bengalgram do not contain these isoflavones, but they are found in germinated seeds, presumably synthesized during germination (6) The hypocholesterolemic effect of Bengalgram is seen even with the use of ungerminated seeds (1-3), and this suggests two possibilities: (a) the seeds contain precursors of isoflavones which, during ordinary cooking conditions, are transformed to active compounds, or (b) the precursors themselves have cholesterol-lowering activity. Results of some studies have shown that p-coumaric acid is a precursor of isoflavones (7). Recently other legumes such as blackgram (8), soybean (9) and kidneybean (10) have also been found to have hypocholesterolemic activity. Studies were, therefore, undertaken to determine the isoflavone profile and content of Bengalgram and two other commonly used pulses effect of cooking and home processing on isoflavone content of Bengalgram, and effect of incorporating these isoflavones or their precursor to diets of rats to titrate their effects on serum and tissue lipids.

METHODS

Commercial varieties of Bengalgram (*C. arietinum*), greengram (*Phaseolus aureus*) and blackgram (*Phaseolus mungo*) were germinated for 72 hr. Fifty g samples of germs in duplicate from each type of seed were extracted with 85% hot methanol. The alcoholic extract was evaporated under vaccum to a thick slurry and taken up in 100 ml of 70% alcohol, extracted with petroleum ether (5 x 150 ml), the aqueous alcoholic phase separated and concentrated under reduced pressure to remove all the alcohol. The residue was repeatedly extracted with ether (6 x 150 ml), adding each time a small amount of NaCl crystals. The combined ether extract was dried over anhydrous sodium sulphate, filtered, and evaporated to dryness to obtain the crude isoflavone fraction. With respect to germ weight, the yield varied between 0.15 to 0.18% in all the pulses studied.

Separation of Isoflavones on Sephadex - G_{25}

Sephadex G_{25} (fine particles, size 20 to 80 μ) was allowed to swell in 0.1 M aqueous ammonium hydroxide overnight and made into a column 45 x 1 cm diameter.

The crude fraction was dissolved in aqueous NH_4OH as described by Nilsson (11). The ammoniacal solution (1 ml) was applied at the top of the column and eluted with 0.1 M NH_4OH at a flow rate of 1 ml/min. The absorption of eluted samples was recorded at 280 nm using a Beckman DB 2 spectrophotometer. A graph was plotted with extinction as abscissa and serial number of tubes as ordinate (Fig. 1).

Identification of Isoflavones

Fractions under eack peak were pooled separately and lypholyzed. The residue was crystallized in alcohol at room temperature. Identification of each isoflavone was made on the basis of Silica Gel G thin layer chromatography using propyl alcohol/ammonia (10:3) as eluant, melting point, infrared and ultraviolet spectrum.

Estimation of Isoflavones

The fractions containing individual isoflavones were combined, and the amount of each isoflavone was measured in a Beckman DU2 spectrophotometer against a blank (0.1 M ammonium hydroxide solution). Authentic

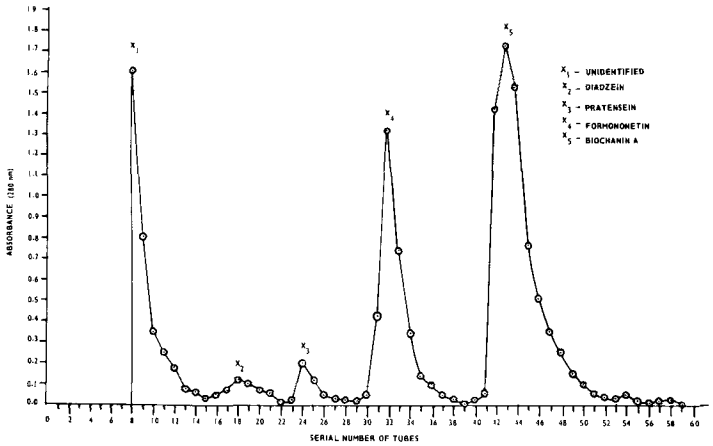

FIG. 1. Elution profile of isoflavones from bengalgram.

standard solutions of individual isoflavones were also measured likewise at maximum absorption.

Isolation and Identification of P-Coumaric Acid

The isolation of p-coumaric acid was carried out in the same manner as for isoflavones. The ether soluble fraction (isoflavone crude) was subjected to thin layer chromatography using Silica Gel G as absorbent and n-propyl alcohol/ammonia (10:3) as eluant. An authentic sample of p-coumaric acid was run concurrently. The spots on the chromatogram were located by ultraviolet light (Mineral light – 365 λ nm) and with spray reagents, diazotized sulphanilic acid being most suitable.

Effect of Cooking, Digestion and Home Processing on Isoflavone Content of Bengalgram

Bengalgram was subjected to the following processing and the isoflavone content was determined: cooked – 100 g of dry Bengalgram seeds boiled in 300 ml of water for 15 min; cooked and digested – the seeds cooked as described above and subjected to in vitro digestion for carbohydrate and proteins (12); home processed – seeds soaked for 24 hr in water and then boiled.

EXPERIMENTAL STUDY

A total of 84 male albino rats of the Institute's colony, weighing between 120 to 150 g, were used to evaluate the effect of crude isoflavone fractions obtained from various pulses on lipid levels. The animals were divided into 7 groups of 12 animals each. During the first 4 weeks, animals in all the groups were fed a hypercholesterolemia-inducing diet (H.I.D.) which consisted of % (w/w) casein 15.0; sucrose 66.6; hydrogenated vegetable oil 10.0; salt mixture (13) 4.0; cellulose 2.0; cholesterol 1.0; and cholic acid 0.2. From week 5 onwards, Group-A was continued on the H.I.D. alone, while the diets of groups B, C, and D were supplemented with 0.15%, 0.07% and 0.030% (w/w) crude isoflavone fraction isolated from Bengalgram. Diets of groups E and F were supplemented with 0.15% isoflavones obtained from greengram and blackgram, respectively. Group G received 0.15% of p-coumaric acid. Food and water were supplied ad lib.

Each rat was caged separately and a record of daily food consuption was maintained. Animals were weighed once a week. After 12 weeks on this dietary regimen, all animals were sacrificed; 4 to 6 ml of blood drawn by direct cardiac puncture and the liver, heart, and aorta removed. Serum cholesterol, both total and free, were estimated by the method of Zlatkis et al. (14), serum lipid phosphorus by that of Fiske and Subbarow (15), and triglycerides by the method of Van Handel and Zilversmit (16). Lipids from liver, heart, aorta were extracted using the Folch (17) technique, and total cholesterol and its fractions, lipid phosphorus and triglycerides were estimated by the methods used for serum.

RESULTS AND DISCUSSION

In Figure 1 is depicted the elution profile of isoflavones from Bengalgram. Fractionation of isoflavones using Sephadex G25 column has certain advantages. The contaminants in the crude extract are eluted before the isoflavones; the various components are clearly separated, and there is no mixing of one component with

TABLE I

Concentration of Isoflavones and p-Coumaric Acid in Various Pulses[a]

Pulses	Isoflavones (mg per 100 g of germ)				P-coumaric acid
	Pratensein	Daidzein	Formononetin	Biochanin A	
Bengalgram	4.8 ± 1.2	5.1 ± 0.8	44.1 ± 7.2	98.6 ± 11.6	D
Greengram	ND	80.7 ± 10.6	ND	ND	ND
Blackgram	ND	88.0 ± 7.8	ND	ND	D

[a]Values are means ± S.D. of 6 samples run in duplicate; D detected in mg concentration; ND not detected.

the others. Recoveries of individual compounds from known amounts of isoflavones were satisfactory. The mean percentage recovery and standard deviation for each isoflavone obtained from five separate determinations over a range of 100 - 200 μ g were as follows: Biochanin A, 98 ± 5.6; Formononetin, 92 ± 3.1; Daidzein, 85 ± 2.2; Pratensein, 85 ± 3.5.

The melting point and spectral data of various isoflavones isolated from these pulses were determined and the constants agreed well with those reported in the literature (18).

In Table I is presented the concentrations of isoflavones and p-coumaric acid in the three germinating pulses used in the experiment.

Bengalgram (germ) contained all the four isoflavones tested, while greengram and blackgram contained only diadzein. These two pulses did not contain any methylated isoflavones. P-coumaric acid was detected in bengalgram and blackgram, but not in greengram.

Seeds of bengalgram, which were subjected to soaking for 24 hr and then boiled (i.e., home processed), contained 12 to 20 mg of isoflavones per 100 g of dry seeds, while seeds which were not soaked, but cooked or cooked and digested *in vitro* did not contain any of these four compounds.

EXPERIMENTAL STUDY

Weight gain and daily diet consumption in the various groups of rats were essentially similar.

As shown in Table II, supplementation of diets with crude isoflavone isolated from germs of Bengalgram at a level of 0.15% in the diet was found to significantly reduce ($P<.01$) the elevated serum cholesterol levels in rats fed the H.I D. This was due mainly to a reduction in the ester fraction. Levels of lipid phosphorus and triglycerides in serum were unaffected. These findings are similar to those reported by Siddiqui and Siddiqui (5). The effect on lipids was dose dependent with no change in animals fed diets containing the two lower levels of crude isoflavones (0.075% and 0.030%).

The addition of crude isoflavone obtained from greengram had no effect on either cholesterol or triglyceride levels, but significantly lowered phospholipid concentration ($P<.05$). On the other hand, the addition of crude extracts from blackgram lowered both total cholesterol and ester cholesterol fractions. However, only the reduction in the ester fraction was statistically significant ($P<.05$).

Addition of p-coumaric acid to the H.I.D. (Group-G) resulted in a significant reduction in both total and esterocholesterol levels ($P<0.01$ and $P<0.001$, respectively). Lipid phosphorus and triglycerides were not affected.

The liver, heart and aorta lipids did not alter significantly ($P>0.05$) in any group as compared to that of the control group.

Data presented here confirm a previous observation that germinating seeds of Bengalgram lower serum cholesterol levels (19). Crude isoflavones obtained from Bengalgram consisted mainly of Biochanin A and Formononetin, both of which are estrogen-like compounds, and are most probably the active compounds which lowered serum cholesterol. Diadsein does not appear to possess hypocholesterolemic property, as evidenced by the fact that isolates from germs obtained from greengram and blackgram, when included in the diets of animals, did not reduce total serum cholesterol levels although some reduction in the ester fraction was seen. In another study recently completed in which pure Biochanin A, Formononetin and diadzein were fed separately to triton-treated rats, similar results were obtained (to be published). The hypocholesterolemic property of Biochanin A and Formononetin may be due to the presence of a methoxyl group in ring B (Fig. 2).

Methylated flavonoids are not only resistant to bacterial attack in the intestine, but are also biologically more active than their methyl-free counterparts (20,21). The mechanism of hypocholesterolemic action is not clear. Probably the several phenolic groups present in the isoflavone molecule enhance its affinity towards lipids and proteins, thereby affecting

TABLE II

Serum Lipid Levels (mg/100 ml Serum) for Various Groups of Rats[a]

Group	Dietary supplements	Level of supplement	Total cholesterol	Free cholesterol	Cholesterol ester	Lipid phosphorous	Triglycerides
A (12)[b]	H.I.D., alone (control)	---	542 ± 21[+]	90 ± 9	452 ± 19	9.0 ± 1.0	248 ± 18
B (12)	H.I.D. ± crude isoflavones from bengalgram	0.15%	435 ± 25[b]	113 ± 11	322 ± 29[b]	10.0 ± 2.2	193 ± 29
				NS		NS	NS[a]
C (12)	-do-	0.075%	485 ± 37	108 ± 9	377 ± 31[a]	8.0 ± 1.3	187 ± 22
				NS		NS	
D (12)	-do-	0.030%	503 ± 32	118 ± 13	385 ± 23[a]	8.0 ± 0.8	239 ± 26
			NS	NS		NS	NS
E (10)	H.I.D. + crude isoflavones from greengram	0.15%	541 ± 46	103 ± 17	438 ± 36	7.2 ± 0.3[a]	265 ± 37
			NS	NS	NS		NS
F (12)	H.I.D. + crude isoflavones from blackgram	0.15%	500 ± 34	116 ± 11	384 ± 26[a]	8.4 ± 0.7	320 ± 41
			NS	NS		NS	NS
G (10)	H.I.D. + p-Coumaric acid	0.15%	440 ± 16[b]	82 ± 9	358 ± 12[c]	8.4 ± 0.8	236 ± 25
				NS		NS	NS

[a]Mean ± Standard Error; Control Vs. Experimental; NS indicates not significant; a indicates <0.05 probably significant; b indicates <0.01 significant; c indicates <0.001 highly significant.

[b]1 Figure in parentheses indicates number of animals.

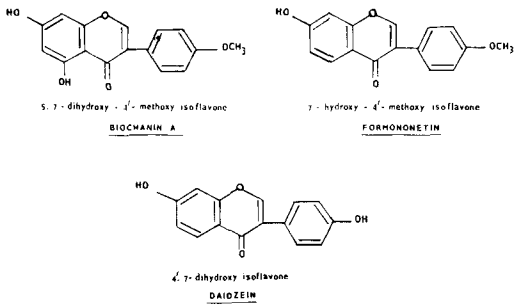

FIG. 2. Molecular structures of isoflavones isolated from pulses.

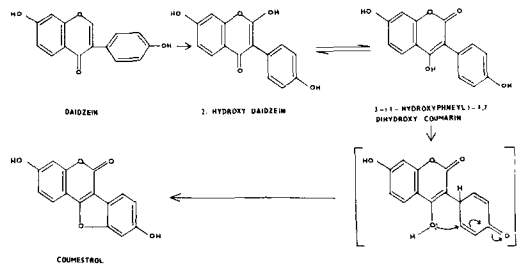

FIG. 3. Derivation of coumestrol (analogous to stilbestrol) by rearrangement of 2-hydroxy daidzein corresponding to daidzein.

lipoprotein metabolism in the animal system.

Addition of crude extract obtained from bengalgram and blackgram resulted in reduction of ester cholesterol. It is now considered that it represents a protective mechanism against development of atheroselerosis.

Esterified cholesterol in the form of low density lipoprotein (LDL) enters the smooth muscle cells of the arteries and, if in excess, accumulates within the cell. It triggers cell proliferation and subsequent disease process (22).

The oestrogen-like activity of isoflavones is considered to be due to the presence of -C = O group in ring-C, which after ring closure assumes a structure resembling that of stilbestrol (23). The estrogenic activity ranged Biochanin A < diadzein < Formononetin (24) (Fig. 3).

It would thus appear, that the hypocholesterolemic property and estrogenisity are two separate entitites and that these functions are dependent upon two different groups present in the molecules.

Isoflavones from bengalgram fed at the lower level (0.075% and 0.030%) could not reduce cholesterol significantly. Extrapolation of these findings to man indicate that one has to consume very large amounts of germinating seeds to have the desired effect on cholesterol. This, however, is contrary to studies in man, wherein habitual amounts (3) of even nongerminating but home processed seeds have been shown to lead to hypocholesterolemia.

In this context, the observation that the addition of p-coumaric acid in the H.I.D. of rats resulted in a significant reduction in cholesterol levels becomes relevant. The presence of this compound in crude extract obtained from bengalgram and blackgram acquires functional significance at least in part for their hypocholesterolemic activity. Probably it explains also the different effects of diets E and F, when they both contain same isoflavone. Phenolic acids can be converted into methyl derivatives by liver enzymes, thereby increasing their biological activity (25). Apparantly, pulses and other vegetarian foods might contain phenolic acids in relatively lesser amount, but many other compounds present in the diet may contribute to this acid pool in animal systems. Flavonoids could be catabolized to such aromatic acids by microbes in the lower intestinal tract and be absorbed (26). The average daily intake of flavonoids through Western diets is more than one gram.

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Variations in the Molecular Species of Lung Phosphatidylglycerol

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ABSTRACT

Approximately 20% of the phosphatidylglycerol of the lung tissue of several animals was found to have both fatty acids saturated. Pulmonary washings from the lung of the rabbit and guinea pig had more saturated phosphatidylglycerol than the washed lung tissue. Lung-saturated phosphatidylglycerol was relatively low in the perinatal period, a time during which saturated phosphatidylcholine accumulated predominantly. This suggests that the metabolism of the saturated species of lung phosphatidylcholine and phosphatidylglycerol, which are considered to be the major pulmonary surfactants, may not be regulated in the same manner, at least in the perinatal lung.

INTRODUCTION

Phosphatidylcholine, especially the dipalmitoyl species, is a major surface active component of pulmonary surfactant, the material which stabilizes the pulmonary alveoli againt collapse (1). Recently, it has been noted that another phospholipid, phosphatidylglycerol, may make an important contribution to the surface activity of the pulmonary surfactant (2-4). This is supported by the following findings: (a) phosphatidylglycerol is one of the characteristic lipids in lung surfactant fraction (5-7); (b) the lipid associated with the apoprotein isolated from canine surface active material showed a high content of phosphatidylglycerol (32% of total) (8); and (c) phosphatidylglycerol from the lung lavage of the Beagle dog had surface tension lowering properties similar to dipalmitoyl phosphatidylcholine (2). However, discrepant observations on the fatty acid patterns of phosphatidylglycerol isolated from the lung surfactant of various mammalian species have been presented. Some reports (2,6) described it as highly saturated, while others (5,9) found that its degree of saturation was much lower than that of phosphatidylcholine. However, no other reports have appeared on the molecular species of lung phosphatidylglycerol other than our original description of rat lung tissue (10). That study revealed that phosphatidylglycerol and diacylglycerol have considerable amounts of saturated species (20-22% of total). The present paper further describes the molecular composition of lung phosphatidylglycerol from several mammalian species. Furthermore, the molecular composition of phosphatidylglycerol in rat lung was compared with that of phosphatidylcholine in the perinatal lung, in which the content of saturated phosphatidylcholine has been known to change predominantly (11).

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EXPERIMENTAL PROCEDURES

Animals

Rats were maintained on a regular Oriental diet (Oriental Co., Tokyo) prior to the experiments. The composition of the diet was as follows: 6% safflower seed oil, 48% starch, 25% casein, 8% cellulose, 5% sucrose, 8% salt mixture and 2% vitamin mixture. The fatty acid fraction of safflower seed oil was 77% in linoleic acid. Rabbits and guinea pigs were maintained on a regular diet supplied by Japan Kurea Co., Tokyo. The content of fat in the diet was 3.3%, 43% of which was linoleic acid. Lungs from cows and pigs were obtained from a slaughterhouse. Fetuses and newborns of rats were obtained as described previously (11). Alveolar washings of rabbits and guinea pigs were collected by the lavage procedure using 50 ml of 0.9% saline five times for rabbits and 15 ml five times for guinea pigs. The lung washing was centrifuged at 600 x g for 10 min to remove cellular debris.

Preparation of Pure Glycerolipids and Resolution of the Glycerolipids into Molecular Species

Lung tissues were homogenized and extracted according to the method of Folch et al. (12). The lipid of the washing was extracted by the method of Bligh and Dyer (13). The lipid classes were separated by column chromatography on DEAE-cellulose into neutral lipids, neutral phospholipids and acidic phospholipids as described elsewhere (10,14). Individual glycerolipid was further purified by thin layer chromatography (TLC). 1,2-Diacylglycerol eluted with chloroform from the column was isolated by means of TLC on Silica Gel G plates with a solvent system of hexane/ether/acetic acid (50:50:1, by vol). Phosphatidylcholine eluted with methanol from the column was purified as described previously (14). After the elution of phosphatidylserine with acetic acid followed by methanol washing of the column,

TABLE I

Fatty Acid Composition of Lung Phosphatidylglycerol in Various Mammalian Species[a]

Fatty acid	Pig (5)[b] Whole lung tissue	Cow (4) Whole lung tissue	Rabbit (4) Washing	Rabbit (4) Washed Lung	Guinea pig (5) Washing	Guinea pig (5) Washed lung
14:0	0.6 ± 0.1	0.6 ± 0.2	0.9 ± 0.8	0.4 ± 0.1	1.6[c]	1.6 ± 0.4
16:0	27.1 ± 4.8	34.4 ± 4.0	61.7 ± 9.2	29.0 ± 6.4	52.8	36.5 ± 6.6
16:1	2.3 ± 0.3	1.0 ± 0.2	3.3 ± 1.5	2.5 ± 1.4	6.6	6.4 ± 0.6
18:0	21.1 ± 3.5	15.2 ± 0.8	4.7 ± 0.9	19.3 ± 5.4	8.3	17.6 ± 5.2
18:1	34.2 ± 5.1	37.3 ± 4.7	24.7 ± 7.2	27.0 ± 3.3	26.0	23.5 ± 5.3
18:2	6.5 ± 1.5	2.5 ± 0.9	4.0 ± 1.0	7.5 ± 1.0	3.6	4.9 ± 0.9
18:3	0.8 ± 0.1	2.4 ± 0.3	—	1.3 ± 0.2	0.3	1.7 ± 0.5
20:1	0.8 ± 0.1	0.6 ± 0.5	—	1.4 ± 0.1	0.2	0.2 ± 0.1
20:3	1.0 ± 0.7	1.1 ± 0.2	—	1.0 ± 0.7	0.6	0.4 ± 0.2
20:4	3.4 ± 0.4	2.7 ± 0.5	—	3.9 ± 0.8	—	3.2 ± 0.8
22:4	0.8 ± 0.3	0.9 ± 0.1	—	2.7 ± 1.0	—	1.6 ± 0.8
22:5	0.5 ± 0.2	1.0 ± 0.6	—	3.0 ± 1.1	—	0.9 ± 0.3
22:6	0.8 ± 0.2	0.6 ± 0.2	—	1.0 ± 0.3	—	0.8 ± 0.2

[a]Values are the mean ± S.D., expressed as percent of total fatty acid in the mixture. Minor components have been omitted.

[b]Numbers in parentheses are numbers of animal analyzed.

[c]The values were obtained from pooled samples from five cases.

the remaining acidic lipids were eluted with chloroform/methanol (2:1) containing 2% of 28% aqueous ammonia. Phosphatidylglycerol was then purified by TLC on Silica Gel H using a solvent system of chloroform/methanol/7N ammonia (65:35:5, by vol). Phosphatidylglycerol was eluted from the gel with chloroform/methanol/formic acid/water (97:97:4:2, by vol) as described by Rooney et al. (5). The yield of phosphatidylglycerol in this isolation procedure was 2.8% (mean of five rat lungs) for total lipid phosphorus. In order to determine the content of phosphatidylglycerol, the acidic lipid fraction was separated by two dimensional TLC using solvent systems of chloroform/methanol/conc. ammonia (65:30:5, by vol) in *x* dimension and chloroform/acetone/methanol/acetic acid/water (4:3:1:1:0.5, by vol) in *y* dimension (15). After detection with iodine vapor, the phosphorus of the phosphatidylglycerol spot was directly determined. Phosphatidylglycerol and phosphatidylcholine thus obtained were converted into 1,2-diacylglycerol by phospholipase C from *Bacillus cereus* and *Clostridium welchii*, respectively. The 1,2-diacylglycerol formed from the phospholipids was extracted three times from the incubation mixture with ether. The ether extracts were evaporated under nitrogen and immediately acetylated with acetic anhydride and anhydrous pyridine. The diacylglycerol acetates were purified and recovered as described by Kuksis et al. (16). The diacylglycerol acetates were resolved into molecular species according to the degree of unsaturation by means of argentation TLC. Silica Gel H plates containing 10% $AgNO_3$ were first developed to a height of 8 cm with chloroform/methanol (95:5), dried under nitrogen, and then further developed to a height of 18 cm with chloroform. The diacylglycerol acetates were recovered from the gel by the method of Arvidson (17). Each diacylglycerol acetate was isolated as the individual molecular species on gas liquid chromatography, using a pyrex column (50 cm x 4 mm o.d.) packed with 1% silicone OV-1 on Gaschrom Q at 290 C. Peak identity of the diacylglycerol acetates was made by comparing with calibration standards composed of dimyristoyl, dipalmitoyl, distearoyl and diarachidoyl acetates.

Analytical Methods

Phosphorus was determined by the method of Bartlett (18). The amount of diacylglycerol acetates was estimated by glycerol determination according to the method of Van Handel and Zilversmit (19). The DNA content was determined by the method of Schneider (20) using samples homogenized in 10% trichloroacetic acid. Fatty acid methylesters obtained by the BF_3-methanol method (21) were analyzed on a 2 m x 4 mm o.d. pyrex column packed with 10% diethleneglycol succinate on Shimalite W at 185 C, in conjunction with Shimadzu chromatopac E-1A.

RESULTS AND DISCUSSION

Table I shows results on the fatty acid analysis of lung phosphatidylglycerol from pig, cow, rabbit and guinea pig. Almost identical fatty acid profiles were noted in phosphatidyl-

TABLE II

Composition of Molecular Classes of Lung Phosphatidylglycerol in Various Mammalian Species[a]

	Pig (5)[b]	Cow (4)	Rabbit (4)		Guinea pig (5)	
	Whole lung tissue	Whole lung tissue	Washing	Washed lung	Washing	Washed lung
Phosphatidylglycerol content (nmoles/mg DNA)	104.5 ± 12.2	121.3 ± 18.6	19.0 ± 5.1[c]	92.0 ± 7.3	19.3 ± 3.2[c]	112.6 ± 19.8
Molecular class (Percent)						
Saturates	15.5 ± 1.0	15.8 ± 3.0	28.0 ± 0.4	14.5 ± 1.6	32.7	19.0 ± 2.7
Monoenes	57.5 ± 2.1	59.1 ± 3.6	58.1 ± 1.1	60.2 ± 3.3	58.4	53.4 ± 4.2
Dienes I (MM)[d]	7.7 ± 2.3	8.8 ± 1.8	4.0 ± 0.9	6.4 ± 0.7	6.2	6.8 ± 1.2
Dienes II (SD)[d]	4.9 ± 2.0	5.8 ± 2.0	4.6 ± 0.2	10.5 ± 1.7		9.7 ± 2.3
Polyenes[e]	14.9 ± 1.9	10.4 ± 2.1	5.3 ± 1.5	18.1 ± 4.2	2.7	10.1 ± 1.8

[a]Values are the mean ± S.D., expressed as percent of total molecular class.

[b]Numbers in parentheses are numbers of animal analyzed.

[c]The values were obtained on the basis of DNA values in the washed lung.

[d]MM and SD represent monoenoic-monoenoic and saturated-dienoic species, respectively.

[e]The polyenes include *trienoic, tetraenoic, pentaenoic and hexaenoic species.*

[f]The values were obtained from samples pooled from five cases.

glycerol of the lung tissue from the mammalian species. The major fatty acids were 18:1, 16:0 and 18:0, in that order. As compared to the results reported for rat previously (10), lower 16:0, 18:2, 20:4 and 22:6, but significantly higher 18:0 and 18:1 were noted. This difference is probably due to the higher linoleate content of the diet used for rat. In all mammalian species examined, ca. 50% of the fatty acids of phosphatidylglycerol from the lung tissue were saturated. On the other hand, in the washings from rabbit and guinea pig, 16:0 accounted for over 50%. This was also much higher than in the washed lung (29-36%). This fatty acid pattern of phosphatidylglycerol from lung tissue and washings generally agrees with previously published data for this phospholipid (5,9).

As seen in Table II, the amount of phosphatidylglycerol in lung tissue was in the range of 105-132 nmoles/mg DNA among mammalian species examined. Approximately one-fifth of the phosphatidylglycerol in lung tissue was found in the washings (rabbit and guinea pig). It has been observed that ca. one-tenth of phosphatidylcholine in lung tissue occurs in the washings (22,23). Hence, phosphatidylglycerol seems to be distributed more in alveolar space than in lung tissue, as compared to phosphatidylcholine distribution in the lung.

Table II gives a comparison of the proportion of molecular classes, characterized by their degree of unsaturation, of lung phosphatidylglycerol from several mammalian species. The molecular class composition of phosphatidylglycerol from the lung tissue were similar to one another among pig, cow, guinea pig and rabbit. The mostly predominant molecular class was monoenes. These profiles of molecular class composition agree with the findings on their fatty acid patterns. The percentages of saturated phosphatidylglycerol (15-19% of total) in the lung tissue were not significantly different among the mammalian species. The percentages of the saturated class in the washing of rabbit and guinea pig were significantly higher (28-33%) than in the lung tissue (15-19%). The major individual molecular species of saturated phosphatidylglycerol was the dipalmitoyl compound in all samples examined (Table III). Palmitoyl-myristoyl and palmitoyl-stearoyl species were in small quantities. This analysis of molecular species of lung phosphatidylglycerol is in good agreement with the tendency obtained with the molecular species of lung phosphatidylcholine, i.e., little variation in saturated species concentration among mammalian species and a higher content of saturated species in pulmonary washing than in lung tissue (23). However, the degree of saturation of lung phosphatidylglycerol seems to be lower than lung phosphatidylcholine. In rat lung tissue, the percentage of saturated phosphatidylcholine is 33-35% (10,14,24,25), while that of saturated phosphatidylglycerol is 22% (10). Rooney et al. (5) also described that 50% of the fatty acids in phosphatidylglycerol are saturated, but over 60% in phosphatidylcholine are saturated in rabbit pulmonary wash. On the other hand, King et al. (8) reported that the

TABLE III

Composition of Major Individual Molecular Species of Lung Phosphatidylglycerol in Various Mammalian Species[a]

Molecular classes	Molecular species[b]	Pig (5)[c] Whole lung tissue	Cow (4) Whole lung tissue	Rabbit (4) Washing	Rabbit (4) Washed lung	Guinea pig (5) Washing	Guinea pig (5) Washed lung
Saturates	16:0/14:0	1.3 ± 0.2	0.8 ± 0.2	2.8 ± 0.6	1.5 ± 0.3	2.3[d]	1.5 ± 0.4
	16:0/16:0	12.3 ± 0.3	13.9 ± 0.4	23.1 ± 0.7	12.0 ± 0.4	30.1	17.1 ± 1.2
	16:0/18:0	0.8 ± 0.2	1.1 ± 0.3	2.0 ± 0.6	1.0 ± 0.3	0.3	0.3 ± 0.1
Monoenes	16:0/16:1	1.8 ± 0.2	3.8 ± 1.0	9.8 ± 0.5	6.9 ± 1.1	9.6	6.4 ± 0.8
	16:0/18:1	19.5 ± 3.0	42.4 ± 2.9	45.5 ± 3.4	33.8 ± 4.1	17.6	31.1 ± 4.2
	18:0/18:1	27.2 ± 3.1	12.9 ± 3.7	2.8 ± 0.4	17.3 ± 4.7	1.1	15.8 ± 3.7
Dienes I (MM)	16:1/18:1	3.1 ± 0.7	1.6 ± 0.4	0.2 ± 0.1	0.5 ± 0.2		1.2 ± 0.4
	18:1/18:1	4.4 ± 0.6	7.1 ± 0.5	1.2 ± 0.1	3.0 ± 0.3	6.2	5.4 ± 0.6
Dienes II (SD)	16:0/18:2	2.4 ± 0.1	4.2 ± 0.4	4.1 ± 0.1	6.7 ± 0.9		6.9 ± 1.2
	18:0/18:2	2.1 ± 0.3	1.6 ± 0.4	0.5 ± 0.1	3.7 ± 0.8		2.7 ± 1.1
Polyenes[e]		14.9 ± 1.9	10.4 ± 2.1	5.3 ± 1.5	18.1 ± 4.2	2.7	10.1 ± 1.8

[a]Values are the mean ± S.D., expressed as percent of total molecular species. Minor molecular species have been omitted.

[b]No positional specificity is implied in the symbolism of molecular species.

[c]Numbers in parentheses are numbers of animals analyzed.

[d]The values were obtained from samples pooled from five cases.

[e]The polyenes include trienoic, tetraenoic, pentaenoic and hexaenoic species.

11,000 dalton apoprotein isolated from canine lung surface active materials contains a uniquely high content of phosphatidylglycerol (32%), the fatty acid composition of which is similar to phosphatidylcholine, containing 16:0 as the major fatty acid (65-70%). The physiological function of phosphatidylglycerol in pulmonary surfactant system remains unknown. Hallman and Gluck (26) suggest that, although phosphatidylglycerol is not an essential component required for formation of the surfactant complex, it is highly important for improving the properties of surfactant, particularly after birth.

The developmental changes of the percentage of molecular classes different in their unsaturation degree of rat lung phosphatidylglycerol, phosphatidylcholine and diacylglycerol are given in Table IV. The developing profile of molecular species of lung phosphatidylglycerol was significantly different from that of phosphatidylcholine, particularly in the saturated species. The concentration of saturated phosphatidylcholine increased sharply during the latter stages of fetal development. In contrast, the concentration of saturated phosphatidylglycerol was very low at the perinatal periods and increased gradually from perinatal periods to adult. These results indicate that the content of saturated phosphatidylglycerol was significantly lower in the perinatal stages when saturated phosphatidylcholine accumulated predominantly in accordance with the appearance of Type II cells in the lung (27). This does not suggest that saturated phosphatidylglycerol is a primary component of surfactant necessary for air-breathing immediately after birth. These results agree with the findings on lung surfactant phospholipids of rabbits reported by Hallman and Gluck (26), which indicated that the most prominent acidic surfactant phospholipid of fetal lung is phosphatidylinositol and that phosphatidylglycerol appears at term and increases after birth.

Table IV also shows data on the molecular species of lung diacylglycerol during perinatal development. Interesting is the finding that, although rather high proportions of polyenes were found in diacylglycerol, the developing profile of the saturated species was very similar to that of saturated phosphatidylglycerol, but different from that of saturated phosphatidylcholine. These findings suggest that the saturated phosphatidylglycerol may be synthesized via phosphatidic acid (28) in the perinatal lung if the pattern of molecular species of diacylglycerol analyzed can be taken to reflect that of phosphatidic acid. Several investigators (14,29-31) have demonstrated that saturated phosphatidylcholine may be synthesized by auxillary mechanisms from unsaturated lecithins formed via a de novo pathway and the synthesizing activity, especially lysophosphatidylcholine/lysophosphatidylcholine transacylase, may increase predominantly in the last stages of gestation (11,30) in order to provide for the increase in dipalmitoyl phosphatidylcholine in the lung. Present results further support the above idea,

TABLE IV

Composition of Rat Lung Phosphatidylglycerol, Phosphatidylcholine and Diacylglycerol at Different Developmental Periods[a]

	Content (nmoles/mg DNA)[b]	Percent composition			
		Saturates	Monoenes	Dienes	Polyenes[c]
Phosphatidylglycerol					
-3 day (2)[d]	18	11.5	41.9	12.0	34.6
-1 day (3)	58 ± 4	14.6 ± 1.5	38.2 ± 3.2	13.8 ± 1.2	33.4 ± 3.5
+2 day (3)	52 ± 3	18.5 ± 1.2	34.4 ± 3.0	15.3 ± 1.4	31.8 ± 3.6
Adult (4)	129 ± 11	22.2 ± 2.3	28.5 ± 3.0	21.2 ± 2.0	28.1 ± 2.5
Phosphatidylcholine					
-3 day (2)	828	19.2	38.4	15.6	26.7
-1 day (4)	1308 ± 24	28.7 ± 2.7	38.3 ± 3.5	14.6 ± 2.8	18.5 ± 1.5
+2 day (4)	1556 ± 121	35.1 ± 1.4	31.3 ± 1.6	12.0 ± 1.3	21.6 ± 3.7
Adult (4)	1790 ± 194	33.5 ± 3.8	23.8 ± 1.0	16.2 ± 1.8	26.5 ± 3.3
Diacylglycerol					
-3 day (2)	11	15.6	24.9	11.3	48.1
-1 day (2)	13	18.3	23.8	8.3	49.5
+2 day (2)	18	19.4	22.3	12.4	45.9
Adult (4)	55 ± 5	24.1 ± 3.9	19.9 ± 1.3	16.1 ± 0.8	39.9 ± 8.4

[a]Values are the mean ± S.D.

[b]The DNA values (mg/g wet tissue) were 8.21 in -3 day, 8.41 ± 0.21 in -1 day, 9.96 ± 0.85 in +2 day and 6.20 ± 0.51 in adult, respectively.

[c]The polyenes include trienoic, tetraenoic, pentaenoic and hexaenoic species.

[d]The numbers in parentheses represent numbers of separate samples analyzed.

since the developing profiles of saturated species of phosphatidylcholine and diacylglycerol are different and the rather lower amounts of saturated diacylglycerol may be unable to supply sufficient amounts of saturated phosphatidylcholine via a de novo pathway alone.

The results reported in the present communication lead us to the idea that the metabolism of the saturated species of lung phosphatidylcholine and phosphatidylglycerol, which can be considered to be the major pulmonary surfactants, may not be regulated in the same manner, at least in the perinatal lung.

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Incorporation of Deuterium-Labeled *cis*- and *trans*-9-Octadecenoic Acids in Humans: Plasma, Erythrocyte, and Platelet Phospholipids

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ABSTRACT

The objective of this study was to follow the uptake and distribution of oleic and elaidic acids into human erythrocytes, platelets, and plasma phospholipids. The use of dual and triple labeling methodology permitted a precise comparison of elaidic and oleic acid utilization. Elaidic acid (EI) was selectively concentrated in all the plasma phospholipids except for lysophosphatidylcholine. Three times more elaidic than oleic acid (OI) accumulated in the 1-acyl position of phosphatidylcholine, as determined by hydrolysis with phospholipase A_2. Rapid incorporation and removal of elaidate were observed for all samples. These results support the concept that enzymes responsible for acylation of phospholipids are sensitive to double bond configuration and the physical properties of the fatty acid moieties. Labeled fatty acid levels in red cell and platelet phospholipids were much lower than for plasma phospholipids, indicating a relatively slow rate for the in vivo incorporation of fatty acids into blood cell membrane phospholipids. No isotope effect was found when oleic acid labeled with deuterium on the double bond was used.

Over 5.5 billion lb. of vegetable oil is hydrogenated annually for use in margarines, salad oils, cooking fats, and shortening (1). This hydrogenation process results in the formation of ca. 15% *trans* and positional fatty acid isomers (2-8). These oils are a major source of dietary fat, and it is estimated the average American diet contains ca. 8% *trans* fatty acid. Metabolism and biological effects of isomeric fatty acids have been studied in rats, monkeys, chickens, swine, rabbits, and man. The uptake and deposition of *trans* isomers into rat (9), swine (10), and human tissue (11) has been documented. Specific geometric and positional fatty acid isomers have been shown to be incorporated into egg yolk lipids (12-14), human blood plasma (15), rat liver (16-20), rat fetuses (21), rabbit serum lipids (22), and *Escherichia coli* membranes (22-27).

Hydrogenated fats have been correlated with an increase in serum cholesterol and triglyceride levels in humans (28-32), increase in fragility of erythrocytes (17), alteration of the lipid transport function of rat mitochondria (17), increase in essential fatty acid requirements (33-34), increase in long chain unsaturated fatty acids content of rat phospholipid (35-36), increases in serum cholesterol levels in swine (37) and rabbits (38,39), alterations in membrane function in *E. coli* (26,27), and increase in rate of de novo fatty acid synthesis from acetate in rat liver (40).

However, contradictory results have been reported by several researchers. For example, serum cholesterol levels in man have been reported both to be elevated and to remain unchanged by diets containing hydrogenated fats. Long term feeding studies have indicated no change in the growth, reproduction, longevity, or size of mice and rats fed hydrogenated fats (41-46).

The results reported in previous investigations with human subjects have all involved hydrogenated fat that is a mixture of isomeric fats. This study specifically compares the utilization of oleic to elaidic acid in human phospholipids and is an extension of previous data reported for human plasma neutral lipids (47).

EXPERIMENTAL

Experimental Design

The metabolism of elaidic (E1) acid and oleic (O1) acid in man was compared by feeding pairs of deuterated analogues of these acids. In these dual-labeled experiments, the deuterium label on at least one of the pairs of fatty acids was placed in a different position, and the number of deuterium labels per mole-

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

TABLE I

Amount and Identity of Dueterated Triolein and Trielaidin Fed to Humans

Subject	Fatty acid fed[a]	Total wt fed (g)	El(%)	01(%)	El/01 ratio
1	El-9,10-d_2 + 01-12,12,13,13-d_4	36.2	48.7	51.3	0.95
2	El-8,8,11,11,-d_4 + 01-9,10-d_2	34.0	46.5	53.5	0.87
3[b]	El-13,13,14,14-d_4 + 01-9,10-d_2 + 01-8,8,13,13,14,14-d_6	35.5	31.4	68.6[c]	0.91

[a]El = elaidic acid and 01 = oleic acid; numbers indicate position of deuterium on the acyl chain of the fatty acid.

[b]El-d_4/01-d_6 ratio fed = 0.91; 01-d_2/01-d_6 ratio fed = 0.98; El-d_4/(01-d_2 + 01-d_6) ratio fed - 0.46.

[c]Contains 34.0% 01-9,10-d_2 and 34.6% 01-8,8,13,13,14,14-d_6.

cule of fatty acid was varied as shown in Table I. This technique was used to detect analytical or biological isotope effects due to the position or number of deuterium labels. In the case of the third subject, 01-d_2, 01-d_6, and El-d_4 were fed in a triple-labeled experiment in order to detect specific deuterium isotope effects that may occur because of the presence of deuterium on the 9,10-double bond position. In dual- and triple-labeled feeding experiments, the same analytical and biological variation must occur equally to both fatty acids since they are fed to the same subject at the same time. Each subject acts as his own control and thus, a comparison of El and 01 incorporation and disappearance in a dual-labeled experiment is more accurate than if only one labeled fatty acid was fed to each subject. Consequently, the effect of dietary, genetic, or biological variation on comparison of fatty acid utilization is reduced in the dual-labeled experiments.

Calculation of selectivity factors. Selectivity factors for comparing the relative utilization of El vs. 01 can be calculated and used to indicate the preference for elaidic acid uptake compared to oleic acid. Such calculations are not simple for experiments in which a single fatty acid is fed to a limited number of subjects, because variations in the fatty acid metabolism between subjects can be so large that the results are not statistically significant. Selectivity factors used in this paper are calculated by dividing the El/01 ratio found in the lipid fraction by the El/01 ratio of the fed mixture. A selectivity factor greater than 1 indicates preferential incorporation of elaidic acid, and a value less than 1 indicates preferential incorporation of oleic acid. Selectivity factors are used also to compare data when subjects are fed mixtures containing different El/01 ratios.

Deuterated Elaidic and Oleic Acids Fed

Oleic acid-9,10-d_2 (15), oleic acid-12,12,13,13-d_4 (15), oleic acid-8,8,13,13,14,14-d_6 (47), elaidic acid-9,10-d_2 (15), elaidic acid-8,8,11,11-d_4 (47), and elaidic acid-13,13,14,14-d_4 (15) were synthesized and converted to their respective triglycerides as previously described.

Feeding and blood sampling. Three male Caucasians, ages 23-27, were given mixtures of labeled triglycerides (Table I) emulsified with casein (30 g), dextrose (30 g), sucrose (15 g), and water (200-250 ml). The subjects were medical students who were in good health as judged by their general appearance, no apparent illness, or congenital ailments. Their weight, blood pressure, serum cholesterol, and triglyceride levels were normal. The diets were not controlled before or after administering the deuterated triglycerides except that food was withheld 10 hr before feeding and the subjects ate nothing for 4 hr after consuming the labeled fats except for juice or coffee. The subjects were requested not to eat high-fat meals for 12 hr after they drank the mixture of deuterated triglycerides.

Blood samples (40 ml) were drawn at 0, 2, 4, 6, 8, 12, 16, 24, and 48 hr intervals after the mixture was consumed. the 0-hr sample was used to determine fatty acid composition before the deuterated fat was ingested. Blood samples (50 to 100 ml) were also drawn at 6, 12, and 24 hr for platelet isolation. Procedures for drawing blood samples, isolating red cells, plasma, and platelets, and extracting lipids have been described previously (15,48).

Separation and Derivatization of Phospholipids

The phospholipids were extracted (15) and separated by preparative thin layer chromatography (TLC) on 2-mm silica gel plates. A chloroform/methanol/petroleum ether/water (8:8:6:1) solvent mixture was used as the developing system (49). Phospholipid bands were visualized by exposing the edges of the TLC plates to iodine vapor. The bands were then identified by comparing them to authentic standards that were chromatographed on the

same plate. The phospholipid bands were scraped from the TLC plate and extracted two times from the silica gel using 2:1 $CHCl_3$/MeOH followed by a single extraction with 3:5:2 $CHCl_3$/MeOH/H_2O (50). After extraction of the phospholipids either from the blood samples or from silica gel, the temperature of the extract was kept below 30 C and never evaporated to dryness. All phospholipid extracts were stored in 19:1 chloroform/methanol at -25 C under nitrogen. These precautions are required to prevent phospholipid oxidation, poor resolution by TLC, and loss of sample. Antioxidants were not needed to prevent autoxidation if the samples were isolated according to the above procedure.

Phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and lysophosphatidylcholine (lyso PC) were methylated by mixing with 1 ml dry benzene and 2 ml 5% HCl-methanol, flushing with nitrogen, and heating at 65 C for 2 hr in screw-cap culture tube. Sphingomyelin (SM) was methylated by mixing with 1 ml dry benzene and 2 ml 10% HCl-methanol and heating at 65 C for 5 hr (51).

Phospholipase A_2 hydrolysis of PC with *Ophiophagus hannah* venom (Ross Allen Reptile Institute, Silver Springs, FL) was used to remove the 2-acyl fatty acid (52). Products from the reaction mixture were purified by streaking the entire reaction mixture on a 2 mm preparative silica gel TLC plate and developing with the chloroform/methanol/petroleum ether/water (8:8:6:1) solvent system. The lysophosphatidylcholine (PC-1) and free fatty acid (PC-2) reaction products from phospholipase A_2 hydrolysis of PC were extracted from the silica gel, recovered, and methylated by the benzene-5% HCl-methanol procedure.

Analysis of Phospholipids

The methyl esters of the separated phospholipids were analyzed for deuterium-labeled fatty acid content by combined gas chromatography-mass spectrometry (GC-MS) using selected ion monitoring of the molecular ion or the molecular ion-31 peaks. A Nuclide Model 12 90 DF mass spectrometer equipped with a silicon rubber membrane separator was employed to separate the carrier gas from the fatty methyl esters. The small sample size and large number of analyses did not permit extensive replication and classic statistical analysis of the data in Table II. The data was determined to be accurate and significant because of three factors: (1) data plotted in Figures 1 through 6 yield curves which are generally smooth; (2) for those samples where there was sufficient sample

TABLE II

Analysis of Plasma Phospholipids

Subject	Phospholipid[a]	Time, hr	Selectivity for E1[b]
1	PE	6	1.50
2	PE	8	1.55
3	PE	6	2.82[c]
1	PS	8	0.80
2	PS	12	1.08
3	PS	8	2.29[c]
1	PC	12	1.40
2	PC	12	1.08
3	PC	16	1.54[c]
1	PC-1	12	3.39
2	PC-1	16	3.25
3	PC-1	12	4.40[c]
1	PC-2	12	0.83
2	PC-2	16	0.31
3	PC-2	12	0.90[c]
1	SM	12	1.24
2	SM	12	3.50
3	SM	12	1.24[c]
1	Lyso-PC	8	0.73
2	Lyso-PC	8	0.92
3	Lyso-PC	8	4.35[c]

[a]Phospholipid abbreviations: PE = phosphatidylethanolamine; PS = phosphatidylserine; PC - phosphatidylcholine; PC-1 = 1-acyl phosphatidylcholine; PC-2 = 2-acyl phosphatidylcholine; SM = sphingomyelin; Lyso-PC = lysophosphatidylcholine.

[b]E1 = elaidic acid.

[c]E1 selectivities for subject 3 phospholipids are based on E1-d_4/01-d_6 ratios.

for replicate analyses, the replicates agreed within several tenths of a percent; and (3) the selective-ion-monitoring analytical procedure has been extensively studied and found to be reliable (53). In a study on the accuracy of the selective-ion-monitoring GC-MS procedure, nine replications were made for each of nine dilutions, which showed that a linear regression line could be drawn through the data such that the percent relative standard deviation is equal to $2.2\sqrt{W}$, where W equals the weight of the deuterated fatty acid in the sample. If this same error is projected to Table II, the expected standard deviations would be in the range of 0.2 to 0.9%, depending on the amount of sample available for analysis and the amount of label in the sample. The larger standard deviation occurs for those samples containing the smallest amount of sample and lowest percentage of deuterated fatty acids.

The total fatty acid profile of the lipid fractions was determined on a Packard Model 7400 gas chromatograph equipped with flame ionization detectors and a 6 ft X 4 mm 10% EGSS-X column. Methyl elaidate and methyl

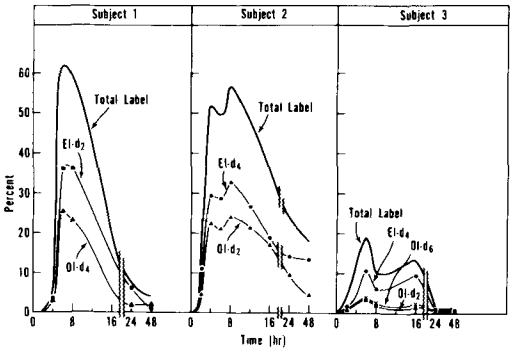

FIG. 1. Incorporation of deuterated oleic acid (O1) and elaidic acid (E1) into plasma phosphatidylethanolamine of three human subjects. The curves identified as O1, E1, and "total label" give the percent deuterated O1, E1, and O1 + E1 contained in the total octadecenoate portion of the samples. See Table I for composition of the deuterated triglyceride mixture fed.

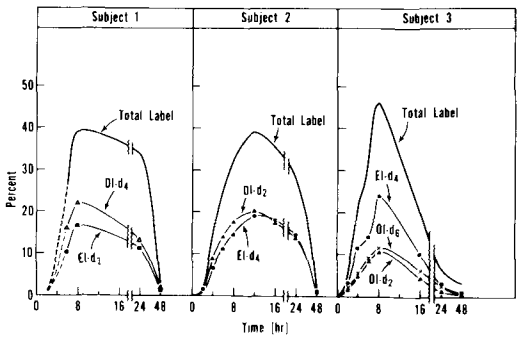

FIG. 2. Incorporation of deuterated O1 and E1 into plasma phosphatidylserine of three human subjects. The curves identified as O1, E1, and "total label" give the percent deuterated O1, E1, and O1 + E1 contained in the total octadecenoate portion of the samples. See Table I for composition of the deuterated triglyceride mixtures fed.

oleate were analyzed on a 20 ft x 4 mm 10% silar 10 C glass column or a 20 ft X 4 mm 15% OV 275 glass column, and their percentages were calculated by integration of the peak areas.

RESULTS AND DISCUSSION

Uptake of Deuterated Fatty Acids by Plasma Phospholipids

Plasma-PE. Plasma-PE data from all subjects in Figure 1 show a definite preference for incorporation of elaidic acid compared to oleic acid. Total label curves in Figure 1 indicate a considerable variation in the uptake of labeled fat by each subject. Deuterated fatty acids accounted for 61.7% of the octadecenoic acids in plasma-PE of subject 1 compared to 19.4% for subject 3. These differences in total uptake reflect variation in our subjects due to unknown causes. This variation did not change the enzyme specificity for double bond configuration since all subjects show similar selectivities for E1. O1-d_2 and O1-d_6 curves in Figure 1 for subject 3 are similar and the O1-d_2/O1-d_6 ratio is the same as in the fed mixture. These data indicate the enzymatic reactions involved in plasma-PE synthesis are not changed by the deuterium on the 9,10-double bond position of O1-d_2.

Plasma-PS. O1 and E1 curves shown in Figure 2 for phosphatidylserine (PS) from subjects 1, 2, and 3 indicate contradictory results. O1 curves for subjects 1 and 2 show a preference for oleic acid incorporation, but the O1 curves from subject 3 indicate a selection for elaidic acid. O1-d_2 and O1-d_6 curves in Figure 2 for subject 3 show slightly less O1-d_2 is incorporated than O1-d_6 due to the deuterium on the double bond. Because O1-9,10-d_2 was fed to subject 2, the selectivity factor for O1 utilization may be slightly diminished by a small dueterium isotope effect. Maximum total labeled fatty acid incorporation is similar for all subjects in Figure 2 although the general shapes of the "total label" curves are different. As previously noted for plasma-PE, differences in the "total label" curves are caused by unknown factors. Lack of published data on labeled fat metabolism in humans prevents comparison of these curves with the results of other studies. These results indicate a specificity that varies from individual to individual for double bond configuration similar to that for plasma-PE, which is sensitive to double bond configuration.

Plasma-PC. Phosphatidylcholine data are given in Figure 3. The data are inconsistent since the preference for elaidic acid utilization observed for subject 1 disappears in the data for subject 2 and is only significant for the 16 hr sample in subject 3. Factors such as diet or difference in metabolic activity or stimulation should have an equal effect on both O1 and E1 utilization and thus are not expected to cause these inconsistencies. Variation in plasma-PC data may reflect differences in enzyme specificities, but the reason for altered enzyme specificities between subjects is unknown. The shape of the "total label" curves and the percentage of labeled fatty acid incorporated are similar except for the dip in the E1-d_4 curve for the 12-hr sample from the third subject. The "total label" curves suggest that the overall lipid metabolism of the subjects are similar, but the E1 curves indicate that there are differences

in the three subjects' abilities to specifically utilize E1.

The E1-d_4 content of the 12-hr sample from subject 3 in Figure 3 would need to be ca. 12% in order to form a smooth curve. The dip in the curve cannot be explained except to postulate that the elaidic acid was specifically mobilized to supply calories or for some other use.

Plasma PC-1. Curves in Figure 4 represent the fatty acid in the 1-acyl position of phosphatidylcholine. Analysis of the fatty acids in the one position of PC allows a more detailed understanding of the uptake of elaidate than can be obtained from the total PC data shown in Figure 3. Unfortunately the opportunity to analyze the positional fatty acids in all the phospholipid fractions was not available because of small sample size. The preferential incorporation of E1 into the 1-acyl position of PC is much larger than was found for total PC in Figure 3. These data are consistent with in vitro studies using rat liver enzymes (52,53) which showed that E1 is preferred over O1 as the substrate for 1-acyl-CoA:phospholipid acyl transferase acylation of the 1 position of PE and PC. These in vivo human studies confirm the validity of using the in vitro data to help predict the metabolism of isomeric fats in humans.

Plasma-SM. Results of the sphingomyelin analyses are given in Figure 5, and comparison of the O1 and E1 curves indicates enzyme specificities for elaidic acid. As observed for the PE "total label" curves in Figure 1, the "total label" curves in Figure 5 also show considerable variation. The 12-hr SM sample from subject 3 in Figure 5 shows a large reduction in total deuterated fat uptake. This result cannot be explained, but it correlates with the reduction in the elaidic acid level in the 12-hr PC fraction for subject 3 in Figure 3. Differences in the total deuterated fatty acid percentages shown by the "total label" curves reflect variation in the subjects' overall lipid utilization, which apparently has little effect on the preferential utilization of E1 or O1 since selectivity for E1 incorporation occurred in all plasma-SM samples. Only 2 data points are plotted for subject 1 in Figure 5 because the GC-MS data were not accurate for the other data points due to small sample size and problems with the esterification procedure. No deuterium isotope effect for O1-9,10-d_2 incorporation into SM was detected based on the O1-d_2 and O1-d_6 curves from subject 3 in Figure 5.

Plasma Lyso-PC. GC-MS analyses of plasma lyso-PC samples are plotted in Figure 6. In this figure, selective incorporation of oleic acid into plasma lyso-PC was observed in subject 1

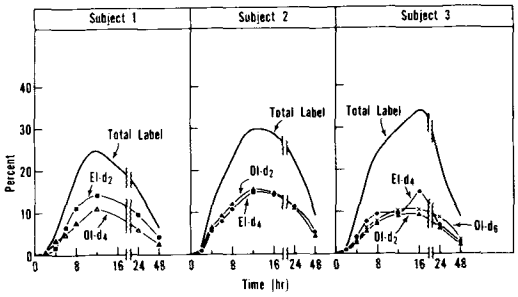

FIG. 3. Incorporation of deuterated O1 and E1 into plasma phosphatidylcholine of three human subjects. The curves identified as O1, E1, and "total label" given the percent deuterated O1, E1 and O1 + E1 contained in the total octadecenoate portion of the samples. See Table I for composition of the deutrated triglyceride mixtures fed.

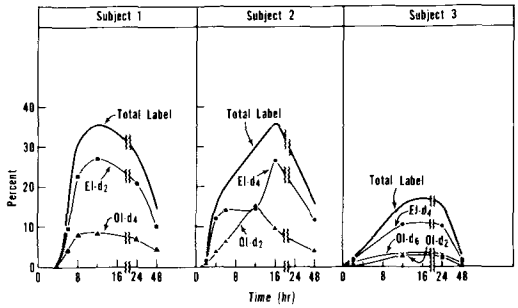

FIG. 4. Incorporation of deuterated O1 and E1 into plasma 1-acyl phosphatidylcholine of three human subjects. The curves identified as O1, E1, and "total label" give the percent deuterated O1, E1, and O1 + E1 contained in the total octadecenoate portion of the samples. See Table I for composition of the deuterated triglyceride mixtures fed.

and 2, but in contrast, a large selectivity for elaidic acid was found for lyso-PC data from subject 3. This inconsistency is similar to the one noted for the PS data in Figure 2 where the E1 selectivity was also reversed for subject 3 compared to the other two subjects. The percent total labeled fatty acid found in the lyso-PC fractions varied from 20 to 43%. The shape of the total label curves was similar in all experiments. These data support the concept that incorporation of individual fatty acids into lyso-PC is effected by double bond configuration. However, these data also indicate that other factors, such as diet or differences in the subject's metabolism, may be involved in controlling the fatty acids utilized in synthesis of lyso-PC.

Selectivity Factors

Selectivity factors are summarized in Table

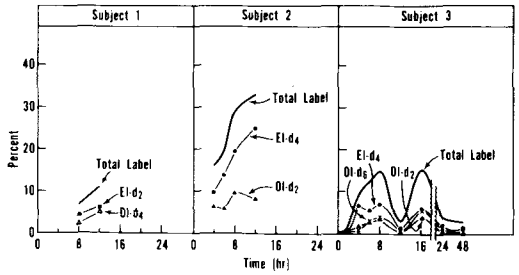

FIG. 5. Incorporation of deuterated O1 and E1 into plasma sphingomyelin in three human subjects. The curves identified as O1, E1, and "total label" give the percent deuterated O1, E1, and O1 + E1 contained in the total octadecenoate portion of the samples. See Table I for composition of the deuterated triglyceride mixtures fed.

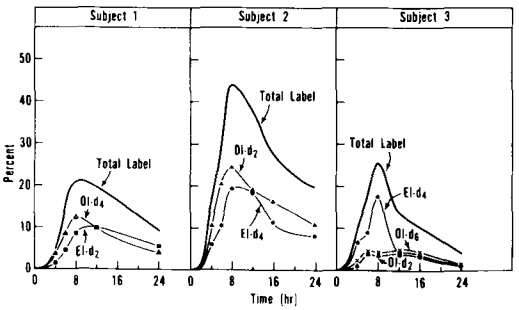

FIG. 6. Incorporation of deuterated O1 and E1 into plasma lysophosphatidylcholine in three human subjects. The curves identified as O1, E1, and "total label" give the percent deuterated O1, E1, and O1 + E1 contained in the total octadecenoate portion of the samples. See Table I for the composition of the deuterated triglyceride mixtures fed.

II for individual phospholipid samples that contained the maximum amounts of labeled fatty acid. All phospholipids selectively incorporated elaidic acid except for PS samples from subject 1, lyso PC samples from subjects 1 and 2, and the 2-acyl phosphatidylcholine samples from all three subjects. The selectivity factors indicate that our third subject had a greater tendency to incorporate elaidic acid than the first and second subjects. The fact that elaidic acid is concentrated in many of the phospholipids indicates dietary elaidic acid may have a larger impact on the structure of the phospholipids than would be predicted based on the *trans* content of hydrogenated fats.

Average E1 selectivity for the total PC samples listed in Table II is 1.34. E1 selectivity for the 1-acyl position of PC averages 3.7 compared to 0.68 for E1 at the 2-acyl position. Since phosphatidylcholine:cholesterol acyl transferase utilizes the fatty acids from the 2-acyl phosphatidylcholine position, the low E1 content of the PC-2 samples is partly responsible for the very low (0.19) E1 selectivity we previously reported for cholesteryl ester samples (48). Thus, incorporation of E1 into plasma cholesteryl ester samples is limited by the reduced activity of 2-acyl-CoA:phospholipid acyl transferase and phosphatidylcholine:cholesteryl acyl transferase for utilization of E1 as their substrate. This concept is supported by the low *trans* content of the cholesteryl ester samples from rabbits fed hydrogenated olive oil containing 37% E1 (55).

trans-Octadecenoic Acid Percentages

The total percent E1 (labeled plus unlabeled) in phospholipids containing the maximum amounts of deuterated fatty acids was determined by gas chromatography and compared to the total percent E1 in the 0-hr phospholipid fractions (Table III).

All 0-hr phospholipids from subjects 1 and 3 contained unlabeled E1. The 0-hr samples from subject 2 contained considerably less E1, and three of the phospholipid fractions (PC, PC-1, lyso PC) contained no unlabeled E1. The source of the E1 in these samples is probably hydrogenated vegetable oil. In all subjects, the total percent E1 (labeled and unlabeled) increase after deuterated E1 was fed. Maximum E1 levels in these phospholipid samples (see Table III) generally coincided with the maximum incorporation of deuterated E1 as determined by mass spectroscopy (see Figs. 1-6). In all the phospholipid samples, the total E1 determined by GC was equal to or greater than deuterated E1 as measured by mass spectrometry. This comparison of GC and MS data indicates there were no large errors in the mass spectrometry data since total E1 as measured by GC cannot be less than deuterated E1 if the mass spectrometry data is correct. In most of the phospholipid samples, total E1 percentage was approximately equal to the 0-hr E1 percentage plus percent deuterated E1. The correlation suggests deuterated E1 is added to the undeuterated E1 already present in the phospholipids. PC fractions generally contained less E1 in the 0-hr samples than other 0-hr phospholipid fractions, and PC fractions also accumulated less total deuterated E1. PC-1 samples had large E1 percentages in the 12- and 16-hr fractions, which reflects the selective incorporation of E1 into the 1-acyl position by 1-acyl-CoA:phospholipid transferase as mentioned earlier.

Fatty acid compositions (16:0, 18:0, 18:1, 18:2, and 20:4) in 0-hr samples were similar to the fatty acid compositions found at maximum deuterated fat incorportion after the data

TABLE III

Percent *trans*-Octadecenoic Acid in Plasma Phospholipid Fractions at 0 hr and at Maximum Deuterium Incorporation

Phospholipid[a]	Subject	E1[a] (%) (0 hr)	E1[a] %	E1[a] Time, hr
PE	1	10.7	37.2	6
PE	2	4.8	32.9	6
PE	3	9.6	17.4	6
PS	1	11.0	21.6	8
PS	2	8.5	24.2	12
PS	3	12.5	38.0	8
PC	1	2.6	13.6	12
PC	2	0.0	16.0	12
PC	3	4.7	15.0	16
PC-1	1	13.1	37.9	12
PC-1	2	0.0	26.6	16
PC-1	3	12.0	25.0	12
SM	1	19.3	35.0	12
SM	2	ND[b]	ND	---
SM	3	13.1	16.7	6
Lyso PC	1	15.1	37.0	8
Lyso PC	2	0.0	20.0	8
Lyso PC	3	7.1	19.5	8

[a]Abbreviations the same as in Table II.
[b]ND = not determined.

are normalized for the increase in deuterated 18:1. Thus, increases in E1 percentages are the result of the deuterated E1 diluting existing fatty acids and are not the result of selective replacement of any one fatty acid in the phospholipid samples.

Platelet and Red Blood Cell Data

Total platelet and red blood cell (RBC) phospholipids were analyzed by GC-MS for deuterated fatty acids and for total E1 by GC. Total deuterated fatty acid incorporation into platelet phospholipids was low (4 to 6% of the total octadecenoates). Platelet phospholipid samples (0-hr) contained 10-15% E1. The deuterated O1 and E1 percentages as determined by GC-MS contained an error of ±2% and, consequently, were not accurate enough to permit meaningful selectivity factors to be calculated. The poor accuracy was caused by a combination of small total sample size and low levels of deuterated fatty acid incorporation into the platelet phospholipids.

Red blood cell (RBC) phospholipids contained smaller levels of deuterated fatty acids than the platelet phospholipid samples. Total deuterated fatty acid percentages in the RBC phospholipids ranged from 0 to 4% of the total octadecenoates with an error of ±1.5%. These data were not useful for calculation of meaningful selectivity factors. Data summarized in this section provide direct evidence for in vivo incorporation of dietary fatty acids into platelet and RBC phospholipids. Since low levels (less than 6%) of deuterated fat were found in platelet and RBC phospholipids, this indicates that either incorporation of deuterated fatty acids or exchange of platelet or RBC phospholipids for plasma phospholipids occurs during the 10- to 12-hr period that plasma phospholipids contain over 10% deuterated fatty acids. The platelet and RBC phospholipid data from these studies are consistent with previous in vitro investigations where human RBC were incubated for 3-5 hr with ^{14}C-labeled fatty acids (56,57) or rat RBC with ^{32}P-labeled phospholipids (58). These in vitro studies were not designed to determine the total amounts of RBC phospholipid fatty acid that were replaced. Neehout (59) reported fatty acid profiles of human RBC phospholipids were not measurably changed during incubation with linoleic acid for 48 hr. Our data provide direct evidence that fatty acid incorporation into human RBC and platelet phospholipids also occurs in vivo at an estimated rate of 2-8% per 24 hr although the rate may be governed by the lipid level of the plasma.

The total E1 percentages for RBC total phospholipids ranged from 0 to 15%, which is approximately the same level as the plasma-0 hr E1 percentages given in Table III. The RBC

and platelet data, summarized in this section, indicate that phospholipid composition of the RBC and platelet membrane phospholipids reflects the plasma lipid fatty acid composition and that the same E1 selectivities observed for individual plasma phospholipids probably occur in platelet and RBC membrane phospholipids.

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Uptake of [^{14}C] Choline and Incorporation into Lung Phospholipid by the Isolated Perfused Rat Lung

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ABSTRACT

We have used the isolated perfused lung (IPL) preparation from the rat to determine whether uptake of choline from the vascular compartment could limit the rate of synthesis of phosphatidylcholine (PC). The uptake of choline was rapid and did not saturate at a concentration of 10 mM. The rate of incorporation of choline into phospholipid was saturated above 0.1 mM choline. Whereas, uptake and incorporation were depressed at 4 C, uptake was neither dependent on the extracellular sodium concentration nor inhibited by equimolar concentrations of hemicholinium-3 (HC-3). We could find no evidence that uptake might limit synthesis of lung lecithin and conclude that uptake is either by free diffusion, or by a carrier-mediated process with a very high Km.

INTRODUCTION

In the mammalian lung, more than 70% by weight of surfactant is phosphatidylcholine (PC) (1). The major route for the de novo synthesis of PC in the lung is via the incorporation of choline, which in turn must either be derived from plasma or be reutilized after degradation of PC within the lung. Evidence for the first source is provided by Chevalier and Collet (2), who demonstrated that an intraperitoneal injection of [^{3}H] choline in mice was rapidly followed by the appearance of radioactive label in the alveolar type II cells. Whereas the uptake of choline from the vascular compartment of the lung has not been investigated, choline uptake in other non-neural tissue is carrier-mediated (3-5). Moreover, inhibition of carrier-mediated transport of choline into rat brain synaptosomes has effectively inhibited the synthesis of acetylcholine (6-8). If choline uptake in lung is also carrier-mediated, we postulate that the process may be rate-limiting in the biosynthesis of surfactant PC. In the present study, we have investigated the characteristics of choline uptake by the isolated perfused rat lung (IPL).

METHODS

Lungs from male Porton rats (200-250 g) were perfused at 10 ml/min with Krebs bicarbonate solution containing 4.5% albumin and ventilated with 5% CO_2 in oxygen (9). In all cases, the lungs were perfused for 10 min before [^{14}C-choline was introducted. Sodium-free medium was prepared as previously reported (10). [^{3}H-methyl] choline chloride (10.1 Ci/mmol) and [^{14}C-methyl] choline chloride (52 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, England). 1-Palmitoyl-2-palmitoyl [9,10-^{3}H] phosphatidylcholine (20Ci/mmol)([^{3}H] DPPC) was obtained from Applied Sciences Laboratories (State College, PA). The purity of labeled compounds was checked regularly by thin layer chromatography (TLC). TLC plates were scanned for radioactivity with a Berthold TLC scanner II (model LB273, Wildbad, Germany). Hemicholinium-3(HC-3) was obtained from Aldrich (Milwaukee, WI). Unlabeled choline chloride was added to the standard medium containing [^{14}C] choline when concentrations of 0.1 mM and greater were required. Choline chloride, dipalmitoyl-DL-α-phosphatidylcholine, mixed PCs and sphingomyelin were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were obtained from normal commercial sources.

Lipids were extracted from the homogenized lung tissue by the method of Folch et al. (11), and samples for counting were taken from both aqueous and organic phases. Samples of the total lipid extract were applied to silicic acid columns (500-750 mg silicic acid in Pasteur pipets), and neutral lipids were eluted with 10 ml chloroform. The phospholipid fraction was eluted with 10 ml methanol, dried at 35 C under reduced pressure, redissolved in methanol (200 μl), and duplicate samples (25 μl) were taken for counting. Samples (10 μl) were spotted on precoated Silica Gel G TLC plates (Macherey Nagel, Germany). A standard carrier mixture of PCs was spotted as a marker with each sample, and [^{3}H] DPPC with unlabeled carrier was run separately as a reference standard. The plates were developed according to Gluck et al. (12). The identity of PC was checked by two dimensional TLC with a second solvent system (13). Lipid spots were visualized by exposure to iodine vapors or to a

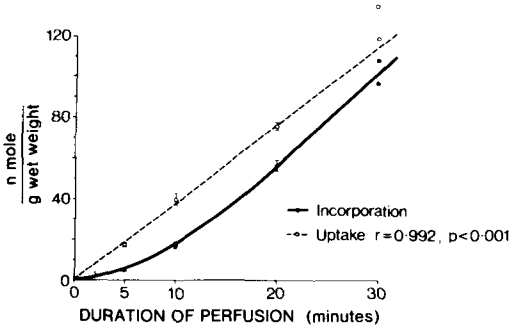

FIG. 1. Uptake and incorporation of [^{14}C] choline into phospholipid plotted against the duration of perfusion of the isolated rat lung with medium containing 10 μM [^{14}C] choline at 37°C. Each point represents the mean ± SE of 5 - 21 experiments.

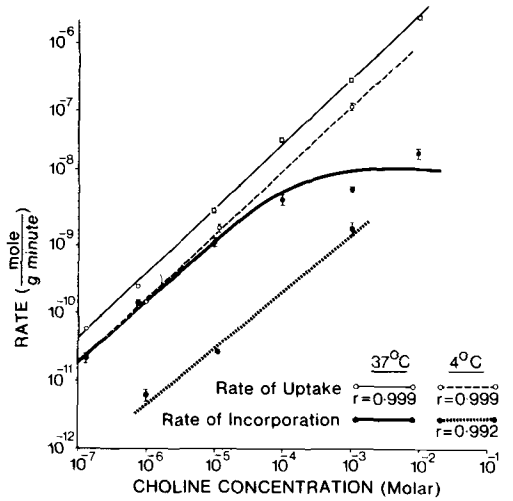

FIG. 2. Rate of uptake and incorporation of choline into phospholipid by the isolated perfused rat lung at 4°C and 37°C plotted against choline concentration in the medium. Each point represents the mean ± SE of 4 - 7 five min perfusions.

spray of aqueous 0.02% dichlorofluorescene. All areas of radioactivity were located and scraped directly into counting vials. The cpm in each spot were expressed either as a percent of total cpm in that lane, or were corrected for quench and recovery (see below) and expressed as percent of label recovered as PC. In most of the lung perfusions, recovery was determined by using [^{3}H] DPPC as an internal standard. Either [^{3}H] DPPC or [^{3}H-methyl] choline were added during homogenization of the tissue. Hence, the efficiency of partitioning choline and PC was analyzed and the recovery of PC was determined at each step in the procedure. Positive identification of PC by TLC was facilitated by comparison of the migration of lung phospholipids with both the internal [^{3}H] DPPC standard standard and the standards run separately. Following extraction, the tissue residues were analyzed for radioactivity after oxidation in a Packard model 306 Tricarb Sample Oxidizer. Of the [^{3}H] DPPC added as an internal standard and tracer, 93.9 ± 0.7% (mean ± SE, n = 117) was recovered. Spillover of tritium into the aqueous methanol phase was negligible. In five extractions, 86.9 ± 1.1% of the added [^{3}H-methyl] choline was recovered in the aqueous phase. Recovery of tritium from the oxidized tissue residue was 11.9 ± 1.2% and spillover of choline into the extracted lipid phase was 1.24 ± 0.06%.

All samples were counted for 10 min on a Searle Analytical Mark III Liquid Scintillation Counter (Amersham, England) using a dual-label program. Efficiency of counting was determined by referring the External Standard Pulse reading for each sample to previously prepared quench curves.

RESULTS

At 37 C the rate of uptake of choline by the IPL at the normal plasma choline concentration in the rat (10 μM) (14) was 3.78 ± 0.05 nmol min^{-1} g^{-1} wet weight (n = 21) and was linear with time for 30 min (Fig. 1). Incorporation of label into phospholipid did not attain steady state within 30 min; however, after 5 min of perfusion, the time-averaged rate was 1.02 ± 0.04 nmol min^{-1} g^{-1} (n = 21). Of the label recovered in the lipid fractions, 87.5 ± 2.7% (n = 15) was identified as PC.

Figure 2 illustrates that during a 5 min perfusion the uptake of choline was linear with perfusate concentrations of up to 10 mM. A maximum incorporation of 10 nmol min^{-1} g^{-1} occurred with 0.1 mM choline in the perfusate. At 4 C the rate of uptake was reduced to 50% and the rate of incorporation reduced to 2% of the values at 37 C.

The rates of uptake and incorporation during a 5 min perfusion with sodium-free medium at 37C and 10 μM choline were increased by 39 ± 5% and 64 ± 10%, respectively, over controls ($p<0.001$). The inclusion of 10 μM HC-3 in the perfusion medium had no effect on the uptake or incorporation of choline into PC.

Forty percent of the [^{14}C] label present in the lungs following a 5 min perfusion with 10 μM [^{14}C] choline then appeared in the effluent following a subsequent 10 min perfusion with 10 μM unlabeled choline (Fig. 3).

DISCUSSION

We have shown that the uptake of choline from the vascular compartment of the IPL is rapid and does not saturate at near toxic concentrations of choline. This finding is consistent with earlier work of Gardiner and Gwee (15) which demonstrated both a high tissue/plasma ratio of free choline in the rabbit lung and an ability of the lung to accumulate three times its free choline content within 3 min of an intravenous injection of 300 μmol kg^{-1} choline. Furthermore, our results are consistent with those of Freeman et al. (16) who found that the uptake of choline by the brain was linear with the carotid arterial plasma level and was not saturated at concentrations of up to 10 mM. In addition, they found that infusion of choline increased the concentration of free choline in the brain and suggested that choline enters the brain by free diffusion or by a carrier-mediated process with a very high Km. Whereas they found a net release of choline from the brain (17), the ($^2H_4$)choline removed from the arterial plasma was not readily released. Freeman et al. concluded that there is both a rapid uptake of choline into the brain and a very rapid turnover of choline within the brain (16).

If we assume that the free choline pool of the lung is similar in the rat and rabbit (15), then the free choline which entered the tissue of the IPL of the rat per min amounted to 1.2% of the total free choline pool. If we then subtract the amount of [^{14}C]choline which was incorporated into PC (27% per 5 min perfusion), then the tissue pool of free choline would turn over every 10 hr. Even though the maximum amount of free [^{14}C]choline would amount to only 4.3% of the total free choline within the lung at the end of a 5 min perfusion, 55% of that label is lost to the effluent during a subsequent 10 min perfusion with unlabeled choline. This may reflect a rapid exchange of choline between plasma and the lung compartment which is a major source of choline for PC synthesis. Although we did not measure labeled or unlabeled free choline in the effluent, we suggest that there may be a high rate of exchange of choline between plasma and lung similar to that observed in the brain (16).

Whereas Figure 2 suggests that the synthetic process was saturated at a choline concentration of 1 mM, Figure 1 shows that the rate of incorporation increased as the perfusion was extended to 30 min. This may be due to an increase in specific activity of that free choline pool which is most readily incorporated into PC within the lung. This is supported by the observation that the high ratio of rate of incorporation/rate of uptake (0.270 after 5 min perfusion, 0.810 after 30 min perfusion) does not compare with the much lower ratio of uptake rate/free choline pool (0.012).

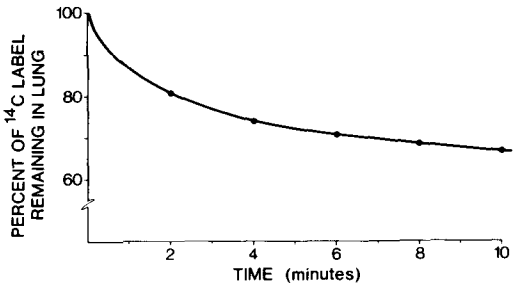

FIG. 3. Efflux of [^{14}C] from the isolated lung after a 5 min perfusion with 10 μM [^{14}C] choline (n = 3). During the 10 min efflux period, the medium contained 10 μM unlabeled choline. Samples of effluent were taken for counting at 2, 4, 6, 8 and 10 min. The amount of [^{14}C] in the aliquot of effluent was integrated with time and is presented as a percent of the label present in the lung at the beginning of the efflux period. The amount of label present in the lung at the beginning of the efflux period was derived from 18 control perfusions.

The inability of equimolar concentrations of HC-3 to inhibit choline uptake in the IPL contrasts with the finding that choline uptake and PC synthesis in HeLa cells were suppressed by a drug similar to HC-3 (18). The mechanism of uptake of choline in the IPL also differs from that in the squid axon, where the K_I for HC-3 was 20 μM (19) and in rat corpus striatum (20) and renal proximal tubule (21) where very significant inhibition occurred at molar ratios (HC-3/choline) of 0.1 and 0.05, respectively.

As has been reported by others (4), we observed that choline uptake and incorporation into PC was temperature-dependent. However, this in itself does not indicate carrier-mediated transport. More significant is the sodium-independence of the system in the lung. In contrast to other reports (4,20), uptake actually increased in the absence of sodium in the perfusing medium, again illustrating that uptake of choline by the IPL is unlike that seen in other tissues.

From the present data we conclude that free choline in the vascular compartment is rapidly taken up and exchanged with pool(s) of free choline in the lung which have ready access to the enzymes of PC synthesis. We suggest that the uptake of choline occurs either by free diffusion or by carrier-mediated transport with a very high Km.

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Serum α-Lipoprotein Responses to Variations in Dietary Cholesterol, Protein and Carbohydrate in Different Non-human Primate Species[1]

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ABSTRACT

Serum α-lipoprotein responses to variations in dietary cholesterol, protein, and carbohydrate were studied in different nonhuman primate species. Chimpanzee, rhesus, green, patas, squirrel and spider monkeys all showed significant interspecies differences in serum total cholesterol responses to 1.84 mg/kcal exogenous cholesterol. Dietary cholesterol significantly increased the α-lipoprotein cholesterol in all species except rhesus and chimpanzee. Among these species, there was no relationship between the basal serum lipoprotein profile and subsequent lipoprotein responses to dietary cholesterol. Although the level of dietary protein at 6%, 12%, and 37% of calories had no appreciable main effect on serum total cholesterol in spider monkeys, very low protein diet (6% of calories) produced a significant elevation in α-lipoprotein cholesterol. Serum α-lipoprotein responses to exogenous cholesterol (1.84 mg/kcal) was highest for the very low protein diet and lowest for low protein diet (12% of calories). Diets with high sucrose (76.5% of calories) and low saturated fat (12.5% of calories) containing no added cholesterol were tested in squirrel and spider monkeys and produced a consistent serum total cholesterol response; the α-lipoprotein response was significantly higher in squirrel monkeys than in spider monkeys. The above findings have implications in experimentally induced and comparative atherogenesis.

Epidemiological studies have indicated an inverse relationship between levels of serum α-lipoprotein and the incidence of coronary heart disease (1,2). The resistance of some species of animals, such as dogs and rats, to experimental atherosclerosis has been attributed to the predominance of cirulating α-lipoproteins (3). Although hyperlipoproteinemia is generally induced by dietary manipulation in a variety of experimental animals, including nonhuman primates, only recently has attention been focused on studying the changes of α-lipoproteins (4-6). Hitherto in the classical high fat-cholesterol dietary model, the induction of hyperlipoproteinemia was assumed to result in increases of only serum total cholesterol and β-lipoprotein fractions. Consequently, serum cholesterol has been considered as a measure of diet-induced hyper-β-lipoproteinemia. Studies on nonhuman primates have indicated considerable species differences in the degree of vascular lesions with elevated levels of serum total cholesterol (7). Since serum total cholesterol represents all of the lipoprotein classes, detailed study of specific lipoproteins and their responses to atherogenic diets is needed for a better appreciation of the relationship to the development of experimentally induced lesions. In comparison to the high fat-cholesterol model, dietary protein and carbohydrates, whose nature and consumption vary widely among different populations, have received far less attention.

We have now conducted a series of nutritional studies in several nonhuman primate species to observe serum lipoprotein responses, and particularly α-lipoprotein responses, to variations in dietary cholesterol, protein, and carbohydrate. The availability of an easy method to quantitate serum lipoproteins repeatedly on small samples of serum (8-11) has made it possible to follow serial lipoprotein changes in individual animals and observe intra and inter species differences in response to dietary changes.

MATERIALS AND METHODS

Animals and Diets

The experiments were conducted as three different models. All species used in the study were adult animals of mixed sexes. Animals were housed in individual cages, and before the experiments all monkeys received Purina Monkey Chow 25 (Ralston Purina Co., St. Louis, MO) ad libitum. The chow contained by calories 68% carbohydrate, 23% protein, and 9% fat. Experimental diets were fed in fixed quantities. The kcal/kg body weight consumed by each species was determined by the daily food intake of the individual animals for 1 week with the following mean results for each species studied: rhesus, green, and spider, 85;

[1]Presented at the Lipoprotein Symposium AOCS meeting, St. Louis, Missouri, May 1978.

TABLE I

Diets with Variable Protein Content

Ingredients (g/100 g)	High protein (25%)		Low protein (8%)		Very low protein (4%)	
	Diet I	Diet II	Diet III	Diet IV	Diet V	Diet VI
Purina Chow 25[a]	41.0	38.0	---	---	---	---
Purina Chow 15[a]	---	---	53	52.5	27.0	27.0
Casein	15.0	15.0	---	---	---	---
Butter	---	5.0	---	5.0	---	5.0
Corn oil	---	---	---	---	1.0	1.0
Dextrin	---	---	---	---	30.0	20.0
Hegsted salt	---	---	---	---	1.0	1.0
Vitamins	---	---	---	---	1.0	1.0
Banana	12.0	12.0	15	10.0	11.0	13.5
Cholesterol	---	0.5	---	0.5	---	0.5
Water	32.0	29.5	32.0	32.0	29.0	31.0
Gross energy (kcal/g)	2.48	2.72	2.36	2.71	2.50	2.54

[a]Purina chow contains 25 and 15% (by weight) protein.

patas, 65; squirrel, 200; and chimpanzee, 122.

High Cholesterol-Low Fat Diet

The lipoprotein responses to high cholesterol diet were studied in chimpanzee (*Pan troglodytes*), rhesus (*Macaca mulatta*), green (*Cercopithecus aethiops*), patas (*Erythrocebus patas*), squirrel (*Saimiri sciurea*) and spider (*Ateles sp.*) monkeys. Five adults of mixed sex for each species were fed the experimental diet for a three week period. The monkey chow-based experimental diet (12) contained by weight: monkey chow 51%, banana 12%, butter 5.0%; cholesterol 0.5% (1.84 mg/kcal); and water 31.5%. In order to relate the lipoprotein responses to dietary cholesterol per se, we have purposely used low levels of fat.

Variations in Dietary Protein

We examined the effect of varying the level of dietary protein on the lipoprotein response in eight adult spider monkeys of mixed sexes. The experimental diets based on Purina Monkey Chow 25 and 15 (25 and 15 denote the percent protein of the respective chows) were arbitrarily chosen to contain high (25% (w/w)), low (8% (w/w)), and very low (4% (w/w)) levels of protein, which represent 37%, 12% and 6% of calories, respectively (Table I). The diets contained either no or 0.5% added cholesterol. Each animal was given the experimental diets without or with cholesterol for three weeks and served as its own control. To minimize any possible influence of the dietary sequence on serum lipoproteins, the eight animals were divided into two groups. While one group was fed high protein diets followed by low protein diets, the second group was fed low protein diets followed by high protein diets. The animals were then grouped together and fed very low protein diets.

High Sucrose-Cholesterol Diets

The lipoprotein responses to diets with a high level of sucrose (69% w/w) and a low level of saturated fat (5% of w/w butter/coconut oil mixture, 1:1) with and without cholesterol were studied in five adult squirrel and five spider monkeys of mixed sexes. In these semi-synthetic experimental diets, the sucrose represented 76.5% of the calories, the protein (casein) 11.0%, and the fat 12.5%. In addition the diets contained by weight 2% vitamin mix, 4% Hegsted salts and 10% cellulose. The exogenous cholesterol represented 0%, 0.036%, or 0.36% (i.e., 0 mg, 0.1 mg and 1.0 mg/kcal). Animals were alternately fed a basal diet for a period of three weeks or high sucrose diets with or without cholesterol for six weeks. In the initial stages of the experiment, one of the five squirrel monkeys died. We subsequently added three monkeys to this group.

Serum Lipoprotein Cholesterol Measurements

Blood (16 hr fasting) was drawn from femoral vein under sedation (Sernylan, Bio-Ceutic Laboratories, Inc., St. Joseph, MO), and serum was prepared and analyzed for lipid and lipoprotein concentrations.

We determined serum total cholesterol and triglycerides in an autoanalyzer (Technicon AutoAnalyzer II, Technicon Instruments Corp., Tarrytown, NY) using isopropanol extracts of the serum (13). Cholesterol in β- and pre-β-lipoprotein fractions was determined by the specific and quantitative precipitation procedure using heparin in the presence of Ca^{++} (8,10). Briefly, the method consists of mixing serum (0.2 ml), distilled water (3.2 ml), heparin (0.25% 140 units/mg, 0.1 ml), and $CaCl_2$ (0.5

TABLE II

Effect of Dietary Cholesterol on Serum Lipoprotein Cholesterol Levels of Six Nonhuman Primate Species

Species		Dietary cholesterol (mg/100 g)	Lipoprotein cholesterol (mean ± S.E.), mg/100 ml			
			Total	α-	β-	Pre-β
Patas	(5)[c]	0	95.2 ± 6.8	44.1 ± 1.1	46.4 ± 5.3	4.8 ± 1.5
		0.5	162.8 ± 31.4[a]	98.0 ± 13.0[b]	62.4 ± 22.3	2.6 ± 1.1
Green	(5)	0	172.6 ± 23.7	76.0 ± 12.9	91.8 ± 11.5	5.0 ± 1.0
		0.5	322.2 ± 32.2[a]	162.7 ± 10.8[a]	156.8 ± 25.4	3.2 ± 2.1
Rhesus	(5)	0	147.6 ± 7.3	65.2 ± 9.1	65.3 ± 6.4	17.3 ± 5.6
		0.5	241.8 ± 31.8[b]	102.3 ± 14.3	124.2 ± 21.2[b]	15.6 ± 2.4
Spider	(5)	0	160.2 ± 14.6	18.6 ± 5.5	131.3 ± 11.6	10.3 ± 2.0
		0.5	212.8 ± 13.2[a]	104.5 ± 9.9[a]	100.2 ± 10.7	8.4 ± 4.0
Squirrel	(4)	0	225.0 ± 17.7	102.1 ± 12.0	110.5 ± 11.1	12.7 ± 4.0
		0.5	362.5 ± 23.9[a]	321.2 ± 18.6[b]	129.4 ± 16.1	2.6 ± 1.0
Chimpanzee	(4)	0	282.0 ± 26.7	120.1 ± 12.9	135.8 ± 11.6	26.5 ± 5.9
		0.5	291.0 ± 29.9	131.6 ± 20.2	146.0 ± 13.5	13.8 ± 3.6
Differences Among Species (p<)			0.001 0.001	0.001 0.001	0.001 0.025	0.005 0.005

[a,b]Significantly different from basal value, a: $p<0.01$; b: $p<0.05$.
[c]Number of animals.

M, 0.5 ml) and centrifuging the precipitate after allowing it to stand for 15 min. The precipitate was analyzed for the corresponding β- and pre-β-lipoprotein cholesterol content. The difference between total cholesterol and β+ pre-β-lipoprotein cholesterol was considered as α-lipoprotein cholesterol.

Densitometric Ratios of β- and Pre-β-Lipoproteins

Electrophoretic separation of serum lipoproteins was done on agar-agarose gel according to the method of Noble with some modification (9). The lipoprotein bands (β- and pre-β-only) were scanned in a densitometer equipped with a digital computer printout (Quick Scan, Helena Laboratories, Beaumont, TX), and the densitometric ratios were corrected as described previously (11).

Estimation of β- and Pre-β-Lipoprotein Cholesterol Concentrations

We calculated the serum content of β-lipoprotein and pre-β-lipoprotein based on the densitometric ratios of β- to pre-β-lipoproteins, β+ pre-β-lipoprotein cholesterol (heparin-Ca^{++} precipitate), and the average cholesterol content per unit β-lipoprotein and pre-β-lipoprotein molecules (9,10). The molecular composition of the lipoprotein classes of nonhuman primates was assumed to be similar to that of humans (14). This method of estimation of human sera was validated by analytical ultracentrifugation (10). The lipoprotein values were converted into the corresponding lipoprotein cholesterol values as follows: β-lipoprotein cholesterol = mg β-lipoprotein X 0.469; pre-β-lipoprotein cholesterol = mg pre-β-lipoprotein X 0.222 (15).

Analysis of Data

Means, standard error, paired t-tests and analysis of variance were computed by standard statistical methods (16).

RESULTS

Serum Lipoprotein Responses to High Cholesterol-Low Fat Diet

The effect of high cholesterol-low fat diet on serum total cholesterol and lipoprotein cholesterol (β-, pre-β- and α-) levels in six different nonhuman primates is shown in Table II. The initial serum total cholesterol levels as well as individual lipoprotein cholesterol levels differed significantly among the six species. Chimpanzees and squirrel monkeys had the highest mean basal serum total cholesterol concentrations, and patas monkeys had the lowest. The distribution of cholesterol among lipoprotein classes showed that cholesterol derived from α-lipoprotein constituted 42% to 46% of total cholesterol in all species except spider monkeys in which α-lipoprotein cholesterol accounted for only 12% of the total cholesterol. The pre-β-lipoprotein cholesterol levels were relatively low in all species except chimpanzees. Chimpanzees, spider and squirrel monkeys had the highest basal concentrations of β-lipoprotein cholesterol, and patas monkeys had the lowest.

The serum total and individual lipoprotein cholesterol levels differed significantly among these species when the monkeys were fed

TABLE III

Effect of Dietary Cholesterol On The Ratio of α- to β-Lipoprotein Cholesterol In Six Nonhuman Primate Species

Species (no. animals)	Ratio of α- to β-lipoprotein cholesterol		%
	Basal diet	0.5% Cholesterol diet	Change
Spider (5)	0.14[a]	1.04	642
Green (5)	0.83	1.04	25
Chimpanzee (4)	0.88	0.90	2
Squirrel (4)	0.92	1.79	95
Patas (5)	0.95	1.57	65
Rhesus (5)	1.00	0.82	-18

[a]Mean values.

TABLE IV

Serum Lipoprotein Cholesterol Levels in Spider Monkeys Fed High Cholesterol Diets Containing Different Levels of Protein[a]

Cholesterol fraction (mean ± S.E. mg/100 ml)	Dietary protein level (g/100 g)			Analysis of variance
	25	8	4	
Total	340.4 ± 26.1	290.1 ± 23.3	534.9 ± 52.9	$p<0.001$
β-lipoprotein	261.9 ± 27.5	216.5 ± 32.7	354.0 ± 44.4	$p<0.05$
Pre-β-lipoprotein	5.6 ± 1.3	7.5 ± 1.6	6.1 ± 1.0	$p>0.1$
α-lipoprotein	73.2 ± 14.3	68.4 ± 13.1	176.0 ± 23.3	$p<0.01$

[a]Number of animals: 8.

cholesterol-containing diets. Exogenous cholesterol at 0.5% level, increased the serum total cholesterol levels significantly in all species except chimanzees. Serum total cholesterol response to dietary cholesterol was reflected in both α- and β-lipoprotein cholesterol. Three weeks of cholesterol feeding significantly increased α-lipoprotein cholesterol in all species except rhesus and chimpanzee. On the other hand, the observed increase in β-lipoprotein cholesterol to exogenous cholesterol was statistically significant only in rhesus monkeys. Although not statistically significant, dietary cholesterol tended to decrease pre-β-lipoprotein cholesterol levels in all species. It can be seen that there is no relationship between the basal serum total cholesterol concentrations and serum cholesterol response to dietary cholesterol. For example, patas monkeys responded more than chimpanzees, although the initial serum total cholesterol concentration was highest in chimpanzees and lowest in patas monkeys. Even within a given species, the initial serum total cholesterol concentration was not reflective of the magnitude of cholesterol response.

The effect of dietary cholesterol on the ratio of α- to β-lipoprotein cholesterol is shown in Table III. While the dietary cholesterol increased the α/β ratio tremendously in spider monkeys, it decreased the ratio in rhesus monkeys. In chimpanzees, the ratio remained almost the same. From these data, however, it is difficult to ascertain whether the initial α- to β-lipoprotein ratio is indicative of subsequent α-lipoprotein response to dietary cholesterol. The response seems to be characteristic of the species rather than related to the basal lipoprotein profile.

Effect of Varying the Level of Dietary Protein

In the absence of exogenous cholesterol, with reduced intake of dietary protein, the serum β+ pre-β-lipoprotein cholesterol tended to increase slightly (139.5± 14.1 mg%, 133.2 ± 11.3 mg%, and 110.4 ± 6.2 mg% at 25%, 8% and 4% protein intake, respectively). Of particular interest is the α-lipoprotein cholesterol changes with dietary protein levels. α-Lipoprotein cholesterol decreased slightly but significantly when the protein intake was lowered from 25% to 8% (38.1 ± 2.8 mg% vs. 34.9 ± 3.2 mg/, $p<0.05$). However, further reduction in dietary protein intake to a 4% level resulted in a significant increase in the α-lipoprotein cholesterol (54.5 ± 2.6 mg%; $p<0.001$).

As expected, the animals responded to exogenous cholesterol (0.5%), but the lipoprotein responses varied significantly at different levels of dietary protein (Table IV). The addition of cholesterol to a 8% protein diet resulted in relatively lower serum total choles-

TABLE V

Effect of Dietary Sucrose and Cholesterol on Serum Lipids in Squirrel and Spider Monkeys

Species (no. animals)	Diet	Mean ± S.E., mg/100 ml	
		Cholesterol	Triglycerides
Squirrel (4)	Basal	191.5 ± 17.0[a]	62.2 ± 13.2[a]
	High sucrose with		
(4)	0% Cholesterol	332.0 ± 9.6[b]	135.5 ± 37.0[a]
(7)	0.036% Cholesterol	333.7 ± 19.0[b]	42.4 ± 14.5[a]
(7)	0.36% Cholesterol	589.4 ± 61.0[c]	49.9 ± 4.0[a]
Spider (5)	Basal	152.8 ± 6.9[a]	73.8 ± 8.2[a]
	High sucrose with		
(5)	0% Cholesterol	208.8 ± 4.2[b]	68.2 ± 9.4[a,b]
(5)	0.036% Cholesterol	211.0 ± 18.0[b]	46.4 ± 4.0[b,c]
(5)	0.36% Cholesterol	327.6 ± 34.8[c]	34.4 ± 8.4[c]

[a,b,c]For each species means within a column without a common superscript differ significantly at the $p<0.05$ level.

terol responses than the 25% protein diet. In contrast, further reduction in dietary protein to the 4% level markedly increased the serum total cholesterol response. Since pre-β-lipoprotein cholesterol levels showed no significant change due to exogenous cholesterol and to the level of dietary protein intake, the observed changes in serum total cholesterol at different levels of dietary protein were reflecting changes in both β- and α-lipoproteins. It should be noted that a markedly increased α-lipoprotein response occurred in the cholesterol-supplemented 4% protein diet. The underlying reasons for the varied effects are not clear, although the synthesis of liver-produced plasma proteins is known to be regulated by dietary proteins and essential amino acids availability (17).

Synergistic Effect of Dietary Sucrose and Cholesterol

The effect of dietary sucrose and cholesterol on serum total cholesterol and triglyceride levels in squirrel and spider monkeys is given in Table V. On basal diet, squirrel monkeys had higher mean levels of serum total cholesterol and lower mean levels of serum triglycerides than spider monkeys. Following six weeks of high sucrose diet without exogenous cholesterol, serum total cholesterol levels increased in both squirrel and spider monkeys, but serum triglycerides increased only in squirrel monkeys. The addition of exogenous cholesterol at the 0.36% level to the sucrose diet resulted in marked increased in the serum total cholesterol levels in both species. The serum total cholesterol response even at three weeks of 0.36% cholesterol-supplemented sucrose diet feeding was relatively high when compared to the previous response observed in the same species (Table II) where no sucrose was used as dietary carbohydrate. These observations are consistent with the consideration that endogenous cholesterol synthesis related to sucrose (18-20) or other dietary components plays an equally important role in modulating the serum total cholesterol levels. Overall, the serum total cholesterol response of squirrel monkeys was significantly greater than that of spider monkeys given the same diets ($p<0.001$). Even low levels of dietary cholesterol (0.036%) tended to decrease the serum triglyceride levels in both species. The observation that exogenous cholesterol has markedly suppressed serum triglycerides in these two species is in agreement with the studies of Illingworth et al. (21) who have shown a reduced secretion of triglycerides in squirrel monkeys when the diets contained 1.0 mg/kcal exogenous cholesterol.

The effect of dietary sucrose and cholesterol on different serum lipoprotein fractions in squirrel and spider monkeys is shown in Table VI. While dietary sucrose with or without exogeneous cholesterol increased the serum β-lipoprotein cholesterol in both species, the above diets had no appreciable effect on serum pre-β-lipoprotein cholesterol levels. α-Lipoprotein cholesterol response was far greater in squirrel monkeys than in spider monkeys, especially when the diets contained exogenous cholesterol.

DISCUSSION

The serum lipoprotein profiles of different nonhuman primate species including chimpanzees indicate significant inter-species differences. As with most mammals, α-lipoprotein seems to be the predominant lipoprotein in the majority of the nonhuman primate species

TABLE VI

Effect of Dietary Sucrose and Cholesterol on Serum Lipoproteins in Squirrel and Spider Monkeys

Species (no. animals)		Diet	Lipoprotein cholesterol (mean ± S.E.), mg/100 ml β-	Pre-β-	α-
Squirrel	(4)	Basal	82.3 ± 9.7[a]	1.0 ± 0.3[a]	108.8 ± 10.8[a]
		High sucrose with			
	(4)	0% Cholesterol	167.8 ± 24.4[b]	2.0 ± 1.1[a]	165.0 ± 20.4[b]
	(7)	0.036% Cholesterol	159.5 ± 16.3[b]	1.2 ± 0.2[a]	158.0 ± 11.1[b]
	(7)	0.36% Cholesterol	260.2 ± 18.2[c]	1.9 ± 0.4[a]	328.9 ± 55.6[c]
Spider	(5)	Basal	102.9 ± 4.2[a]	2.0 ± 0.2[a]	48.1 ± 4.7[a]
		High sucrose with			
	(5)	0% Cholesterol	141.8 ± 2.3[b]	3.3 ± 0.5[b]	64.2 ± 4.2[b]
	(5)	0.036% Cholesterol	153.9 ± 15.9[b]	3.4 ± 1.0[a]	54.2 ± 10.4[a]
	(5)	0.36% Cholesterol	242.2 ± 24.5[c]	2.4 ± 0.4[a]	80.4 ± 12.5[a]

[a,b,c]Means within a column without a common superscript differ significantly at the $p<0.05$ level.

studied. The differences in relative proportions of different classes of lipoproteins among these species might reflect their status of lipid and lipoprotein metabolism. The present study also indicates that dietary changes in cholesterol, protein, and carbohydrate affect the serum α-lipoprotein levels as much as, if not more than, the other serum lipoproteins. Prolonged feeding of high cholesterol-high fat diets is generally considered to lower serum α-lipoprotein levels in nonhuman primates (22-24). However, studies of cholesterol metabolism in baboons have indicated that both exogenous and endogenous cholesterol are generally transported nonpreferentially by serum α- and β-lipoproteins (25). The current observations show that the high cholesterol diet had varied effects on serum α-lipoprotein responses of different nonhuman primate species, suggesting a species-related phenomenon. These findings may have some bearing on comparative atherogenesis. Whether the high α-lipoprotein response observed in certain nonhuman species is a transitory response to dietary changes is not known although such a transitory hyper-α-lipoproteinemia has been observed by Morris and Greer (26) in rhesus monkeys.

Recently the occurrence of an HDL-like lipoprotein (HDL_c) in the low density fractions has been reported in cholesterol-fed miniature swine, dog, and patas monkeys by Mahley and coworkers (4-6) and in rats by Lasser and colleagues (27). Identification of this newly recognized α-lipoprotein subfraction is not possible by the polyanionic method used in this study, and ultracentrifuge studies will be needed in similar dietary programs to evaluate the components of HDL (28).

In view of the precursor-product relationship among the lipoprotein classes and the role of α-lipoproteins in lipid-clearing and cholesteryl esters transport through their respective enzymes, lipoprotein lipase and lecithin/cholesterol acyltransferase (14,29), species- and diet-related differences in α-lipoprotein response warrant further investigation. At this juncture, we can only speculate on the mechanisms. It is likely that the increase in α-lipoprotein results from active lipid transport, necessary to cope with increased availability of endogenous and/or exogenous cholesterol. While diet-induced β-lipoproteinemia may promote the flux of cholesterol into extra-hepatic tissues, the increased levels of α-lipoprotein may facilitate the transport of cholesterol from peripheral tissues to the liver for subsequent metabolism and excretion (29). The present studies provide a basis for exploring these possibilities under various dietary conditions in selected nonhuman primate species.

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Plasma Lipoprotein Changes in Suckling and Weanling Rabbits Fed Semipurified Diets

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ABSTRACT

The distribution and composition of the plasma lipoproteins were studied in suckling rabbits and in rabbits either weaned to or fed as young adults cholesterol-free, semipurified diets containing protein of animal (casein) or plant (soy protein isolate) origin. The raised cholesterol levels of the suckling period resulted in an increase of very low density and intermediate density lipoproteins in a manner similar to that seen in adult rabbits fed a high fat diet supplemented with cholesterol. Young rabbits fed the cholesterol-free, semipurified diet containing casein also became hypercholesterolemic but, in this case, the increased cholesterol was found primarily in lipoproteins of intermediate density. Cholesterol levels remained low in those rabbits fed the soy protein isolate diet, and the lipoprotein distribution was somewhat similar to that of chow-fed animals. It appears that the raised cholesterol levels during the suckling period result in different lipoprotein patterns to those produced in young adult animals by a cholesterol-free, semipurified diet.

INTRODUCTION

It is well known that adult rabbits become hypercholesterolemic when fed diets containing added cholesterol and that certain characteristic changes occur in the distribution and composition of the plasma lipoproteins (1-10). Cholesterol levels also increase in rabbits during the suckling period but decrease at weaning (11). This physiological hypercholesterolemia has been shown to depend on the cholesterol content and, to some extent, the fat content of mother's milk (11,12). These two cholesterolemic states are produced by the presence of exogenous cholesterol. Another type of hypercholesterolemia can be produced without the addition of exogenous cholesterol by feeding semipurified diets containing animal protein (13-15). Similar diets containing plant proteins result in normal low plasma cholesterol levels being maintained (13-15).

It has been reported that, in rabbits fed cholesterol for short time periods, more than two-thirds of VLDL cholesterol is derived from chylomicron cholesterol that is of dietary origin (16) and that the clearance of this exogenous cholesterol is delayed, with the accumulation of chylomicron remnants in the VLDL fraction (10). It is also thought that the major breakdown product of hepatic (or endogenous) VLDL is lipoprotein of intermediate density ($1.006 < d < 1.019$, IDL) (17). This study reports the lipoprotein patterns in cholesterolemic states produced with and without exogenous cholesterol. Classical "LDL" (1.006-1.063) which includes IDL was subdivided to isolate both IDL and LDL (1.019-1.063) in order to determine the relative contribution of each to the hypercholesterolemia. The lipoprotein patterns in rabbits fed chow or a semipurified diet containing plant protein are also reported.

MATERIALS AND METHODS

Animals and Experimental Design

Young adult male New Zealand White rabbits (1.3-1.5 kg body weight) and mature female rabbits were obtained from a local breeder. The males were housed and maintained as previously described (13). The young adult rabbits were fed ground chow for 1-2 weeks after their arrival and then fed the experimental diets for a period of 4 weeks. The diets were either commercial chow or low fat, cholesterol-free, semipurified diets containing either casein or soy protein isolate (27% w/w), dextrose (62% w/w), celluflour (5% w/w), molasses (2% w/w) and corn oil (1% w/w) together with appropriate amounts of vitamins and salt mix (13). The females were housed in breeder cages of galvanized iron with sawdust-covered bases and were mated with males from the breeding colony. After the young were born, the mother remained with them until they were 4½ weeks of age and was then removed. During this period, the mother had access to commercial chow.

Sampling of Blood and Analysis of Lipoproteins

Animals were bled by heart puncture into syringes containing EDTA (0.4M, pH 7.4) and

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plasma separated by centrifugation (600 g x 10 min). Weanling rabbits and young adult rabbits were bled after an overnight fast. This was not possible for the suckling animals and therefore chylomicrons were removed by a preliminary spin of the plasma overlayed with saline (d = 1.006, 24,000 g x 30 min). Lipoproteins were separated by a discontinuous density gradient ultracentrifugal method (18). The lipoproteins were analyzed on pooled samples from 2 day old rabbits and 4 week old rabbits, and on individual samples from 7 week old rabbits weaned to the semipurified diets or to chow. After 4 weeks on diet, lipoproteins from individual young adult rabbits were analyzed.

Analytical Methods

Lipids from the different lipoprotein classes were extracted and protein precipitated as previously described (18). Cholesterol was analyzed after saponification (19) by the method of Zlatkis and Zak (20). Triglyceride and phospholipid were analyzed by the methods of Neri and Frings (21) and Eibl and Lands (22), respectively. The protein precipitate was dissolved in NaOH (IN) and assayed by direct Nesslerization of Kjeldahl digests (23).

RESULTS

Plasma Cholesterol Changes with Age and Diet

The plasma cholesterol level of the young increased during the suckling period and declined sharply after weaning (Fig. 1). The level remained low in those animals weaned to chow or to the purified diet containing soy protein isolate but started to increase again by 7 weeks of age in those animals consuming the casein diet. Young adult rabbits had significantly higher ($P<0.001$) plasma cholesterol levels after 4 weeks on the diet containing casein (341 ± 37 mg/dl, mean ± S.E.) than after 4 weeks on the diet containing soy protein isolate (48 ± 6 mg/dl).

Lipoprotein changes with age and diet

Suckling rabbits. The mean concentrations of cholesterol, triglyceride and protein were significantly ($P<0.05$) increased in VLDL and IDL during the suckling period, but phospholipids were little changed (Tables I and II). The proportion of total lipoproteins circulating as VLDL and IDL increased from ca. 50% to over 70% by 4 weeks of age (Table III).

Weanling rabbits. Compared to the suckling animals at 4 weeks of age, the weanling rabbits had reduced levels of all components of the lipoproteins at 7 weeks of age (Tables I,II).

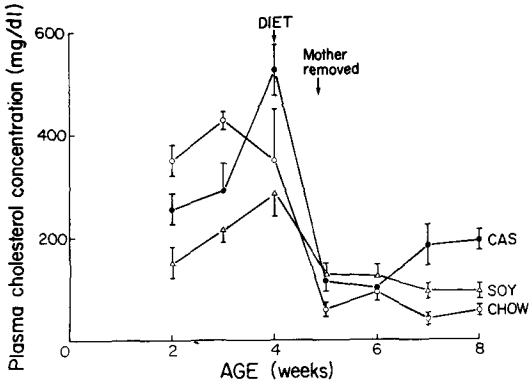

FIG. 1. Changes in mean plasma cholesterol concentrations with age in rabbits abruptly weaned to low fat diets. Food was offered from 4 weeks and the mother removed at 4½ weeks. Animals were weaned to low fat, cholesterol-free diets containing casein (CAS ●——●, 3 animals) or soy protein isolate (SOY △——△, 5 animals) or to commercial chow (CHOW ○——○, 4 animals). The vertical bars represent the standard error of the mean at each time point. At week 8, those animals fed casein had significantly higher ($P<0.001$) plasma cholesterol levels than those fed soy protein isolate or commercial chow.

There were major differences, however, between the dietary treatments. Animals weaned to chow had higher ($P<0.01$) VLDL triglyceride and phospholipid than those weaned to soy protein isolate (Tables I,II), and the proportion of lipoproteins circulating as VLDL was increased ($P<0.01$ Table III).

Animals weaned to the semipurified diet containing casein had similar levels of VLDL cholesterol, triglyceride, phospholipid and protein to those weaned to soy protein isolate, but higher ($P<0.05$) levels of the components of IDL. The increased plasma cholesterol was almost all in IDL (Table I), the result of which was a marked increase ($P<0.05$) in the proportion of circulating lipoprotein appearing as IDL (Table III).

Young adult rabbits. Rabbits fed the casein diet had increase ($P<0.05$) levels of VLDL cholesterol, phospholipid and protein and of all components of IDL and LDL ($P<0.05$) compared to those fed soy protein isolate (Tables I,II). The increases in the components of IDL were especially marked, producing a result similar to that in the weanling animals fed casein, i.e., an increase ($P<0.01$) in the proportion of circulating IDL to ca. 50% (Table III). There were differences, however, between the animals weaned to soy protein isolate and those fed it as young adults. The cholesterol levels of VLDL, IDL and LDL were higher ($P<0.05$) in the weanling animals fed soy

TABLE I

Cholesterol and Triglyceride Content of Plasma Lipoproteins[a] in Rabbits: Variation with Age and Diet[b]

	Mean concentration mg/dl ± S.E.[c]							
	VLDL		IDL		LDL		HDL	
	Cholesterol	Triglyceride	Cholesterol	Triglyceride	Cholesterol	Triglyceride	Cholesterol	Triglyceride
Suckling[d]								
2-3 days (3)[e]	36 ± 7**	77 ± 15	52 ± 8*	37 ± 14*	45 ± 10	19 ± 8	20 ± 3*	13 ± 3
4 weeks (3)	225 ± 14	157 ± 33	264 ± 63	94 ± 3	83 ± 17	25 ± 6	42 ± 4	18 ± 2
Weanling[f]								
Chow (4)	24 ± 7	86 ± 14**	17 ± 4*	28 ± 5*	11 ± 3*	19 ± 3	7 ± 1	9 ± 3
Soy (5)	28 ± 7	27 ± 4	54 ± 14	14 ± 3	33 ± 6	14 ± 1	11 ± 2	11 ± 3
Casein (3)	31 ± 1	35 ± 17	111 ± 21*	33 ± 7*	26 ± 2	16 ± 7	12 ± 2	5 ± 3
Young adult[g]								
Soy (6)	11 ± 2***	38 ± 7	19 ± 6****	15 ± 2***	7 ± 1***	6 ± 1*	8 ± 2	10 ± 2
Casein (6)	72 ± 2	35 ± 10	187 ± 8	48 ± 6	59 ± 1	14 ± 3	18 ± 6	7 ± 1

[a]The lipoproteins were separated into 4 classes, d<1.006 (VLDL), d=1.006 - 10.9 (IDL), d=1.019 -1.063 (LDL), d<1.21 (HDL).

[b]During the suckling period, the young only received mother's milk. The soy and casein diets were similar to the low fat, cholesterol-free, semipurified diets used in previous studies (13).

[c]Differences between means within each age group were analyzed for significance by Students' t-test. Within the weanling group, comparisons were made between soy and chow groups and between soy and casein groups. *P<0.05; **P<0.01; ***P<0.001.

[d]At 2-3 days of age, samples of blood from 3 animals were pooled for each lipoprotein analysis. At 4 weeks of age, samples of blood from 2 animals were pooled for each lipoprotein analysis. At all other times samples were obtained from individual animals.

[e]Number of samples for lipoprotein analysis.

[f]Weanling animals were offered the diets from 4 weeks of age and lipoproteins analyzed after 3 weeks on diet, at 7 weeks of age.

[g]The young adult rabbits were weaned to chow and fed the semipurified diets from about 7 weeks of age for a period of 4 weeks.

protein isolate than in the young adults fed that diet (Table I). The phospholipid and triglyceride content of LDL was also higher ($P<0.001$) and the protein content of VLDL lower ($P<0.001$) (Tables I,II). The result of these differences was a higher ($P<0.01$) proportion of circulating LDL and a lower proportion ($P<0.05$) of VLDL in the weanling rabbits fed soy protein isolate than in the young adult animals fed the same diet (Table III).

DISCUSSION

As has previously been reported (11,12), rabbits became hypercholesterolemic during the suckling period, and became normocholesterolemic after weaning. The changes in lipoproteins which occur during the suckling period are essentially an increase in the proportion of circulating VLDL and IDL with a concomitant decrease in LDL and HDL. The increase in VLDL and IDL is primarily caused by the increase in amount of circulating cholesterol and triglyceride. The cholesterolemia during this period is mainly the result of exogenous cholesterol and fat supplied in the milk (11,12) and may be compared with that produced by adding cholesterol (2 g/day) to the diet of adult rabbits. Under these conditions and at similar plasma cholesterol concentrations, Garlick et al. (3,24) showed that "VLDL" ($d<1.1019$, that is VLDL+IDL) and LDL increased, while HDL decreased as a proportion of circulating lipoprotein. While the lipoprotein pattern of cholesterol-fed adult rabbits is similar to that of the suckling rabbit in that VLDL levels are elevated, the composition of VLDL differs markedly. In the suckling rabbit, VLDL cholesterol and triglyceride are increased, whereas VLDL cholesterol is increased at the expense of triglyceride in cholesterol-fed rabbits (7,8). The lipoprotein pattern in the suckling rabbits is more analogous to that in adult rabbits fed a high fat diet containing cholesterol (8).

The hypercholesterolemia produced by the cholesterol-free, casein diet results in little change in VLDL but a marked increase in IDL to ca. 50% of the circulating lipoprotein, both when the cholesterol level was only moderately elevated (weanling rabbits, ca. 170 mg/dl) and when it was considerably elevated (young adults, ca. 340 mg/dl). Brattsand (25), using a somewhat similar semipurified diet containing 15% coconut oil, showed that "LDL" (d 1.006-1.063) transported most of the serum cholesterol when the serum total cholesterol levels were between 175-600 mg/dl. Above this level, VLDL became the major carrier of cholesterol. Since "LDL" was not subdivided into IDL and LDL, it is not

TABLE II

Protein and Phospholipid Content of Plasma Lipoproteins[a] in Rabbits: Variations with Age and Diet[b]

	Mean concentration mg/dl ± S.E.[c]							
	VLDL		IDL		LDL		HDL	
	Protein	Phospholipid	Protein	Phospholipid	Protein	Phospholipid	Protein	Phospholipid
Suckling[d]								
2-3 days (3)[e]	8 ± 4*	24 ± 6	17 ± 8**	56 ± 20	22 ± 2	69 ± 22	28 ± 1	40 ± 2
4 weeks (3)	36 ± 8	46 ± 7	75 ± 5	70 ± 25	79 ± 33	67 ± 19	44 ± 16	37 ± 9
Weanling[f]								
Chow (4)	12 ± 4	30 ± 4*	9 ± 2	17 ± 5	6 ± 2*	27 ± 6	19 ± 4	22 ± 3
Soy (5)	5 ± 1	11 ± 2	9 ± 2	15 ± 2	19 ± 4	31 ± 3	36 ± 11	30 ± 5
Casein (3)	6 ± 1	17 ± 6	34 ± 6*	48 ± 6**	15 ± 2	17 ± 3*	25 ± 6	14 ± 5
Young adult[g]								
Soy (6)	30 ± 4	15 ± 3*	21 ± 6**	14 ± 3***	12 ± 2*	10 ± 2*	37 ± 7*	23 ± 6
Casein (6)	31 ± 6	33 ± 5	86 ± 15	109 ± 20	41 ± 9	34 ± 6	58 ± 6	42 ± 8

[a]See footnotes to Table I.

TABLE III

Proportions of Total Lipoproteins[a] as Individual Lipoprotein Classes in Rabbits: Variations with Age and Diet[b]

	Total lipoprotein % mean ± S.E.[c]			
	VLDL	IDL	LDL	HDL
Suckling[d]				
2-3 days (3)[e]	26 ± 3	28 ± 6	26 ± 4	19 ± 7
4 weeks (3)	35 ± 7	36 ± 4	18 ± 4	10 ± 1
Weanling[f]				
Chow (4)	45 ± 4**	20 ± 2	18 ± 2**	17 ± 1*
Soy (5)	20 ± 3	25 ± 4	29 ± 3	25 ± 3
Casein (3)	20 ± 5	51 ± 6*	16 ± 1*	12 ± 2*
Young adult[g]				
Soy (6)	34 ± 4	25 ± 5**	13 ± 2	28 ± 4*
Casein (6)	21 ± 4	49 ± 4	16 ± 3	14 ± 2

Total Lipoprotein = TG+TC+PL+PR for each lipoprotein class.

$$\% \text{ VLDL} = \frac{\Sigma_{\text{VLDL}} \text{ TG+TC+PL+PR}}{\Sigma_{\text{TOTAL}} \text{ VLDL+IDL+LDL+HDL}}$$

[a]See footnotes to Table I.

possible to say if most of this increase was confined to IDL as shown in the present study.

Age may also be a factor in the different lipoprotein patterns of suckling, weanling and young adult rabbits. However, as the lipoprotein pattern of suckling rabbits appears similar to that of adult rabbits fed a high fat, cholesterol-supplemented diet (8), it would seem that diet is the more important factor. In addition, rabbits fed the casein semipurified diet respond in a similar manner whether fed from weaning or as young adults. Thus, diet appears to be the more important variable at least for weanling and young adult rabbits.

Comparison between animals fed casein or soy protein isolate shows major differences in the distribution of the lipoproteins. The proportion of circulating IDL is not increased in those fed soy but, unlike animals fed the casein diet, there are differences between those weaned to soy and those fed it as young adult animals. The proportion of VLDL is higher in young adult animals than in the weanling rabbits. It should be noted that at 7 weeks of age, the plasma cholesterol level of those animals weaned to soy protein was still somewhat elevated (124 mg/dl) compared to those weaned to chow (59 mg/dl). It is possible, therefore, that the plasma lipoprotein profile is in a transitional state at this time. The lipoprotein distribution in the young adult rabbit fed soy protein, in which the cholesterol level is at the normal low level (48 mg/dl), is somewhat similar to the distribution in the chow-fed weanling rabbits, with most of the circulating lipoprotein as VLDL (Table III).

The reasons for the differences between the lipoprotein patterns in the two hypercholesterolemic states are not clear. It has been shown that the clearance of exogenous cholesterol is delayed in cholesterol-fed rabbits and that there is an accumulation of cholesterol-rich chylomicron remnants in the VLDL fraction (10,16). These are presumably metabolized to IDL (17) accounting for the increased VLDL and IDL found in suckling and cholesterol-fed rabbits. As there is no dietary cholesterol being absorbed in animals on the casein diet, VLDL cholesterol, rather than chylomicron cholesterol, is probably the major source of plasma cholesterol. It is possible, therefore, that the accumulation of cholesterol-rich IDL which occurs under these conditions is analogous to the accumulation of cholesterol-rich VLDL in cholesterol-fed animals. Studies of the turnover and metabolism of these lipoproteins will determine if this supposition is correct.

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Reproduction and Survival of Rainbow Trout (*Salmo gairdneri*) Fed Linolenic Acid as the Only Source of Essential Fatty Acids[1]

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ABSTRACT

A semipurified test diet containing 1% linolenate as the sole dietary essential fatty acid was fed to a group of rainbow trout (*Salmo gairdneri*) for 34 months. The fish matured and the eggs produced were hatched. The second generation fry were fed our laboratory diet for 3 months. The growth of these fry was normal. Histologic examinations revealed no abnormality in liver, heart and kidney tissues of the fry during the three month period.

INTRODUCTION

Previous studies (1-3) have demonstrated that 1% or more of ω3 fatty acids (ω3 FAs) are required in the diet of rainbow trout if maximum growth is to be attained. Similar results were reported by other investigators (4-6). These experiments were conducted for restricted periods of 14 to 18 weeks. It has never been determined whether rainbow trout can grow to maturity, reproduce, and produce viable offspring on a diet containing only ω3 FAs and without ω6 FAs which are essential for land animals. Experiments were designed to answer these questions by a 34-month feeding experiment with two groups of rainbow trout. One group was fed a test diet containing only linolenic acid (18:3ω3). The second group was fed the same diet with the addition of linoleic acid (18:2ω6). Fish growth and feed efficiency during the first 14 weeks of feeding are presented. The fish were maintained on these diets for 34 months at which time the fish reached maturity. Reproductive products were produced and viability for the succeeding generation was established.

MATERIALS AND METHODS

Diet A contained 1% 18:3ω3 fatty acid (FA) and Diet B contained 1% 18:3ω3 + 1.5% 18:2ω6 FAs. A calculated quantity of ethyl laurate (12:0) was added to make the lipid content in each diet 6%. The diet ingredients, casein, gelatin, dextrin and α-cellulose were extracted with 4 exchanges of warm isopropanol to remove trace lipids. The composition of test diets is presented in Table I. Ethyl linolenate, linoleate and laurate were used as sources of 18:3ω3, 18:2ω6 and 12:0 FAs. These esters were obtained from Nu Chek Prep. Inc., Elysian, MN. Gas chromatographic (GC) analyses showed them to be at least 99% pure with the linolenate free of linoleate family.

The diet ingredients including esters were mixed with hot (65 C) water. The soft diet (60% water) that formed upon cooling was cut to appropriate size cubes (7). The diet was prepared monthly and stored at -20 C. The daily ration was thawed prior to feeding.

Eggs produced from the broodstock maintained in our laboratory were hatched and the fry fed a fat-free diet for one month to deplete the essential lipids carried over from the eggs. Fifty fish (average initial weight 0.43 g) were then randomly selected and stocked in each of four 60-liter fiberglass tanks. The water temperature was 11.5 C and the flow rate ca. 8 liters/min. Oxygen content in each tank, determined weekly, was in excess of 8 ppm. Each test diet was fed *ad libitum* to duplicate lots of fish 5 times daily. Fish growth, feed consumption and mortality were recorded biweekly during the first 14 weeks.

At the end of the 14th week, each lot was transfered to a 380 l. fiberglass tank and fed twice daily. For economic reasons, and to lessen the consumption of the pure fatty acid esters, the number of fish in each lot was reduced to 25. At the 12th month, the fish number was further reduced to 15 per lot. Finally, the duplicate lots were combined and only 8 females and 5 males were maintained on each test diet. Due to repeated reduction of fish population, feed consumption, and fish weight gain were not recorded after the initial 14 weeks.

When the fish were mature, eggs were taken from each female and divided into two lots. Each lot was fertilized by milt collected from a selected male. For comparative purposes, male trout from our laboratory broodstock main-

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TABLE I

Percentage Composition of Diets and Growth Response of Fish

Ingredients (dry)	Diet No. A	Diet No. B	Laboratory Control[a]
Casein (fat-free)	51.0	51.0	49.5
Gelatin	9.0	9.0	8.7
Dextrin	16.8	16.8	15.6
Ethyl linolenate	1.0	1.0	---
Ethyl linoleate	0	1.5	---
Ethyl laurate	5.0	3.5	---
Salmon oil	0	0	10.0
Carboxymethylcellulose	1.0	1.0	1.0
α-cellulose	9.0	9.0	8.0
Mineral and vitamin mix[b]	7.2	7.2	7.2
Average initial fish weight (g)	0.43	0.43	see Table III[a]
Average fish weight at 14 weeks (g)	4.84	4.03	
Feed conversion efficiency (gain/feed)	1.14	0.99	
Accumulated mortality (%)	4.0	2.0	

[a]Laboratory control diet was used to feed the second generation fry. See Table III.
[b]Percent in diet: mineral mix, 4.00; vitamin mix 2.00; choline chloride (70%), 1.00; vitamin E mixture, 0.20 (660 IU/kg); total 7.2%. Mineral mix: Bernhart-Tomarelli salt mix (9). Vitamin mix: supplies (mg/kg of diet): thiamin, 64; riboflavin, 144; niacinamide, 512; biotin, 1.6; capantothenate, 288; pyridoxine, 48; folic acid, 19.2; menadione, 16; cobalamine, 0.16; i-inositol, 2500; ascorbic acid, 1200; PABA, 400; vitamin D_2, 4000 IU/kg; vitamin A, 25,000 IU/kg.

tained on commercially prepared Oregon moist pellets (OMP) (8,9) were used to cross fertilize the eggs produced by the test diet fish. The OMP fish eggs were also fertilized by the test diet males.

The size, number and viability of the eggs produced by each test diet female fish were determined. The fry hatched from eggs by A diet females X A diet males were combined, and 550 fry were stocked in a 380 l. tank. The same number of fry from A females X OMP males; B females X B males; B females X OMP males and OMP females X OMP males were similarly stocked in individual tanks. The fry were fed laboratory control diet (Table I) for 3 months. Weight gain and mortality of these second generation fish were determined. At the end of the third month, a histologic examination was conducted on six fish from each group.

After collection of eggs and milt from the 34-month-old test diet fish, they were sacrificed. Total lipid was extracted from the whole fish following the method of Folch et al. (10). Phospholipids were isolated from the extracted lipid by using a silicic acid column (11). Methyl esters were prepared from the phospholipids by transesterifications with boron-trifluoride (12). GC separation and identification of the component fatty acids were carried out as described previously (11).

RESULTS AND DISCUSSION

The two test diets promoted rapid fish growth during the initial 14 weeks. During this period, the fish weight increased 10-fold. The diet A fish showed no ill effects due to lack of dietary 18:2ω6 FA. Fish weight gain and feed conversion efficiency of diet A fish were comparatively greater than that of diet B fish (Table I). Both groups had very low mortality.

At the 22nd month, two females from each diet group matured. Eggs produced were fertilized and hatched. The fertility of the eggs was estimated to be between 74% and 93%. However, hatching was not successful because of bacterium *Sphaerotilus* infection that caused suffocation of the embryos.

Feeding of the test diet to fish was continued. All fish matured at the 34th month. The eggs produced by these fish were pale yellow in color and were devoid of the familiar carotenoid pigments. The egg size varied from 8.5 to 15.0 ml/100 eggs. One of the OMP diet females produced the largest eggs; however, the other OMP fish produced the smallest eggs. The OMP female also produced the greatest number of eggs (6300) compared with the least number eggs produced by an A diet female (1272). Fry hatched from eggs of diet A female no. 1 were poor in quality and were discarded. The number, size, and viability of the eggs produced by

TABLE II

Viability of Trout Eggs

Eggs from	Fertilized with	Egg size ml/100 Eggs	Eggs total No.	Fertile eggs, %	Viable fry, %	g/100 Fry	Quality of fry
A Female (1)	A Male	14	671	0.04	0.02	---	Slow development
	OMP Male	14	671	0.07	0.03	---	Slow development
A Female (2)	A Male	12	1100	68.2	51.1	14.6	Normal
	OMP Male	12	900	66.7	62.2	13.1	Normal
A Female (3)	A Male	11	727	87.4	71.5	11.6	Normal
	OMP Male	11	545	99.8	85.5	11.6	Normal
A Female (4)	A Male	11	1636	73.3	39.9	12.2	Normal[a]
	OMP Male	11	1181	99.9	55.1	12.2	Normal[a]
OMP Female	A Male	15	3200	97.9	93.8	14.0	Normal
	OMP Male	15	3100	98.9	96.8	13.8	Normal
B Female (1)	B Male	13	1384	52.9	38.5	13.8	Normal
	OMP Male	13	1230	57.5	43.1	14.2	Normal
B Female (2)	B Male	11	1363	100	73.1	12.8	Normal[b]
	OMP Male	11	1545	87.1	73.2	12.0	Normal[b]
B Female (3)	B Male	12	1000	100	98.0	13.0	Normal
	OMP Male	12	916	100	98.3	12.6	Normal
B Female (4)	B Male	12	750	77.7	20.7	13.6	Normal
	OMP Male	12	750	88.8	38.4	11.8	Normal
OMP Female	B Male	8.5	1294	66.4	64.0	11.8	Normal
	OMP Male	8.5	1765	48.7	45.7	11.8	Normal

[a]A Few fish showed ascites.
[b]A Few fish showed scoliosis.

TABLE III

Growth of the Second Generation Fry

Fry hatched from	Average initial wt. (g)	Average wt. 3 months old fish (g)	Mortality (%)
A Female X A Male	0.15	3.04	14.6
A Female X OMP Male	0.13	2.48	3.0
OMP Female X OMP Male	0.16	3.39	1.0
B Female X B Male	0.15	2.80	11.0
B Female X OMP Male	0.15	2.69	5.5

each fish are summarized in Table II.

Table III presents the growth and survival rate of the second generation fry produced by the test diet parents. During the 3 month period, the growth was normal for all groups of fish. The accumulated mortality was 14.6% for fry produced by A females X A males and 11.0% by B females X B males. These mortality figures are not considered excessively high in the practice of artificial propagation.

Results of the histologic examination showed that the kidney, heart and liver tissues were normal in the second generation fish produced by all male and female crosses.

Based on fish growth, reproduction capability and the well-being of their succeeding generation, the data strongly support prior findings (1-4) that $\omega 3$ FA are the essential dietary FAs for trout. Addition of a $\omega 6$ FA-containing lipid to a trout diet formulation is unnecessary. Prior experiments (3,4) indicated that a high percentage of dietary $\omega 6$ FA would adversely effect the growth of trout.

The fatty acid composition of phospholipids extracted from the diet A and B fish carcasses and eggs are presented in Table IV. A high percentage of total $\omega 3$ FAs was present in all samples. The $\omega 3$ FA content in phospholipids of the diet A females and males was considerably higher than that of the diet B fish. The high $\omega 6$ FA content (1.5%) in diet B could have, to a certain extent, inhibited the

TABLE IV

Percentage Fatty Acid Composition of Trout Eggs and Carcass Phospholipids

Fatty acids	Diet A			Diet B		
	Female	Male	Egg	Female	Male	Egg
12:0	0.7	1.2	0.4	0.2	0.4	0.4
14:0	2.7	3.7	3.7	1.7	1.9	2.6
16:0	19.5	21.0	15.0	19.5	21.7	16.0
16:1	6.2	7.0	7.1	3.1	4.0	4.1
18:0	5.5	4.8	10.5	7.0	8.4	10.9
18:1	18.6	23.4	24.2	14.0	18.7	16.1
18:2ω9+ω6	1.7	1.9	1.2	---	---	---
18:2ω6	---	---	---	11.4	10.9	4.1
18:3ω6	---	---	---	0.3	0.2	0.2
18:3ω3	6.3	6.2	0.3	3.2	1.9	0.3
20:1ω9	0.7	0.9	3.0	0.8	0.8	2.4
18:4ω3	1.0	0.9	0.4	0.5	0.3	0.6
20:2ω9	0.8	0.8	1.3	0.4	0.3	0.6
20:2ω6	---	---	---	0.9	0.8	2.3
20:3ω9	0.7	0.8	4.4	1.4	1.0	1.5
20:3ω6	---	---	---	2.8	2.0	3.5
20:4ω6	0.4	0.5	0.5	8.4	7.6	8.8
20:4ω3	1.5	2.3	2.2	0.4	0.2	0.9
20:5ω3	4.2	3.0	3.4	1.9	1.6	2.2
22:4ω6	---	---	---	0.3	0.8	---
22:5ω6	---	---	---	2.2	2.0	1.5
22:5ω3	1.2	1.4	1.5	0.9	trace	0.8
22:6ω3	28.4	20.2	20.8	18.7	14.2	19.9
Total ω3 FAs	42.6	34.0	28.6	25.6	18.2	24.7
Total ω6 FAs	0.4	0.5	0.5	26.3	24.3	20.4

metabolism of the ingested ω3 FA and thereby reduced the incorporation of the ω3 FAs into phospholipids. This is in agreement with the competitive inhibition between linolenate and linoleate metabolism suggested by Rahm and Holman (13).

A small quantity of 20:4ω6 (arachidonic acid) was found in the phospholipids of the diet A male, female and eggs. A possible source of the ω6 FAs could be incompletely extracted diet ingredients such as dextrin, casein, and gelatin.

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Hydroxy Acids and Estolide Triglycerides of *Heliophila amplexicaulis* L.f. Seed Oil

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ABSTRACT

Thirty percent of the fatty acids from *Heliophila amplexicaulis* seed oil are hydroxy acids, primarily lesquerolic acid (14-hydroxy-*cis*-11-eicosenoic acid) with a trace of a new fatty acid, 16-hydroxy-*cis*-13-docosenoic acid. The hydroxy acids in the oil are found exclusively in the 1 and/or 3 positions of the triglycerides and are completely acylated with C_{20} or C_{22} saturated or monoenoic acids.

INTRODUCTION

Significant levels of erucic acid have been found in the seed lipids of nearly three-fourths the reported Cruciferous species (1). In addition, hydroxy acids have been reported in several genera of the family, most notably in the genus *Lesquerella.* These species contain relatively large amounts of C_{18} or C_{20} hydroxy acids (2). To date, no species containing both high levels of hydroxy acids and long chain acids such as erucic was known. Some of the hydroxy acids in the triglycerides of one species, *Lesquerella auriculata,* were found acylated with normal C_{16} and C_{18} fatty acids forming tetraglycerides (3). We now report that the seed oil of *Heliophila amplexicaulis* collected in Spain contains ca. 30% hydroxy acids, all of which are acylated with the C_{20} and C_{22} fatty acids.

MATERIALS AND METHODS

Oil was extracted from ground seeds and analyzed as previously described (4). Infrared (IR) spectra were recorded from liquid films on sodium chloride disks or 1% $CHCl_3$ solution; nuclear magnetic resonance (NMR) spectra were recorded from deuteriochloroform solutions with a Varian HA-100 spectrometer. Methyl esters were prepared from the oil by the BF_3-methanol procedure described by Kleiman et al. (5). Methyl esters of the free acid fractions from lipolysis of the oil were prepared using diazomethane. Gas liquid chromatography (GLC) analyses of esters were completed on polar (5% LAC-2R446 or 3% Silar 5CP) and nonpolar (5% Apiezon L) columns at oven temperatures of 200 C. GLC analyses of lipolysis mixtures were carried out on 3 ft x 1/8 in. stainless-steel columns, packed with 3% OV-1, temperature programmed from 150-400 C at 4 C/min. The monohydroxy esters were isolated by preparative thin layer chromatography (TLC) on 2mm Silica Gel G layers with hexane/ether (70:30) as the developing solvent.

Lipolysis of the glycerides were carried out as reported by Kleiman et al. (3). Lipolysis products were separated into four classes: free acids, monoglycerides, diglycerides, and unreacted glycerides. This was accomplished by preparative TLC on 2-mm Silica Gel G layers with hexane/ether (70:30). Methyl esters prepared from the free acid fraction were analyzed and separated using a 3.8 mm x 30 cm RP-8 reverse phase high pressure liquid chromatography (HPLC) column eluted with CH_3-CN. Detection of components was made by differential refractometry. A standard methyl ester estolide was made by reacting methyl ricinoleate with palmitoyl chloride as reported by Payne-Wahl et al. (6). Mass spectra were obtained at 70 eV using the gas chromatographic (GC) inlet on the Bendix 2625-Dupont 21-492-1 gas chromatography-mass spectrometer (GC-MS) system.

The whole oil was analyzed by HPLC on three columns: a 3.8 mm x 30 cm μ-Porasil column with an isooctane/ether acetic/acid (98:2:1) solvent system; a 7.9 mm x 30 cm Waters triglyceride analysis column with a CH_3CN/THF (3:1) solvent system; and a 7.9 mm x 30 cm C_{18} μ Bondapak column with an acetone/acetonitrile (3:1) solvent system.

RESULTS AND DISCUSSION

Heliophila amplexicaulis seed oil has an IR spectrum not unlike any normal triglyceride oil. No free OH absorbance is observed at 3500 cm^{-1}. TLC analysis of the oil showed essentially one spot with an R_f similar to that observed for normal triglycerides. However, TLC of the

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

methyl esters from the seed oil on silica plates showed two classes of components with R_fs indicating normal methyl esters and monohydroxy methyl esters. The hydroxy esters were isolated by preparative TLC and shown to be methyl lesquerolate (14-hydroxy-*cis*-11-eicosenoate), 99.8% and 16-hydroxy-*cis*-13-docosenoate by GC-MS and NMR. The NMR spectrum of this fraction is virtually identical to that of methyl ricinolate (12-hydroxy-*cis*-9-octadecenoate). Signals are observed at δ = 5.46 m(2H) for the double bond protons, δ = 3.60 s+m(4H) for the ester protons and the methine on C_{14} (C_{16} in the C_{22} homolog), δ = 2.0-2.2 m(6H) for the protons α to the carbonyl and the protons α to the double bonds; δ = 1.4 for the long chain methylene protons and δ = 0.88 t for the terminal methyl protons. Double resonance experiments show coupling between the methine proton and the farthest downfield portion of the multiplet at δ = 2.2 signal, which is from the protons on C_{13}. Coupling between the double bond protons and both of the signals in this multiplet arising from the protons on C_{10} and C_{13} was also observed. The mass spectrum of the 20:1-OH component from *H. amplexicaulis* was identical to that obtained for authentic methyl lesquerolate. The silylated derivatives were also identical.

Spectra of the trimethylsilyl (TMS) derivative of unsaturated hydroxy acids are more easily interpreted (7). The spectrum of TMS derivative of 20:1-OH component was again identical to the TMS derivative of authentic methyl lequerolate with intense fragments at m/e 187 and 227 and a TMS rearrangement ion (7) at m/e 298 indicating a hydroxyl on C_{14} and a double bond at C_{11}. The spectrum of the TMS derivative of 22:1-OH component had intense fragments at m/e 187 and 355 with a TMS rearrangement ion observed at m/e 326 and a M-15 ion at m/e 425, which indicated that the hydroxyl group and the double bond were located at C_{16} and C_{13}, respectively. Table I shows the composition of the total methyl esters from *H. amplexicaulis* seed oil.

The presence of large amounts of hydroxy acids in the saponified seed oil, with the absence of free OH adsorption in the IR and no evidence of hydroxy components by TLC, suggested that the hydroxy components were acylated in the original oil. The absence of an acetate band in the IR spectrum eliminated structures like those found in *Nerium oleander* (8) and suggested that the hydroxyls might be acylated by normal fatty acids. GLC analysis of the oil showed no evidence of normal triglycerides. Reverse phase HPLC of the seed oil, under conditions used to elute normal triglycerides (9), failed to elute any components. Increasing the strength of the solvent system to acetone/acetonitrile (3:1) eluted a cluster of components with a K′ of ca. 4.

TABLE I

Composition of Methyl Esters from *Heliophila amplexicaulis* Seed Oil by GLC

Component	Area, %
14:0	tr
16:0	1.1
16:1	0.2
18:0	0.6
18:1	12.1
18:2	5.2
18:3	11.1
20:0	1.8
20:1	7.9
20:2	0.7
22:0	3.5
22:1	25.0
22:2	0.6
24:0	tr
24:1	tr
20:1-OH	30.1
22:1-OH	tr

The NMR spectrum of *H. amplexicaulis* oil showed the signals observed in normal triglycerides with the addition of a multiplet at δ = 4.84 which arises from the methine proton at an estolide linkage. For comparison of NMR data, synthetic tetra-, penta-, and hexa-acyl triglycerides were prepared from the reaction of the 1-, 2-, and 3-hydroxy fractions of *Lesquerella globosa* seed oil [a source of lesquerolic acid containing triglycerides (10)] and erucoyl chloride. The synthesis and complete HPLC and TLC examination of these synthetic multiacyl triglycerides are reported separately (6). Comparison of the integrations of the signal, δ = 4.84 (1H/estolide linkage) from the methine proton on the carbon of the estolide linkage to the signal, δ = 4.2 (4H/molecule) from the α glycerol protons indicated a ratio of estolide signal per glycerol signal of 0.37. This corresponds to an average of ca. 1.5 estolide linkages per molecule. Ratios of 0.23 for the synthetic tetraglyceride, 0.50 for the pentaglyceride, and 0.78 for the hexaglyceride agree with the theoretical values of 0.25, 0.50, and 0.75, respectively.

HPLC and GLC analyses of the methyl esters from lipolysis of *H. amplexicaulis* oil show the presence of small amounts of normal fatty acids. In addition, the bulk of the fraction is four estolide esters, whose structures were established by GC-MS and their NMR spectra. Figure 1 shows the HPLC chromatogram of the esters of the free acid fractions. Components were eluted from a 30 cm x 3.8 mn RP-8

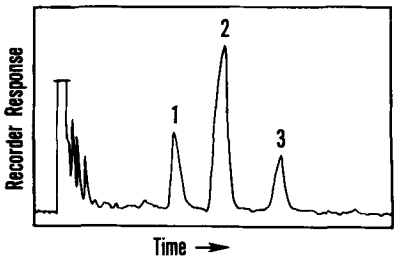

FIG. 1. HPLC chromatogram of 25 μl of 10% solution of methyl esters of free acids from lipolysis of *Heliophila amplexicaulis* seed oil. Column and solvent were as follows: 30 cm x 3.8 mm RP-8 column eluted with acetonitrile at 1.0 ml/min. Peaks were detected by differential refractometry. Normal esters eluted in solvent tail while peaks 1-3 were identified as estolides by GC-MS and NMR.

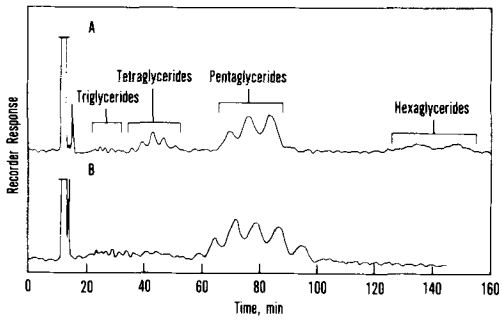

FIG. 2. HPLC chromatogram of a mixture of synthetic triglycerides, tetraglycerides, pentaglycerides, and hexaglycerides from *Lesquerella globosa* seed oil (A) and *Heliophila amplexicaulis* seed oil (B). Column and solvent were as follows: 30 cm x 7.8 mm Waters "Triglyceride analysis" column eluted with acetonitrile tetrahydrofuran (3:1) at 2.0 ml/min.

column with acetonitrile. The three major components were collected and identified as methyl eicosenoyloxy lesquerolate, a mixture of methyl eicosenoyloxy lesquerolate and methyl docosenoyloxy lesquerolate, and methyl docosanoyloxy lesquerolate by GC-MS and NMR. The NMR spectra had the multiplet at $\delta = 4.88$ representing the methine proton on the carbon atom of the estolide linkage. The integrated signal from this proton had a value of 1 relative to the methoxyl protons (3) and the terminal methyl protons (6). GC-MS of these four estolides showed ions at m/e 600, 602, 628, and 630, respectively, which arise from loss of 32 (CH_3OH) from the molecular ion. Intense ions were observed for the loss of the nonhydroxy acyl group with a hydrocarbon rearrangement at m/e 322. The other prominent ion was the nonhydroxy acyl ion (RC = O+) at 291, 293, 219, and 321, which arises by cleavage at the estolide linkage.

The monoglyceride fraction from the lipolysis of the *Heliophila* oil contained only monoglycerides of C_{18} unsaturated fatty acids. No hydroxy acids, longer chain normal acids, or estolides were detected. The lipolysis and NMR data indicated that *H. amplexicaulis* seed oil contains a mixture of tetraglycerides and pentaglycerides with a normal C_{18} unsaturated acid in the 2-position. Comparison of the HPLC chromatogram of *H. ampleicaulis* seed oil with the tetraglyceride and pentaglyceride standard (Figure 2) supports this conclusion. The tetra- and pentaglycerides overlap more in the *H. amplexicaulis* seed oil than the standards because the estolides in the natural oil are more complex, having tetraglycerides with C_{16}-C_{22} normal acids in two positions and estolides with four different fatty acids (20.0, 20.1, 22.0, and 22.1), whereas the standard estolides had only C_{22} estolides and only C_{16} and C_{18} normal acids in the other two positions.

Several triglyceride oils containing hydroxy acids are known to have more than three fatty acids per glycerol molecule. Acetic acid was the only acid esterified to the hydroxy acids in *Nerium oleander* (9), *Cardamine impatiens* seed oil (11), and accounts for approximately one-fourth of the estolide acids in *Chamaepeuce afra* (12). *trans*-2-*cis*-4-Decadienoic acids were found bound to the allenic hydroxy acid in *Sapium sebiferum* (13) seed oil while oil from *Monina emarginata* (14), *Mallotus philippinensis* (15), *C. afra,* ergot (16), and *Lesquerella auriculata* (3) are reported to contain normal C_{16} and C_{18} fatty acids. Only in ergot is estolide formation reported to be complete, yielding an oil with no free hydroxyl groups.

This is the first estolide-containing seed oil reported to have complete acylation of all of the hydroxy acids present. The specific distribution of the fatty acids in the glycerides of this seed oil, with the elongated normal acids being the only ones that are so completely acylated to the hydroxy acids, raises interesting questions about the biological system operating in triglyceride synthesis in this plant. *Heliophila amplexicaulis* is the first crucifer to have large amounts of both hydroxy fatty acids and elongated fatty acids. Perhaps it represents some link between the two enzymatic systems that accounts for the two types of crucifers previously reported.

ACKNOWLEDGMENT

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Identification of Novel Octadecadienoic Fatty Acids in the Seaweed *Cladophora rupestris* through Oxidative Ozonolysis of the Alcohols Prepared from the Acids

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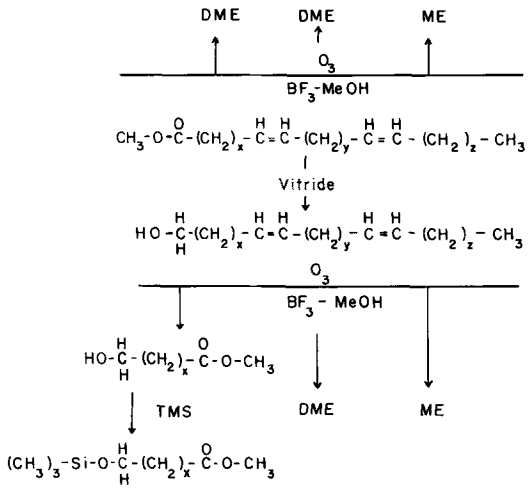
ABSTRACT

The BF_3-MeOH reagent for ozonolysis of ethylenic unsaturation does not oxidize alcohols. It is therefore feasible to determine the position of ethylenic unsaturation in long chain fatty alcohols of synthetic or natural origin by recovering the methyl ester products intact and silylating the alcohol function of half-ester, half-alcohol, products prior to gas liquid chromatographic analysis. The C_3 fragment from methylene-interrupted alkyl chains is not recovered, but, by first reducing carboxyl ester groups to alcohols, the terminal difunctional products can be identified in nonmethylene-interrupted dienoic fatty acids. The seaweed *Cladophora rupestris* is shown to contain Δ5,Δ11-, Δ8,Δ11-, and Δ11, Δ14- as well as Δ9,Δ12-octadecadienoic acid.

INTRODUCTION

The positions of the ethylenic bonds in certain mono- and polyethylenic fatty acids influence elution patterns for methyl esters in both gas liquid chromatography (GLC) and argentation thin layer chromatography ($AgNO_3$-TLC). In our work, fairly complex procedures must be applied to conclusively identify all isomers present in natural mixtures. A new combination of methods has now been illustrated through study of the octadecadienoic fatty acids of a seaweed, *Cladophora rupestris*. The long chain monoethylenic fatty alcohols of wax esters in marine crustacea (1-3) are an important lipid component, and their biochemical conversion to fatty acids is probably responsible for the pecularity that the docosenoic acids of marine origin are predominantly of the *cis* Δ^{11} or cetoleic structure instead of the *cis* Δ^{13} or erucic structure (3). Oxidative fission to determine the position of the ethylenic bond in these alcohols was required as part of studies of marine lipids (4), and it was discovered that the alcohol function was stable in the presence of BF_3-MeOH and ozone under conditions used to establish the positions of ethylenic bonds (5,6). Derivatization of alcohol functions for GLC was earlier achieved through acetylation (3), but can be conveniently achieved through silylation of the half-alcohol, half-ester product. Exploratory silylation steps were carried out initially after thin layer chromatographic (TLC) separation of the half-alcohol, half-ester product, but silylation could also be carried our directly on the mixture of all ozonolysis products recovered from the methylcyclohexane extractant initially used for recovery of ester products (5), or the chloroform introduced later (6).



SCHEME 1.

The original ozonolysis method has now been extended (6) to *cis,cis*-methylene-interrupted acids (i.e., Δ9,Δ12-octadecadienoic acid) and herein to specifically determine the difunctional structures of *cis,cis*-nonmethylene-interrupted octadecadienoic acids (e.g., Δ5,Δ11-18:2) by first reducing the carboxyl group to an alcohol, and then ozonizing in BF_3-MeOH, thus splitting the ethylenic bonds to give methyl ester products. The prior reduction of acid to the stable alcohol distinguishes the terminal difunctional product (C_5 in this case) obtained as an alcohol-methyl

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ester, from the central difunctional product (C_6) obtained as the dimethyl ester.

MATERIALS AND METHODS

Alcohols employed included octadecanol and *cis*-9-octadecenol (both purchased from Nu-Chek-Prep, Elysian, MN), a mixture of monoethylenic fatty alcohols prepared by reduction of the methyl ester of technical erucic acid with Vitrile* reducing agent (Eastman Kodak, Rochester, NY, 70% sodium bis-(2-methoxyethoxy) aluminum hydride in benzene), and a natural mixture of octadecadienols prepared by similarily reducing octadecadienoic esters isolated from the fatty acids recovered from a seaweed, *Cladophora rupestris*.

The seaweed lipid was saponified, non-saponifiables were removed, and the fatty acids recovered and converted to methyl esters. A pure C_{18} fraction was collected from preparative GLC on SE-30 and subjected to $AgNO_3$-TLC (7). The diene band, R_f 0.20, was relatively broad, contained methyl Δ9,Δ12-octadecadienoate and three other components, and was arbitrarily divided into three sections which were reduced and ozonized as described below.

For reduction, 20 mg or less of methyl esters were dissolved in benzene (1 ml) in a screw cap (Teflon*lining) centrifuge tube (10 ml). To this was added a 70% solution of Vitride in benzene (1 ml for 20 mg of 18:1 esters). After 20 min of agitation, the mixture was added slowly to a cool (0 C) 20% solution of H_2SO_4 in water (5 ml) in a 40 ml centrifuge tube immersed in an ice bath. The quenched mixture was agitated for 10 min and extracted with benzene (3 x 4 ml). The pooled extracts were washed with water (3 x 5 ml), dried, and the solvent removed under nitrogen. TLC showed only one product, R_f 0.48, corresponding to long chain fatty alcohols (Applied Science Prekotes; developed in petroleum ether/diethyl ether, 1:1).

Ozonolysis was executed in BF_3-MeOH as described elsewhere (5,6) with recovery of methyl ester or half-ester, half-alcohol, products by extraction with methylcyclohexane. Either the half-ester, half-alcohol recovered from TLC, or the total ozonolysis product was dissolved in dry pyridine in a capped tube. Hexamethylenedisilazane (200 μl) and trimethylchlorosilane (100 μl) (both from Applied Science Laboratories, State College, PA) were added, the mixture agitated for 1 min, and then stood for 5 min.

GLC was carried out in wall-coated, open tubular columns, 47 m x 0.025 mm I.D., of stainless steel coated with SILAR-5CP liquid phase (5). For ozonolysis silylation products operation was either isothermal at 120 C or isothermal at 120 for 24 min followed by programming at 24 C/min to 150 C and then isothermal operation at 150 C, depending on the chain lengths of the products to be expected. Other analyses were conducted at 180 C for SILAR-5CP and 190 C for Apiezon-L columns.

*Trade Mark

TABLE I

Compositions of Difunctional Products from Ozonolysis of Technical Erucic Acid[a] or Corresponding Alcohols

Chain length of difunctional product	Mole %	
	Dimethyl ester	Methyl ester-silyl ether
C_9	7.1	7.4
C_{11}	12.8	12.2
C_{13}	78.0	78.4
C_{15}	2.1	2.0

[a]Apparently simply crystallized from rapeseed oil and therefore mostly docosenoic acid, with eicosenoic and octadecenoic acids as major impurities.

RESULTS AND DISCUSSION

The exposure of the octadecanol to the oxidative steps with ozone in BF_3-MeOH did not result in any conversion of pure alcohols to aldehydes, acids or methyl esters, as shown by careful TLC and GLC analyses. The pure *cis*-9-octadecenol gave the expected products, methyl nonanoate and the corresponding C_9 half-acid ester, half-alcohol silyl ether in yields of 98% as shown with octadecanol as internal standard. As expected from other studies (6), two minor byproducts (i.e., the C_9 aldehyde and the corresponding C_9 acetal of the aldehyde) could be detected in both monofunctional and difunctional products at about 1% of the main products.

The technical erucic acid was ozonized in the form of both the methyl ester of the acids and of the alcohol derivative. Table I compares the relative recoveries of difunctional products. These, in fact, correspond to rapeseed oil main components, respectively, Δ13-docosenoic, Δ11-eicosenoic, Δ9-octadecenoic and Δ15-tetracosenoic acids, with minor additions from other monoethylenic isomers (8). The results from the two methods are closely comparable. Monofunctional products were not evaluated.

The products from ozonolysis of Δ9,Δ12-octadecadienol did not include the C_3 diester fragment, confirming studies carried out in

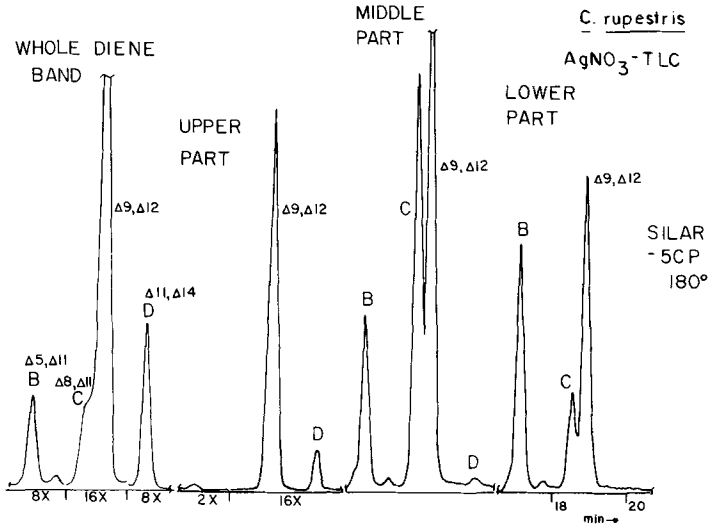

FIG. 1. Sections of charts of gas liquid chromatographic analyses, on a stainless-steel open-tubular column coated with SILAR-5CP, of $AgNO_3$-TLC diene band and respective segments from a separate preparation. Note attenuation changes in whole diene band section.

parallel with Δ9,Δ12-octadecadienoic acid (6). Reductive ozonolysis to give aldehydes is often used to distinguish terminal difunctional products (half-ester, half-aldehyde) from internal difunctional products from ozonolysis of fatty acids (9). Prior reduction of carboxyl groups to alcohols is equally satisfactory except for the absence of the C_3 fragment. Low recoveries of C_3 fragment are also reported for the procedure in which the carboxyl ester is retained and the ozonides are reduced to alcohols (10).

The dienoic fatty methyl esters from *C. rupestris* (Fig. 1) included two components (B and C) which on SILAR-5CP eluted ahead of the major isomer, Δ9,Δ12-18:2, and one component (D) eluting later. Table II shows the results of the ozonolysis of the seaweed fatty acids after reduction to alcohols. From this study and the analytical GLC of the isomeric dienes in each of the three segments of the $AgNO_3$-TLC band (Fig. 1), it is clear that the upper third of the band contained mostly Δ9,Δ12-octadecadienoic acid with some Δ11, Δ14-octadecadienoic acid (D) and a trace of Δ5,Δ11-octadecadienoic acid (B). The center part of the diene band was dominated by Δ9,Δ12-octadecadienoic acid, but included most of the Δ8,Δ11-octadecadienoic acid (C) and was also enriched in Δ5,Δ11-octadecadienoic acid (B). The lower third of the band was rich in both B (Δ5,Δ11-) and Δ9,Δ12-octadecadienoic acids and included a lesser proportion of Δ8,Δ11-octadecadienoic acid (C). Three of the octadecadienoic acid isomers identified (including the very common Δ9,Δ12-octadecadienoic acid) share the same basic methylene-interrupted structure. The position of the total ethylenic unsaturation would be expected to be meaningful in influencing $AgNO_3$-TLC mobility.

The respective enrichments of C and D relative to Δ9,Δ12-18:2 (Fig. 1) are in agreement with the findings for a series of synthetic *cis,cis*-octadecadienoic acids with methylene-interrupted unsaturation (11) and correspond to other observations on similar types of mono- and polyethylenic isomer elution (12-14). In the *cis*-monoethylenic isomers, the R_f values for ω6 and ω4 isomers are almost identical (12), whereas those for the complementary Δ9 and Δ12 isomers show a considerable difference, the Δ12 isomer being more mobile. The additive effect for two monoethylenic bonds may be reduced, but is still sufficient to help explain the greater mobility of isomer D relative to the Δ9,Δ12-18:2 isomer. On the other hand, in respect to isomer C, both the Δ8 and Δ11 monoethylenic isomers are less mobile than the corresponding Δ9 and Δ12 pair, yet isomer C is not concentrated in the lower part of the $AgNO_3$-TLC band relative to the Δ9, Δ12-18:2 to the extent which would be expected. Evidently, too much stress cannot be placed on additive effects and instead, as in GLC (15), the total effect of a chain substituent may be critical. Proximity of the ethylenic unsaturation to the end of the chain (16) and

TABLE II

Ozonolysis Products of the Three Segments of the Diene Band Obtained from C_{18} Methyl Ester Fraction of *C. rupestris* after Reduction to Alcohols

Sample $AgNO_3$-TLC band divided into three segments	Half methyl ester-half silyl ether products and origin if other than Δ9,Δ12		Diester from B	Monoester products and origin if other than Δ9,Δ12
		Mole %	Mole %	Mole %
Upper segment	$CH_3CO_2(CH_2)_8$ OTMS (C_9)	86.1	---	C_6 94.3
	$CH_3CO_2(CH_2)_{10}$ OTMS (C_{11},D)	13.9	---	C_4 5.7 (D)
Middle segment	$CH_3CO_2(CH_2)_4$ OTMS (C_5,B)	2.9	DMC_6 100	C_7 52.8 (B + C)
	$CH_3CO_2(CH_2)_7$ OTMS (C_8, C)	37.9	---	C_6 47.4
	$CH_3CO_2(CH_2)_8$ OTMS (C_9)	58.3	---	C_4 0.1 (D)
	$CH_3CO_2(CH_2)_{10}$ OTMS (C_{11},D)	0.9	---	---
Lower segment	$CH_3CO_2(CH_2)_4$ OTMS (C_5,B)	32.7	DMC_6 100	C_7 53.1 (B + C)
	$CH_3CO_2(CH_2)_7$ OTMS (C_8,C)	7.7	---	C_6 46.9
	$CH_3CO_2(CH_2)_8$ OTMS (C_9)	59.6	---	---

conformation of the molecule to the absorbent (12) may be factors in applying a complex and little understood, although very useful, chromatographic tool. The reason for the retardation on $AgNO_3$-TLC of the nonmethylene-interrupted Δ5,Δ11 isomer is not clear from observations on a variety of *cis,cis*-octadecadienoates (13,14) but is in all probability primarily due to the Δ5 bond which occurs in the most retarded *cis*-18:1 isomers (12). Equivalent chain length (ECL) values for methyl esters of Δ5,Δ11-; Δ8,Δ11-; Δ9,Δ12- and Δ11,Δ14 octadecadienoates were, respectively, 18.55, 18.75, 18.83, and 19.03 on SILAR-5CP and 17.33, 17.48, 17.48 and 17.63 on Apiezon-L. The methylene-interrupted acids elute in the correct order and with ECL values comparable with published figures (17). It was possible to verify the ECL value of 18.55 on SILAR-5CP for the Δ5,Δ11-18:2 as the sum of monoethylenic fractional chain lengths for the Δ5 and Δ11 *cis*-18:1 isomers, ca. 0.15 and 0.42 on the same column, plus the ECL base of 18:00, since there is little or no additional increment for interaction of the ethylenic bonds (18). The calculation (19) of ECL value for the Apiezon column (8) was equally satisfactory, the calculated ECL value for Δ5,Δ11-18:2 being 17.26 = (18.00 - [(18.00 - 17.64) + (18.00 - 17.62)]) vs. 17.33 observed.

The Δ8,Δ11- and Δ11,Δ14-octadecadienoic acid isomers are plausibly derived from the corresponding hexadecadienoic isomers (respectively, Δ6,Δ9 and Δ9,Δ12) which are known in marine plants (20-22); and the Δ5 desturase also operates in the C_{20} chain lengths to produce the Δ5,Δ11-eicosadienoic, Δ5,Δ11, Δ14-eicosatrienoic and Δ5,Δ11,Δ14,Δ17-eicosatetraenoic acids known to occur in *C. rupestris* (22), some other seaweeds (22,22), and in other types of plants (23). This type of origin satisfactorily explains the occurrence of only *cis* ethylenic unsaturation in the materials studied, although *trans* ethylenic bonds are not excluded from consideration in Δ5 unsaturation (8). The Δ11,Δ14-octadecadienoic acid can accumulate in animals (24,25).

Reduction of fatty alcohol ozonides to aldehydes and analysis of half-aldehyde, half-silyl esters is feasible (26), as well as the reduction to alcohols already mentioned (10). The oxidative ozonolysis route which we have evaluated is flexible and convenient and avoids the toxic-reducing agents sometimes used to achieve similar mixed function products (27). Preferential losses of shorter chain monoester product (Table II) can be minimized by using chloroform in lieu of methylcyclohexane (6). Suitable methyl ester qualitative standards are readily available, and others can usually be prepared as needed from cheap materials such as the technical erucic acid.

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Hypolipidemic Effects in Monkeys of ML-236B, a Competitive Inhibitor of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase[1]

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ABSTRACT

The fungal metabolite ML-236B, a competitive inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, has been shown to be significantly effective in lowering serum cholesterol levels in cynomolgus monkeys at doses of 20-50 mg/kg per day. Levels of serum phospholipids and triglycerides were, however, not significantly changed by the administration of the drug. Of the serum lipoprotein fractions, a β-lipoprotein corresponding to low density lipoprotein was preferentially reduced by the drug treatment. Fecal excretion of neutral sterols was unaffected but that of bile acids was slightly elevated by the administration of ML-236B.

INTRODUCTION

The fungal metabolite ML-236B, a potent inhibitor of cholesterol biosynthesis, has been isolated from the culture broth of *Penicillium citrinum* in this laboratory (1). The compound was shown to specifically inhibit 3-hydroxy-3-methylglutaryl CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, in a competitive fashion with respect to the substrate 3-hydroxy-3-methylglutaryl CoA (2). ML-236B is highly inhibitory to sterol synthesis both in vitro (3-5) and in vivo (5).

Hypocholesterolemic effects of ML-236B have been shown in rats treated with the detergent Triton WR-1339 (6) and in dogs (7). The present experiments show the hypocholesterolemic activity and the effects on the excretion of fecal neutral sterols and bile acids of this drug in monkeys.

MATERIALS AND METHODS

Animals

Cynomolgus monkeys weighing 3.8-4.5 kg from our biological testing colony were housed individually. The animals were maintained on 80 g (male) or 60 g (female) of a commercial monkey food (Type AB, Oriental Yeast Co., Japan). All animals received a daily piece of orange (50 g) to supply vitamin C.

ML-236B concealed in a 30 g piece of banana was given twice a day (at 10:00 a.m. and 4:00 p.m.) at doses of 20 and 50 mg/kg per day for 11 days. Control animals received banana without drug. The daily food intake and initial and final body weight were determined. Blood samples were taken at 9:00 a.m. where indicated. Total feces were collected for 48 hr on 5 days before initiation of drug treatment (pretreatment) and on 9 days on drug. To ensure complete recoveries, feces were collected several times per day, weighed and kept at -40 C until analyzed for neutral sterols and bile acids.

Chemicals

The lactone form of ML-236B was isolated from culture broth as described previously (1). [4-^{14}C]Cholesterol (54.0 Ci/mol) was from New England Nuclear (Boston, MA) and [carboxy-^{14}C]deoxycholic acid (50 Ci/mol) from Radiochemical Centre (Amersham, England). Cholesterol, coprostanol, desmosterol, campesterol, stigmasterol, β-sitosterol and lanosterol were from Applied Science Laboratories Inc., (State College, PA). 7-Ketodeoxycholic and 7-ketolithocholic acids were from Stearoid (Wilton, NH), and 5α-cholestane and methyl esters of deoxycholic, lithocholic, chenodeoxycholic and cholic acids from Analabs (New Haven, CT).

Lipid determinations

Serum cholesterol, triglyceride and phospholipids were assayed as described previously (7). Serum lipoproteins were stained with Sudan Black B and separated by polyacrylamide gel electrophoresis (gel concentrations were 1.875% for condensation and 3.0 and 5.0% for separation) by the method of Narayan et al. (8). For determination of lipoprotein composition, tubes were scanned in a Gelscan model 39375 (Gelman Instrument Co., Ann Arbor, MI), and percent composition of lipoprotein fractions was calculated from the respective area in the densitogram.

For extraction, isolation and analysis of fecal neutral sterols and bile acids, the methods of Grundy et al. (9) and Parkinson et al. (10)

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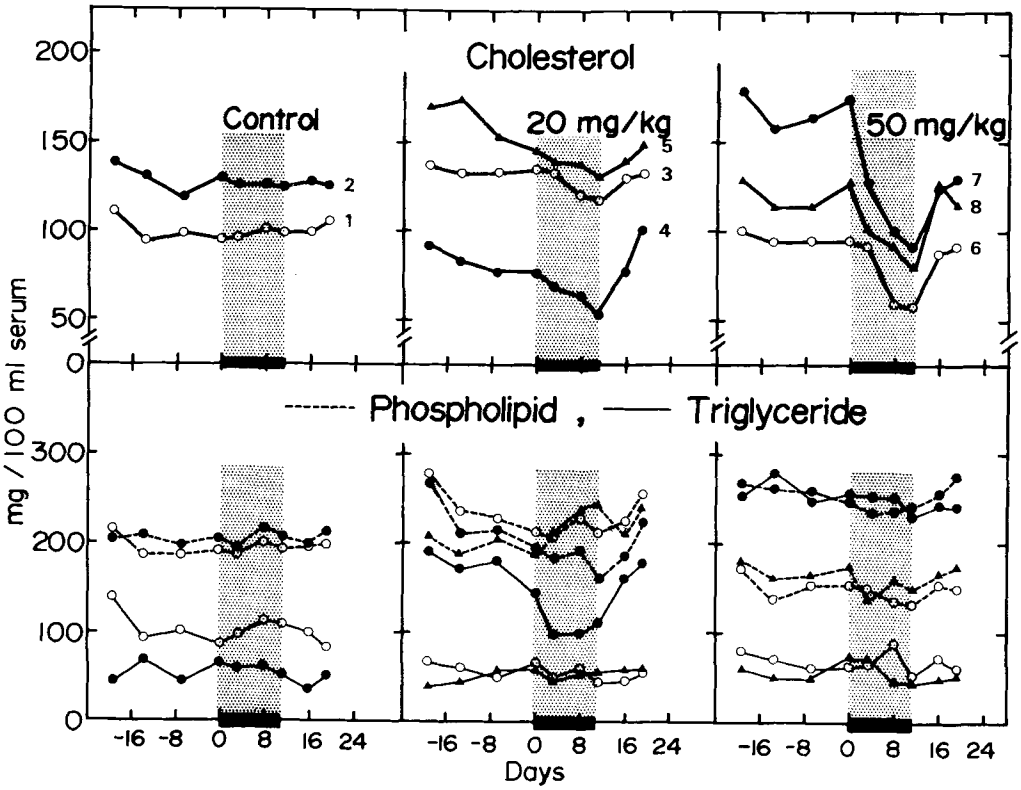

FIG. 1. Hypolipidemic effects of ML-236B in monkeys. Monkeys 1 (o, male) and 2 (•, female) in A were control animals, monkeys 3 (o, male) 4 (•, female) and 5 (▲, female) in B received ML-236B at a dose of 20 mg/kg per day for 11 days (from day 0 to 10, *stippled*), and monkeys 6 (o, male), 7 (•, female) and 8 (▲, female) in C received the drug at 50 mg/kg per day for 11 days.

TABLE I

Hypocholesterolemic Effects of ML-236B in Monkeys[a]

Dose (mg/kg/day)	Monkey No.	Percent decrease in serum cholesterol
20	3	12.4
	4	31.4
	5	17.7
(Mean) ± (S.D.)		20.5 ± 9.8
50	6	32.6
	7	44.9
	8	30.4
(Mean) ± (S.D.)		26.0 ± 7.8

[a]Experimental conditions were described in Figure 1. Values for each monkey represent percent decreases from the pretreatment levels (average of 4 determinations of intervals as shown in Fig. 1) after 11 days of treatment.

were combined and modified as described previously (7). Ultracentrifugation of serum lipoproteins was carried out by the method of Hatch and Lees (11).

RESULTS

As shown in Figure 1 and Table I, serum cholesterol levels were rapidly lowered in all monkeys treated with ML-236B at doses of 20 and 50 mg/kg per day regardless of beginning cholesterol levels. On day 11, percent decreases from pretreatment levels were 12-31% at 20 mg/kg per day and 30-45% at 50 mg/kg per day, respectively (Table I). The reduced levels

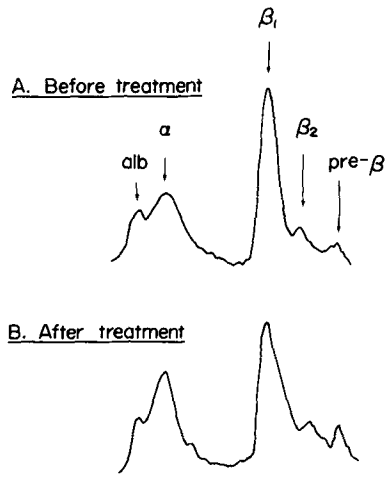

FIG. 2. Disc gel electrophoretic patterns of serum lipoprotein of monkey before (A) and after (B) treatment with ML-236B. Venous blood samples taken from monkey 7 (Fig. 1C) on day 0 and 11 were submitted to disc gel electrophoresis as described in Materials and Methods. Peaks alb, α, β_1, β_2, and pre-β represent albumin and α-, β_1-, β_2- and pre-β-lipoproteins, respectively (see text).

of serum cholesterol returned to pretreatment levels in 10-20 days after the termination of drug treatment in all animals (data for day 30 were not shown). Control animals showed no consistent changes in serum cholesterol levels during experimental period. Serum levels of triglyceride and phospholipid were not significantly lowered by the administration of ML-236B in all animals except monkey No. 4, which had far higher normal levels of serum triglyceride than the rest of the animals, showed a marked reduction in serum triglyceride levels by the treatment with ML-236B (Fig. 1). No changes were noted in levels of serum triglyceride and phospholipid in control animals during the period of the experiments. Weight gain and food intake were unaffected by the administration of ML-236B.

On disc gel electrophoresis, serum lipoproteins of cynomolgus monkeys were separated into 4 major bands, one with α-mobility, two with β-mobility (tentatively designated β_1 and β_2) and one with pre-β-mobility (Fig. 2). On the other hand, of the serum lipoprotein fractions isolated by fractional ultracentrifugation (11), both very low and low density lipoprotein (VLDL and LDL) showed a single band on disc gel eletrophoresis, corresponding to pre-β- and β_1-lipoprotein, respectively. The high density lipoprotein (HDL) was, however, separated into two bands: one with α-mobility and the other with β_2-mobility. Of the serum lipoprotein fractions, only β_1-lipoprotein was considerably decreased, and in opposition α-lipoprotein fraction was relatively increased in all animals treated with ML-236B (Table II).

Gas liquid chromatographic analyses showed the presence of 8 neutral sterols in feces of cynomolgus monkeys, of which 5 major peaks were identified as cholesterol, desmosterol, campesterol, stigamsterol and β-sitosterol, and lithocholic, deoxycholic, cholic and 7-keto-lithocholic acids were found to be major fecal bile acids. The fecal excretion of neutral sterols in cynomolgus monkeys was not affected by the treatment with ML-236B at a dose of 50 mg/kg per day. Thus, both relative contents of individual sterols and total sterol levels in feces of each monkey, which were measured at pretreatment and on day 9 (Fig. 1), showed no changes in treated animals as well as in control

TABLE II

Changes in Serum Lipoprotein Composition of Monkeys after Administration of ML-236B[a]

Dose mg/kg/day	Monkey No.	Day	Lipoprotein composition (%)			
			α	β_1	β_2	Pre-β
Control	1	0	34.3	40.7	12.9	12.1
		11	30.5	40.0	11.4	18.1
	2	0	44.5	42.2	8.9	4.3
		11	42.0	35.0	16.0	7.0
50	6	0	20.4	42.4	23.9	13.3
		11	32.2	31.4	23.5	12.9
	7	0	12.9	48.2	16.5	22.3
		11	30.7	27.9	17.1	24.4
	8	0	36.0	54.0	7.0	3.0
		11	43.6	43.9	7.7	4.9

[a]For all monkeys (Fig. 1), blood taken on days 0 (before treatment) and 11 (after treatment) was assayed as described in Materials and Methods. Details are shown in Figures 1 and 2.

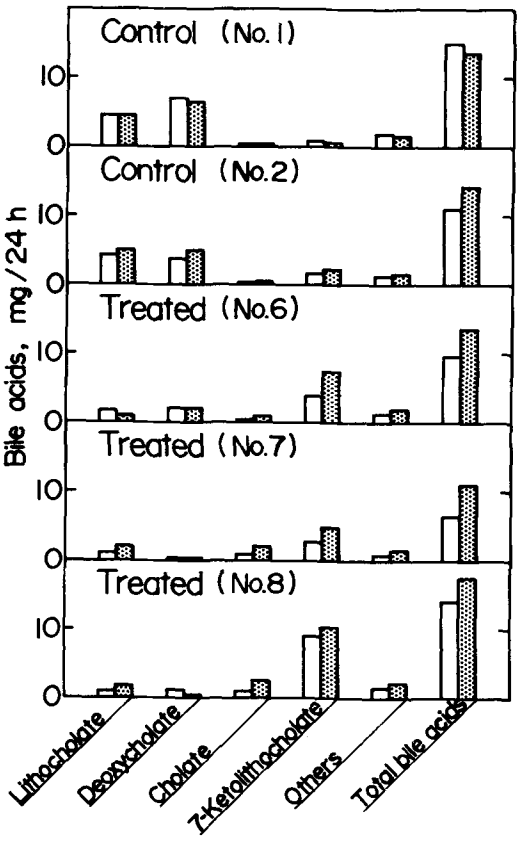

FIG. 3. Excretion of fecal bile acids in cynomolgus monkeys before and after treatment with ML-236B. Feces of 2 control monkeys (No. 1 and No. 2) and 3 treated animals at a dose of 50 mg/kg (Nos. 6, 7 and 8) collected at pretreatment and 9 days were assayed for bile acids as described previously (7). Open and *stippled* bars represent values for pretreatment and treatment, respectively.

group. The fecal excretion of total bile acids was, however, slightly elevated by the administration of ML-236B in all the animals treated (Fig. 3). Increases were 30-50% after 9 days of drug treatment at a dose of 50 mg/kg.

DISCUSSION

The present experiments have shown that ML-236B, a potent inhibitor of cholesterol biosynthesis, is highly effective in lowering serum cholesterol levels in monkeys at doses higher than 20 mg/kg per day. Serum triglyceride and phospholipids were, however, not consistently lowered by the drug.

Serum lipoproteins of cynomolgus monkeys were separated into four distinct components, one α-, two β- and one pre-β-lipoproteins, on disc gel electrophoresis. Of these fractions, one located in the β-region corresponding to LDL was preferentially decreased, as compared to the rest of the components, by the treatment with ML-236B. The results are very similar to those obtained with dogs and suggest that the drug is effective in the prevention and treatment of atherosclerosis, since LDL is thought to be responsible for the development of this disease (12).

Major bile acids excreted in cynomolgus monkeys were lithocholic and deoxycholic acids in some monkeys, but 7-ketolithocholic acid was predominent in the others. Thus, differences between individual animals are evident. The total amounts of fecal bile acids in all control samples (feces collected on day 5 for all animals and those collected on day 9 for control group) were, however, rather similar in all the monkeys except monkey 1, ranging from 10 to 15 mg/day. The values are comparable to those reported by Barnhart et al. (13), who studied the effects of the hypolipidemic agent probucol on the excretion of fecal bile acids in cynomolgus monkeys. The fecal excretion of bile acids is increased in dogs by the administration of ML-236B (7). The observed increase in fecal bile acids in the present experiments may be at least partly responsible for the hypocholesterolemic action of the drug, as previously shown with dogs (7).

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METHODS

A Simplified Procedure for the Quantitative Extraction of Lipids from Brain Tissue

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ABSTRACT

A method is described for the quantitative extraction of lipid from brain tissue with chloroform/methanol (C/M) that eliminates secondary purification of the lipid extract by dextran-gel chromatography or aqueous washing of the organic extract. Nonlipid substances that generally contaminate C/M lipid extracts are separated by pre-extraction of the tissue with dilute (0.25%) aqueous acetic acid. The residual tissue is extracted twice with 40 volumes of C/M (1:1, v/v). Approximately 97% of the lipid is recovered in these extractions. A third extraction which yields ca. 1% more lipid is performed if the process is discontinued at this stage in a shortened version of the method. The remainder of the lipid is recovered after treatment of the tissue with 1 N HCl by two additional extractions, the first with 40 volumes of C/M (1:2, v/v) and the second with 40 volumes of methanol. The method, which was demonstrated with pig brain, gave a complete extraction of the lipid, including gangliosides, free of nonlipid substances.

INTRODUCTION

The most widely used methods for the extraction of lipids from animal tissues are based on the use of chloroform/methanol by one or another modification of the Folch procedures (1,2). Historical and technological developments encompassing the Folch procedure leading to present methods of lipid extraction have been reviewed recently by Nelson (3). The major drawback of methods employing chloroform/methanol is that, while they provide essentially complete extraction of the lipid, they also extract substantial amounts of nonlipid substances (3,4). These substances are generally separated by solvent partition, washing with aqueous solvents (1,2,5-7), or dextran-gel chromatography (3,4,8-12).

Reported here is a method for the quantitative extraction of lipids from brain tissue by means of chloroform/methanol that does not require secondary purification by dextran-gel chromatography or aqueous washing of the lipid extracts. Nonlipid substances that generally are contaminants of C/M extracts of tissue are removed in a pre-extraction of the tissue with dilute acetic acid. The method also provides complete extraction of the gangliosides.

MATERIALS AND METHODS

Tissue

A fresh whole pig brain was obtained from a local slaughterhouse, immediately ground to a fine pulp, quick frozen with dry ice and stored at -50 C in a sealed container under an atmosphere of nitrogen to provide uniform samples for all experiments.

Solvents

Chloroform, methanol and low boiling petroleum ether (40-60 C) were purified by distillation. Glacial acetic acid and ammonium hydroxide were reagent grade and used as purchased.

Thin Layer Chromatography (TLC)

TLC was carried out with filter paper-lined tanks containing an atmosphere of nitrogen and solvents saturated with nitrogen with glass plates coated with 0.3 mm of Silica Gel H (Ag. Merck, Darmstadt, Germany) activated by heating them for 1 hr at 110 C. One dimensional TLC of polar lipids was carried out with chloroform (C), methanol (M), 2.5 N ammonium hydroxide (NH_4OH) (60:35:8, v/v/v). Two dimensional TLC was carried out using 65:35:5 C/M/conc. NH_4OH (v/v/v) in the first dimension and C/acetone/M/acetic acid/water (5:2:1:1:0.5, v/v/v/v/v) in the second dimension after drying the plate in an atmosphere of nitrogen for ca. 30 min. The spots were made visible by charring them with chromic-sulphuric acid (13); phospholipids were detected with a molybdenum blue reagent as described by Dittmer and Lester (14) and gangliosides with a resorcinol reagent (15). A ninhydrin spray reagent (16) (0.2% in butanol plus 5% acetic

acid) was used to detect nonlipid substances by TLC as previously described (13). In this procedure the presence of nonlipid substances is indicated by the detection of ninhydrin positive spots that do not char or which upon charring are not identifiable with any known lipid.

The chloroform/methanol extraction procedure reported by Nelson for rat brain (3) was used for comparison with our method. In this procedure two extractions were made with a solvent to sample ratio of 50:1 with chloroform/methanol (2:1, v/v), and a third extraction with a solvent to sample ratio of 25:1 with 2:1 (v/v) chloroform/methanol acidified with 1% HCl.

Dextran-gel column chromatography was carried out according to the procedure described by Wuthier (12) using Sephadex G-25 fine bead purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

PROCEDURE

One gram of wet tissue is homogenized for ca. 1 min with a Tekmar Super Dispax Tissuemizer Model SDT with SDT-100 N shaft at minimum speed in 5 ml of aqueous 0.25% acetic acid in a 40 ml centrifuge tube under an atmopshere of nitrogen. The mixture is allowed to stand 15 min at room temperature under an atmosphere of nitrogen, centrifuged 5 min at 3000 rpm and the supernatant decanted. The pellet of tissue is rehomogenized and extracted again in the same manner. These extracts contain no lipid (illustrated by TLC analysis in the Results section) and are discarded.

The extracts should be perfectly clear, as illustrated in Figure 1, which shows the supernatant obtained upon extraction of the tissues with different concentrations of acetic acid; otherwise, lipid is lost in the dispersed particles. Measurement of the pH of the supernatant indicated that it should be 4.0 to 4.4.

The pellet is slurried by means of a small stainless steel spatula in the same centrifuge tube with 40 ml of C/M (1:1, v/v) and allowed to stand for 5 min to insure complete mixing with the solvent. Then, the slurry is homogenized with the Tekmar homogenizer at maximum speed for 30 sec, allowed to stand for 10 min, centrifuged for 5 min at 3000 rpm and the C/M extract decanted. A second extraction is performed in a similar manner yielding ca. 97% of the lipid. A third extraction will increase the yield further by 0.5 to 1%, but is only performed when the procedure is stopped at this point in a shortened verion of the method.

Tightly bound lipid which consists mostly of gangliosides is recovered after treatment of the tissue with 1 N NCl as follows. The residue in the centrifuge tube is slurried by means of a spatula with 2 ml of 1 N HCl, allowed to stand 15 min and then centrifuged at 3000 rpm. The supernatant contains no lipid and is discarded. Residual HCl must be removed from the tissue, and this is accomplished by repeating the above procedure twice with 5 ml of distilled water. The final traces of HCl are removed from the tissue by means of a third washing with 2 ml of dilute 0.05 N ammonium hydroxide. Upon completion of these washings, the tissue is extracted once with 40 ml of C/M (1:2, v/v) containing 10 μl of glacial acetic acid by homogenization with the Takmar homogenizer at maximum speed for ca. 30 sec. The mixture is allowed to stand for 1 hr and centrifuged as before. The residue is then extracted with 40 ml of methanol in a similar manner. These extracts are combined with the first two C/M extracts and the solvent evaporated under reduced pressure at room temperature, care being taken to avoid evaporation to dryness and exposure of the extracted lipid to the atmosphere. The moist residue is taken up in 5 ml of C/M (2:1, v/v); insoluble material which consists mostly of fine particles of tissue is removed from the solution by passing it through a Pyrex microanalysis filter with a glass frit support (Millipore Cat. No. XX10,02500) using a Whatman GF/A glass fiber filter disc. The amount of the lipid extract is determined by weighing the residue recovered from a small aliquot of this solution on an analytical or micro-balance.

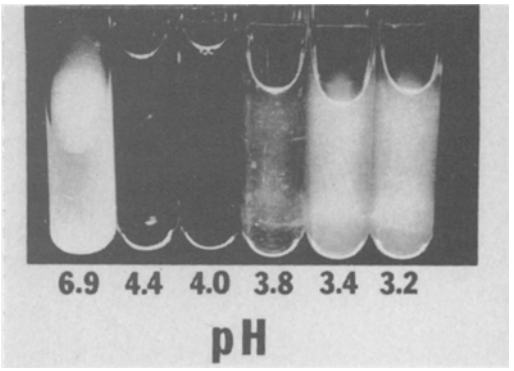

FIG. 1. Supernatant after homogenization of 1 g brain tissue in various concentrations of acetic acid.

RESULTS

The course of the extraction of the lipid of pig brain tissue by the procedure described

TABLE I

Extraction of Lipid from Pig Brain[a]

Extractions	New procedure[b]		C/M procedure	
	Solvent	mg/g	Solvent	mg/g
First	40 vol C/M, 1:1 (v/v)	93.4 ± 1.1[c]	50 vol C/M, 2:1 (v/v)	110.3 ± 1.0
Second	40 vol C/M, 1:1 (v/v)	4.4 ± 0.2	50 vol C/M, 2:1 (v/v)	0.5 ± 0.1
Third	40 vol C/M, 1:2 (v/v)	2.9 ± 0.2	25 vol C/M, 2:1 (v/v) plus 1% HCl	8.9 ± 1.1
Fourth	40 vol methanol	0.2 ± 0.1		
Total extract		100.9 ± 1.3		119.7 ± 2.0
Sephadex chromatography of total extract[d]		97.9 ± 1.6 [97.0][e]		98.7 ± 0.4 [82.5][e]

[a]Three separate analyses on 1 g samples.

[b]The tissue was pre-extracted twice with 0.25% acetic acid before the first two C/M extractions and the residue of the second extraction treated with 1 N HCl before the third and fourth C/M extraction (see text for details).

[c]M ± SD.

[d]Lipid fraction of Wuthier method (12).

[e]Percent of total extract.

above is compared to that of a standard chloroform/methanol procedure in Table I. These results show that the first two C/M extractions of the new procedure contained ca. 97% of the total lipid extract. The remainder of the lipid was recovered by two further extractions after treatment of the tissue with HCl, one with C/M (1:2, v/v) and the other with pure methanol. Extraction of the residual tissue with petroleum ether and chloroform after digestion with KOH followed by acidification gave only an additional 0.06 and 0.16 mg, respectively, of lipid. Hence, for all practical purposes, the new procedure gave an exhaustive extraction of all non-covalently bound lipid.

In contrast to the new procedure, much more material was extracted by the standard chloroform/methanol procedure, as shown in Table I. Slightly more material was obtained from the residual tissue after KOH digestion and acidification with HCl by extraction with petroleum ether and chloroform, 0.21 and 0.44 mg, respectively, but the tissue was essentially exhaustively extracted of non-covalently bound lipid. Much more material was removed from the total extract obtained with the standard C/M procedure than that with the new procedure by Sephadex chromatography, 17.5% compared to 3%, because the former contained considerable amounts of nonlipid substances. However, the total lipid free of nonlipid impurities recovered after Sephadex chromatography was not significantly different for the two procedures.

TLC analysis of the fractions (Fig. 2) provided an insight into the mode of extraction of the lipid classes. The first two extracts of the new procedure contained all of the lipid classes, including gangliosides, as evidenced by comparison with the total lipid extracts obtained by the standard C/M method. A third C/M (1:1, v/v) extract which was performed specially for TLC analysis as an extension of the first part of the new procedure showed that it contained only small amounts of phosphatidylserine, some glycolipids and ganglioside. After treatment of the tissue with HCl, small amounts of most of the lipid classes, including all of the gangliosides, were extracted with C/M (1:2, v/v). The final methanol extract contained mostly ganglioside indicating that essentially complete extraction of the lipid had been achieved by the new procedure

Comparison of the two dimensional TLC analysis of the total lipid extract obtained by the new method by the ninhydrin and charring techniques of spot detection, Figures 3A and 3B, respectively, showed that it was free of nonlipid substances inasmuch as the only

ninhydrin positive substances detected in the extract were phosphatidylserine (PS) and phosphatidylethanolamine (PE). The apparent positive spot at the origin of the plate in Figure 3A was not a true ninhydrin color but off-yellow that showed up positive in black and white photography. In contrast, the acetic acid extracts contained many substances that gave a positive ninhydrin test as shown by the TLC analysis in Figure 3C. None of the spots on this chromatoplate charred or corresponded to any known lipid. Hence, these extracts were devoid of lipid as far as could be determined.

The difference between the amount of material removed by Sephadex chromatography by the two procedures was due to the fact that the extract obtained by the new procedure was devoid of nonlipid substances, as shown by the TLC analysis in Figure 3.

TLC analyses of the total lipid extract obtained by the standard C/M procedure (Fig. 4) showed that it contained many nonlipid substances as evidenced by comparison of the spots that gave a positive ninhydrin test with those that charred (Figs. 4A and 4B, respectively). Moreover, the pattern of the ninhydrin positive spots (Fig. 4A) was very similar to that observed in the analysis of the acetic acid extracts obtained in the new procedure (Fig. 3C), except for PE and PS.

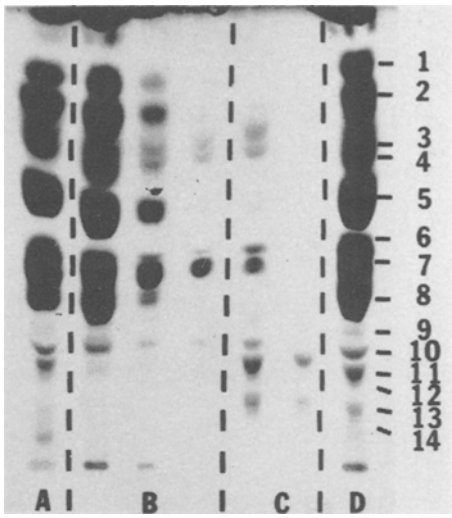

FIG. 2. Thin layer chromatogram on Silica Gel H with chloroform/methanol/2.5 N ammonium hydroxide (60:35:8, v/v/v). A, total extract by standard C/M procedure; B, first three C/M (1:1, v/v) extracts by new procedure; C, two extracts after HCl treatment by new procedure; D, total extract of new procedure. 1, cerebrosides with normal fatty acids; 2, cerebrosides with hydroxy fatty acids; 3, sulfatides with normal fatty acids; 4, sulfatides with hydroxy fatty acids; 5, phosphatidylethanolamine; 6, phosphatidylinositol; 7, phosphatidylserine and phosphatidylcholine; 8, sphingomyelin; 9, G_{M2}; 10, G_{M1}; 11, G_{D1a}; 12, G_{D2}; 13, G_{D1b}; 14, G_{T1}.

DISCUSSION

In order to obtain complete extraction of lipid from animal tissue, present methods generally employ two C/M (2:1, v/v) extractions, a third extraction with C/M (2:1, v/v) saturated with 28% (w/w) aqueous NH_3 and a fourth extraction with C/M (2:1, v/v) acidified with 1% HCl (3). Thus, the total lipid extract contains considerable amounts of nonlipid substances that must be removed to obtain a quantitative analysis of the lipid content of animal tissue.

The present investigation shows that purification of the chloroform/methanol extracts of brain tissues by dextran-gel chromatography or

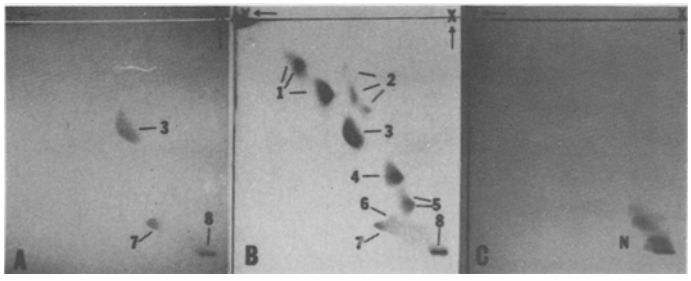

FIG. 3. Two dimensional thin layer chromatograms: X, first dimension development in chloroform/methanol/concentrated ammonium hydroxide (65:35:5, v/v/v); Y, second dimension development in chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5, v/v/v/v/v). A, total lipid extract by new method, chromatogram visualized with ninhydrin reagent; B, total lipid extract by new method, chromatogram visualized by charring; C, dilute acetic acid extracts, chromatogram visualized with ninhydrin reagent. 1, cerebrosides; 2, sulfatides; 3, phosphatidylethanolamine; 4, phosphatidylcholine; 5, sphingomyelin; 6, phosphatidylinositol; 7, phosphatidylserine; 8, origin; N, ninhydrin positive spots that do not char.

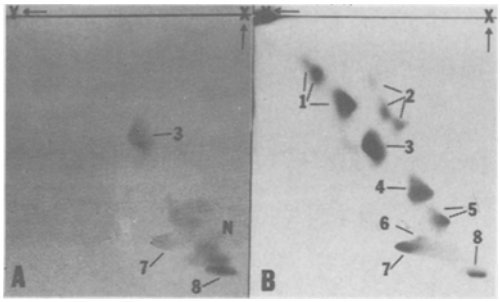

FIG. 4. Two dimensional thin layer chromatograms: X, first dimension development in chloroform/methanol/concentrated ammonium hydroxide (65:35:5, v/v/v); Y, second dimension development in chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5, v/v/v/v/v). A, total lipid extract by standard C/M method, chromatogram visualized with ninhydrin reagent; B, total lipid extract by standard C/M method, chromatogram visualized by charring. 1, cerebrosides; 2, sulfatides; 3, phosphatidylethanolamine; 4, phosphatidylcholine; 5, sphingomyelin; 6, phosphatidylinositol; 7, phosphatidylserine; 8, origin; N, ninhydrin positive spots.

aqueous washing of the lipid extract can be eliminated by the simple expediency of pre-extraction of the tissue with dilute acetic acid. The key to extraction of the tissue with acetic acid is a clear upper phase upon centrifugation. Otherwise, some lipid will be lost in the fine dispersion of particles in the supernatant. With a concentration of acetic acid less than 0.25%, tissue particles containing lipid are dispersed in the solution and are difficult to centrifuge. With high concentrations of acetic acid, some of the lipid particles together with nonlipid substances are solubilized or they cannot be readily separated because they are very finely dispersed. The optimum pH for separation of the lipid particles and extraction of the nonlipid substances appears to be between 4.0 and 4.4. Under these conditions, all of the nonlipid substances are extracted and the dispersed tissue containing lipid is readily centrifuged into a pellet. Mineral acids cannot be used to adjust the pH because these acids catalyze hydrolysis of the vinyl ether lipids.

Chloroform/methanol of a 1:1, v/v, mixture is used instead of the traditional 2:1, v/v, mixture in order to facilitate centrifugation as well as to insure complete extraction of the more highly polar lipids. It has been reported that not all of the lysophosphatides are extracted with C/M (2:1, v/v) (17); in more recent work, Nelson (3) has recommended a C/M ratio of 1:1 (v/v) for the extraction of lipid from animal tissues.

Although only ninhydrin positive substances are detected in the technique employed here to detect nonlipid substances, their detection in conjunction with the detection of lipids by charring and the molybdenum blue test for phospholipids appears to give a valid test for the presence of nonlipid substances inasmuch as the ninhydrin test is very sensitive and all of these substances should have similar solubility properties. Otherwise, they should not be extracted with mixtures of chloroform and methanol as well as aqueous solvents. In accessory experiments, it was found that the acetic acid extracts contained substances that gave a positive test with α-naphthol. However, this test is not as sensitive as the ninhydrin test and does not appear to be as good an indicator of nonlipid substances.

There is no evidence that treatment of the tissue with dilute (0.25%) acetic acid hydrolyzes ether lipids. However, compounds (salts) with vinyl ether structures remaining in the tissues after exhaustive extraction with C/M (1:1, v/v) would be split by the HCl treatment. No such compounds were detected in the present study inasmuch as no lysophosphatides were detected in the final two extracts. Whether any such compounds do exist is not known. It is, of course, very important that all of the HCl be removed from the tissue prior to the final extractions because upon combining these extractions with the initial C/M extracts, traces of HCl will promote hydrolysis of plasmalogens.

The advantage of the procedure described here is that it is simple, faster than conventional methods, and provides a quantitative extraction of the lipid, including the gangliosides, free of nonlipid impurities. If yields of lipid of the order of 98% are satisfactory, the shortened version of the procedure is very simple and rapid, and provides an extract that is free of nonlipid substances. The conditions described here were worked out specifically for brain tissue, which contains large amounts of highly polar lipids. The principle applied here can be used as the basis of a simple method for the quantitative extraction of the lipid from other tissues. However, the conditions of the acetic acid extraction must be varied slightly with some tissues to insure separation of the nonlipid substances without loss of lipid and to obviate the effects of hydrolyzing enzymes that are a problem in some tissues. These aspects of the application of the method are currently under investigation with a variety of other tissues.

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The Conversion of Phosphatidylethanolamine into Phosphatidylcholine Labeled in the Choline Group Using Methyl Iodide, 18-Crown-6 and Potassium Carbonate

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ABSTRACT

A chemical method for the conversion of phosphatidylethanolamine into phosphatidylcholine is described. Methyl iodide in the presence 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) and potassium carbonate in benzene is used to alkylate phosphatidylethanolamine in 2.5 hr at 37 C to give an isotopically enriched phosphatidylcholine. The product is purified conventionally and is obtained in 75% yield.

At present, a very limited number of published procedures are available for the chemical labeling of the choline moiety of phosphatidylcholine, and each of these has severe limitations including large excesses of reagent, long reaction times and undesirable byproducts. The procedure of Stoffel et al. (1) involves the mono-demethylation of phosphatidylcholine to the corresponding N,N-dimethylphosphatidylethanolamine, followed by methylation with methyl iodide. This procedure calls for methylation with barium oxide or sodium hydroxide in methanol and is often accompanied by undesirable deacylation, producing lysophosphatidylcholine as a side product. The procedure of Stockton et al. (2) for the trimethylation of phosphatidylethanolamine requires a large excess of methyl iodide and a rather long reaction time (14 days) to obtain a high yield of labeled phosphatidylcholine. Smith et al. (3) have recently published two procedures; one involved the trimethylation of phosphatidylethanolamine with silver carbonate (Fetizon's reagent) and methyl iodide; the other involved methylation of the same substrate with diazomethane in tritiated water to obtain a low yield of phosphatidylcholine.

We report here a mild and efficient procedure for the trimethylation of phosphatidylethanolamines. This procedure involves the 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) catalyzed trialkylation of phosphatidylethanolamine with methyl iodide in the presence of anhydrous potassium carbonate. Typically, the reaction is complete in 2.5 hr at 37 C. We have used this method to synthesize phosphatidylcholines labeled with ^{13}C and ^{2}H in the choline methyl group, obtaining yields of isolated products of 70% or greater. This method has also been used to synthesize 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine labeled in the quaternary ammonium group from purified egg phosphatidylethanolamine in the following three-step reaction sequence: 1) labeling egg phosphatidylethanolamine with perdeuterated methyl iodide to obtain the deuterated egg phosphatidylcholine, 2) deacylating with methanolic tetrabutylammonium hydroxide (4), and 3) reacylation with myristic anhydride using 4-pyrrolidinopyridine as catalyst (5).

The enhanced solubility in organic solvents of inorganic ions by cyclic crown ethers lead us to investigate the use of 18-crown-6/K_2CO_3 as a base for the alkylation of phosphatidylethanolamine. Using this reagent, we have demonstrated that phosphatidylethanolamines can be methylated directly to give labeled phosphatidylcholines. Subsequently, the labeled phosphatidylcholine can be deacylated to $(GPC)_3$ $(CdCl_3)_2$ which can be reacylated to obtain any desired phosphatidylcholine. Since the reaction time is short and the isolated yield of the products is greater than 70% in each step, we feel this procedure offers a marked advantage over previously published methods.

EXPERIMENTAL

Materials

Egg phosphatidylethanolamine was purified from crude egg yolk lipids as described previously (6). Dimyristoylphosphatidylethanolamine was prepared by phospholipase D exchange as described by Yang and Morrisett (7). Phospholipids were purified either by silicic acid column chromatography or on a Waters Prep LC/500, equipped with radially compressed silica gel columns, and eluted with chloroform/methanol/water (60:30:4). Purity

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was judged by thin layer chromatography (TLC) on Brinkmann silica plates eluted with chloroform/methanol/water (60:30:4) and visualized with iodine and/or phospholipid spray (8). Methyl[^{13}C]iodide was purchased from Koch Isotopes (Cambridge, MA); perdeuteromethyl iodide and 18-crown-6 were obtained from Aldrich (Milwaukee, WI). Myristic anhydride was obtained from Nu-Chek (Elysian, MN); Rexyn I-300 was from Fisher (Pittsburgh, PA).

Procedures

Synthesis of egg phosphatidylcholine from egg phosphatidylethanolamine. Dry benzene (4 ml) was added to 256 mg (2.56 mmole) of K_2CO_3 (anhydrous powder) and 1.34 g (5.12 mmole) of 18-crown-6. After 20 min, a solution of 900 mg (1.28 mmole) of egg phosphatidylethanolamine in 5 ml of benzene was added (oil bath temperature, 37 C), followed by 747 mg, 5.15 mmole, of perdeuteromethyl iodide and the tube sealed under a N_2 atmosphere. After the reaction mixture had stirred for 2 hr, TLC showed phosphatidylcholine to be the major product. After stirring an additional half hour, the reaction was worked up by adding 25 ml of water and extracting with 3 x 25 ml of benzene. The solvent was evaporated and the residue purified by chromatography on a silicic acid column (1.5 x 40 cm) to give 720 mg (75.5%) of phosphatidylcholine which was identical to authentic material by TLC. In a similar manner, egg phosphatidylethanolamine and dimyristoylphosphatidylethanolamine were converted with $^{13}CH_3I$ and CD_3I to the corresponding phosphatidylcholines in 74% and 76% yields, respectively. The nuclear magnetic resonances (NMR) spectra were identical to the reported spectra.

Synthesis of 1,2-Dimyristoyl-sn-glycero-3-phosphocholine-[N-(CD$_3$)$_3$]. Phosphatidylcholine labeled by the above procedure was deacylated to *sn*-glycero-3-phosphocholine (GPC) according to the procedure of Brockerhoff and Yurkowski (4). The GPC was dissolved in 5 ml of 90% ethanol and added to a solution of $CdCl_2$ (570 mg) in 90% ethanol. After standing 4 hr at 0 C, the white precipitate was collected by filtration and dried in a drying pistol at 55 C under vacuum over P_2O_5; 800 mg (74%) of $(GPC)_2(CdCl_2)_3$ was obtained.

The labeled $(GPC)_2(CdCl_2)_3$, 678 mg (0.6 mmole), was dissolved in 3 ml of dry dimethylsulfoxide at 45 C and a solution of 2.1 g (4.8 mmole) of myristic anhydride and 182 mg (1.22 mmole) of 4-pyrrolidinopyridine in 3 ml of benzene was added. After stirring for 5 hr under N_2, TLC indicated that a large amount of phosphatidylcholine had formed. The reaction mixture was diluted with 20 ml of $CHCl_3$/MeOH/H_2O (5:4:1) and passed through a pre-equilibrated ion exchange column (Rexyn I-300). The eluent was evaporated and the residue chromatographed on a Waters Prep LC/500 as previously described (6). After evaporation and lyophilization, a 73% yield of phosphatidylcholine was obtained.

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COMMUNICATIONS

Argentation Chromatography on Silver Sulfamate-Impregnated Silica Gel Layers

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ABSTRACT

Silver sulfamate was used in place of silver nitrate as argentation and charring reagent in thin layer chromatography (TLC). This reagent permits thin layer chromatographic separations of fatty acid esters with differing degrees of unsaturation as well as of *cis/trans* isomeric fatty acids, and allows for direct charring of fractions. Examples given for separations on silica gel plates impregnated with silver sulfamate include cholesterol esters and fatty acid methyl esters.

INTRODUCTION

Because of its simplicity, charring of lipid fractions on thin layer chromatograms is frequently used for both qualitative and quantitative analyses (1-10). Charring after spraying with destructive reagents, such as sulfuric acid, has certain disadvantages; treatment with sulfur trioxide (11) or sulfur chloride (12) represents a better approach but also necessitates suitable laboratory equipment. Inclusion of charring reagents, such as $(NH_4)_2SO_4$, into the chromatographic layer is an alternative provided the agent does not interfere with the chromatographic separation process.

We have previously suggested (13) inclusion of sulfamic acid into chromatographic layers, a compound which decomposes upon heating to 200 C. The sulfuric acid formed in the process allows lipid zones to be evenly charred. Sulfamic acid usually does not interfere with the separation of lipid classes, for example, from serum lipids; however, separation of compounds differing in their degree of unsaturation cannot be achieved on layers containing sulfamic acid and silver nitrate.

This paper reports a method for the separation of various unsaturated lipids on silica gel layers containing the silver salt of sulfamic acid which also allows for direct charring of the fractions.

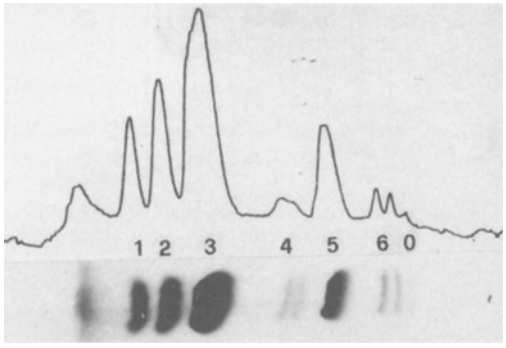

FIG. 1. Chromatography of serum cholesterol esters on silver sulfamate-impregnated silica gel layers: 1, CE of saturated FA; 2, CE of monoenoic FA; 3, CE of dienoic FA; 4, unidentified fractions; 5, cholesterol arachidonate; 6, triglycerides and free cholesterol (contaminations); O, origin.

EXPERIMENTAL PROCEDURES

The silver salt of sulfamic acid was prepared according to the method of Sakellarides (14). For this purpose, 8 g of silver nitrate was dissolved in 170 ml of distilled water, and a 10% solution of sodium hydroxide was added dropwise until the silver oxide was precipitated (pH 9-9.5). Ag_2O was filtered off and repeatedly washed with distilled water until neutral. Sulfamic acid, 4 g, in 30 ml of distilled water was used to dissolve the silver oxide, and residual solids were removed by filtration. The solution (pH 6.8-6.9) was evaporated under vacuum at 30 C to give colorless silver sulfamate prismatic needles (yield, 95-96%; purity, 99%; m.p., 225-245 C with decomposition).

Equal parts of Silica Gel G and Silica Gel HR (Merck), 2 g, were slurried with 5 ml of a 6% aqueous solution of silver sulfamate, and five glass plates, 190 x 40 mm, were uniformly coated (ca. 0.2 mm thick) by means of a spreader. The plates were allowed to air-dry and were kept in a desiccator over calcium chloride prior to use.

Cholesterol esters (CE) were prepared from human plasma by preparative TLC. A standard mixture of methyl esters of palmitic, stearic,

TABLE I

Quantitation of Cholesterol Esters by TLC/Densitometry or Gas Chromatography[a]

Cholesterol ester fatty acids	TLC mean ± SD	GC mean ± SD	F (GC/TLC)
Saturated esters	11.1 ± 0.3		
16:0		10.7 ± 0.2	1.018
18:0		0.6	
Monoenoic esters	13.2 ± 0.6		
16:1		1.0	0.962
18:1		11.7 ± 0.5	
18:2	64.5 ± 2.8	66.8 ± 2.2	1.036
20:4	11.2 ± 0.7	9.2 ± 0.4	0.821

[a]TLC/densitometry (TLC) and gas chromatographic (GC) data were determined in four independent assays. F values are expressed as GC data versus TLC data. GC data are not corrected for molecular weight differences.

oleic, linoleic, and linolenic acid (No. 189-1) was purchased from Sigma (St. Louis, MO, USA) to which elaidic acid was added. Cholesterol esters or fatty acid (FA) methyl esters were applied in *n*-hexane (1 mg/ml), ca. 0.2 mg per fraction, and the plates were developed with *n*-hexane/petroleum ether (bp 30-50 C)/diethyl ether/acetic acid, 35:12:2:1 (by vol) at room temperature (CE) or at 12-15 C (FA methyl esters).

After evaporating the solvents, the chromatograms were exposed to iodine vapors for 3-5 min. The plates were then kept over a hot plate for 4 min at 100 C and then for 4 min at 200 C. Lipid fractions appeared as dark brown or black bands on a white background.

Quantitations were carried out by densitometry (Zeiss-Scanner, double beam, reflected light). Densitometric results were compared with those obtained by gas chromatography of the methyl esters (Pye-Unicam; 250 cm x 0.5 cm glass column packed with 14% DEGS on 80/100 mesh Chromosorb W-DMCS; isothermal at 198 C; carrier gas, N_2; flow rate, 50 ml/min; flame ionization detector). Results were calculated by triangulation (rel. %). CE were converted to FA methyl esters with methanol/HCl.

RESULTS

Figure 1 shows a chromatogram and the corresponding densitometric profile of isolated cholesterol esters from human plasma. F values for the cholesterol esters tested, i.e., the ratios of data obtained by GC vs. those obtained by densitometry of thin layer chromatograms, are given in Table I.

Figure 2 shows a chromatogram and its corresponding densitogram of a standard mixture of fatty acid methyl esters. The respective F values were for saturated fatty acid methyl esters 2.58, for *trans*-monoenes 1.058, for *cis*-monoenes 1.055, for dienes 0.976, and for trienes 0.880 (from mean values, n = 4).

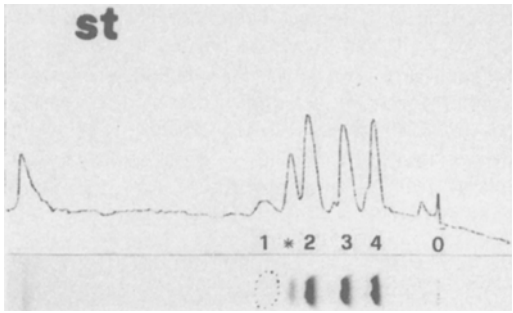

FIG. 2. Chromatography of fatty acid methyl esters on silver sulfamate impregnated silica gel layers: 1, saturated esters, *, elaidic acid methyl esters; 2, monoenoic esters; 3, dienoic esters; 4, trienoic esters; O, origin.

DISCUSSION

The use of an absorbent mixture consisting of Silica Gel G and Silica Gel HR was found to produce more intense darkening of fractions which could not be achieved with Silica Gel G alone. For developing, petroleum ether was added to the solvent system described by Dudley and Anderson (15), and single-development was used. When chromatography was performed at 12-15 C, the R_f values of FA methyl ester fractions were lower and well reproducible. TLC separation of CE can well be carried out at room temperature.

Exposure of chromatograms to iodine vapors leads to dissociation of silver sulfamate to free sulfamic acid necessary for charring at ca. 200 C. Under these conditions the background remained quite colorless. Heating to 100 C for

4 min is intended to eliminate iodine and solvents.

Use of silver sulfamate allows the TLC separation of various fatty acid esters differing in their degrees of unsaturation and permits direct charring of separated zones. Saturated FA methyl esters (F = 2.58) however, were not readily charred by our procedure. Privett and Blank (16) could achieve charring of saturated FA methyl esters by spraying with 80% sulfuric acid which was saturated with potassium dichromate, whereas 50% sulfuric acid produced similar results as silver sulfamate impregnation.

Analysis of cholesterol esters on silica gel layers impregnated with silver sulfamate gave well reproducible results for both saturated and unsaturated CE (see Table I). The procedure works well also for total plasma lipids. As can be seen in Figure 1, the R_f values of cholesterol arachidonate (0.30) and those of triglycerides (0.16-0.22) are sufficiently different to permit direct analysis. The procedure should prove useful for the quantitation of serum lipids as they are done in clinical laboratories.

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Separation of Tetra-, Penta-, and Hexaacyl Triglycerides by High Performance Liquid Chromatography

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ABSTRACT

Synthetic triglycerides with more than three acyl groups were prepared by forming estolides from triglycerides containing one, two, or three monohydroxy fatty acyl moieties. These tetra-, penta-, and hexaacyl triglycerides were examined by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). HPLC separation by the number of acyl groups was obtained with both conventional phase and reverse phase columns. In all systems, triglycerides were eluted first, followed in sequence by tetra-, penta-, and hexaacyl triglycerides. Within each glyceride class, further separation occurred due to variations in chain length and degree of unsaturation among the component acids. TLC migration on silica gel was found to be a function of the number of acyl groups and the length of component acid chains.

INTRODUCTION

Glycerides containing more than three acyl groups have been identified in several natural oils (1-4). These multiacyl triglycerides may be more prevalent in nature than indicated by the literature, since conventional analytical methods are not suitable for detection of estolide triglycerides composed of long chain fatty acids ($\geqslant C_{16}$) in oils containing hydroxy fatty acids. They are not eluted during gas chromatography (GC) under normal conditions (5) and are not easily separated from normal triglycerides by thin layer chromatography (TLC), although separation of estolide triglycerides by multidevelopment TLC has been reported (3).

In the study of the composition of natural seed oils, it is important that estolide linkages not be overlooked in those oils that contain hydroxy fatty acids. Therefore, a rapid, reliable procedure for detecting and isolating estolide triglycerides is needed. High performance liquid chromatography (HPLC), an effective technique for separating normal triglycerides (6), appeared promising for this analysis. To demonstrate HPLC separation of estolide triglycerides, we analyzed tetra-, penta-, and hexacyl triglycerides prepared from natural triglycerides with one, two, or three monohydroxy acyl groups (hereinafter, mono-, di-, and trihydroxy triglycerides).

MATERIALS AND METHODS

Isolation and Identification of Hydroxy Triglycerides

Linum mucronatum (7) oil was the source of monohydroxy triglycerides containing one ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid) moiety. One gram of the oil was placed on a 32.5 cm x 2.2 cm column of 60/200 mesh Hi-Flosil (Applied Science Labs) and eluted with 500 ml each of 5, 10, and 15% ether/hexane. Fifty milliliter fractions were collected, and those containing monohydroxy triglycerides were identified by TLC on 0.25 mm thick layers of silica gel with hexane/ether (70:30) as developing solvent. The di- and trihydroxy triglycerides of castor oil were isolated in a similar manner.

Mono-, di-, and trihydroxy triglycerides containing lesquerolic acid (14-hydroxy-*cis*-11-eicosenoic acid) are present in *Lesquerella globosa* oil (1). The hydroxy fractions were isolated from the whole oil by HPLC (8) on a Waters ALC 200 High Performance Liquid Chromatograph equipped with a differential refractometer using a 61 cm x 12.7 mm ID column packed with Porasil A (35-75 μ, Waters Assoc.) and eluted with a solvent gradient from isooctane to 50% ether in isooctane at 4 ml/min. Ten milliliter fractions were collected and the mono-, di-, and trihydroxy triglyceride fractions were detected by TLC. Small portions (5-10 mg) of each of the hydroxy fractions were separated by chain length and degree of unsaturation by HPLC on a 30 cm x 7.8 mm μ-Bondapak C_{18} column (Waters Assoc.) with acetonitrile/acetone (2:1) as solvent at 1 ml/min. The fatty acid composition of these subfractions was determined from methyl esters made by saponification of the subfractions in 0.5 N alcoholic KOH and esterification with 10% BF_3 in methanol. These methyl esters were silylated with BSTFA (Regis Chem. Co.) and analyzed on a Packard Model 7401 gas chromatograph equipped with a 10.2 cm x 6.4

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

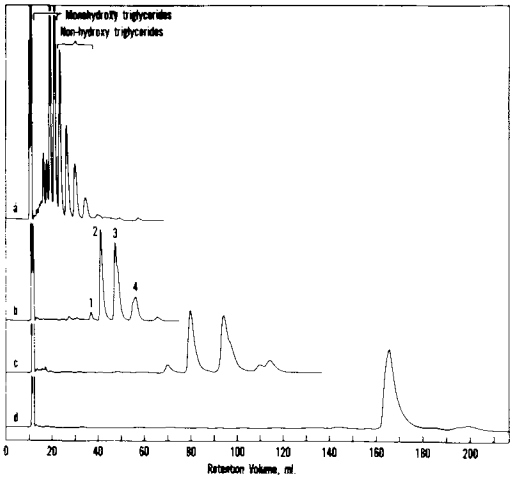

FIG. 1. HPLC of: (a) *Linum mucronatum* oil, (b) tetraacyl triglycerides, (c) pentaacyl triglycerides, and (d) hexaacyl triglycerides. Separations were obtained on a 30 cm x 7.8 mm μ-Bondapak C-18 column eluted with acetonitrile/acetone (2:1) at 1 ml/min.

mm glass column packed with 5% Apiezon L. on Chromasorb W-DMCS and a 25.4 cm x 6.4 mm glass column packed with 5% LAC-2-R 446 on the same support.

Estolide Preparation

Estolides of the *Linum* and castor hydroxy triglycerides were prepared by mixing 100 mg of hydroxy triglycerides in 10 ml of benzene with 0.4 ml palmitoyl chloride in 0.3 ml pyridine. The reaction mixutre was allowed to stand for 15 min. Estolides of the hydroxy triglycerides from *Lesquerella globosa* oil were prepared by reacting them with erucoyl chloride under the same conditions. The estolides were recovered from the reaction mixture by first concentrating it under a stream of dry nitrogen. The concentrate was then taken up in ca. 30 ml of diethyl ether and filtered. The filtrate was transferred to a separatory funnel, washed three times with water, and reconcentrated. The estolides were isolated by preparative TLC on 2-mm thick layers of silica gel with hexane/ether (70:30) as the developing solvent. Intact oils from *Linum mucronatum*, castor, and *L. globosa* seeds were also subjected to the estolide formation and purification procedures, yielding mixtures of estolide triglycerides and normal triglycerides.

Analysis of Estolide Triglycerides

The estolide products were analyzed by HPLC with the following columns and solvents: 7.8 mm x 30 cm triglyceride column (Waters Assoc.) with acetonitrile/tetrahydrofuran (3:1) at 2 ml/min; 7.8 mm x 30 cm μ-Bondapak C_{18} with acetone/acetonitrile (2:1 and 3:1) at 2 ml/min; 3.9 mm x 30 cm μ-Porasil with isooctane/ether/acetic acid (98:2:1 and 100:1:1) at 1 ml/min. Typically 1–2 mg of sample was injected as 50% solution in $CHCl_3$. TLC analysis of multiacyl triglycerides was performed on 0.25 mm layers of silica gel developed three times with benzene.

RESULTS AND DISCUSSION

Estolides of Ricinoleoyl Triglycerides

Linum mucronatum oil has the fatty acid composition: 6% 16:0; 3.6% 18:0; 24% 18:1; 48% 18:2; 2.0% 18:3; 0.4% 20:1; and 15% ricinoleic (7). Figure 1a shows the HPLC chromatogram of *L. mucronatum* seed oil on a μ-Bondapak C_{18} column. The triglycerides are separated by carbon number and degree of unsaturation as previously reported (6). First to elute are the monohydroxy triglycerides with various degrees of unsaturation. The monohydroxy triglyceride fraction of *Linum mucronatum* oil is composed of triglycerides that contain a ricinoleyl moiety. The remaining two glycerol positions are filled by various combinations of the fatty acids present in the oil. Since the major fatty acid components are ricinoleic (15%), oleic (24%), and linoleic (48%), the most probable combinations for the monohydroxy fraction are:

ricinoleic, linoleic, linoleic
ricinoleic, linoleic, oleic
ricinoleic, oleic, oleic

The tetraacyl triglycerides were prepared by reacting this fraction with palmitoyl chloride to form estolides with the ricinoyl acyl group of the monohydroxy triglycerides. Therefore, the predominant components are:

estolide, linoleic, linoleic
estolide, linoleic, oleic
estolide, oleic, oleic

These are the three largest peaks on the chromatogram in Figure 1b. Since shorter chain and less saturated compounds elute earlier on reverse phase HPLC, the first peak in Figure 1b is composed of an estolide, an 18 carbon diene (18:2) and an 18 carbon triene (18:3). It is a small peak, since linolenic acid comprises only 2.0% of the total fatty acid content of the oil.

Peak 2 represents the tetraacyl triglyceride composed of an estolide and two linoleic acids. The predominant tetraacyl triglyceride in peak 2 contains 70 carbons and 5 double bonds, 34

carbons with 1 double bond from the estolide and 18 carbons with 2 double bonds from each of the linoleic acids. Other tetraacyl triglycerides with different component acids that also total 70 carbons with 5 double bonds would elute with these tetraacyl triglycerides. Similarly, tetraacyl triglycerides of 68 carbons and 4 double bonds would elute under this peak. The effects of chain length and degree of unsaturation on HPLC retention time have been discussed elsewhere in detail (6). Components in Peak 3 total 70 carbons with four double bonds and 68 carbons with three double bonds. Similarly, Peak 4 is due to 70 carbons with three double bonds and 68 carbons with two double bonds.

A double bond does not have exactly the same effect on HPLC retention volume as a shortening of chain length by two carbons. Consequently, some broadening of the peaks exists due to slight differences in retention times of the components. The HPLC column, however, is not efficient enough to resolve these components. Overall, the tetraacyl triglycerides are retained longer on the column than the triglycerides in Figure 1a.

Castor oil, composed primarily of di- and trihydroxy triglycerides, served as the foundation for building the penta- and hexaacyl triglycerides (Figures 1c and 1d). The hexaacyl triglyceride is composed solely of glycerides containing three ricinoleic-palmitic estolides and, consequently, appears as a single peak on the chromatogram.

The chromatograms in Figure 1 demonstrate that separation is obtainable between the hydroxy triglyceride, normal triglyceride, tetraacyl triglyceride, pentaacyl triglyceride, and hexaacyl triglyceride classes, with some separation also occurring within each class. Glycerides containing more acyl groups are retained longer than those with fewer acyl groups.

Estolides of Lesqueroyl Triglycerides

A second set of multiacyl triglycerides was prepared by reacting hydroxy triglycerides containing lesquerolic acid with erucoyl chloride. These multiacyl triglyceride standards are analogous to the naturally occurring multiacyl triglycerides in *Heliophila amplexicaulis* oil (8) and were made to determine the effects of increased chain length on HPLC retention of multiacyl triglycerides.

Lesquerella globosa oil contains the following fatty acids: 2% 16:0, 0.7% 16:1, 0.6% 18:0, 10% 18:1, 9% 18:2, 4% 18:3, 7% ricinoleic, and 66% lesquerolic (9). The mono-, di-, and trihydroxy triglyceride fractions of *Lesquerella globosa* oil were isolated by HPLC and analyzed by GC. All of the ricinoleic acid was found in the trihydroxy triglyceride fraction. Consequently, the estolides made from the mono- and dihydroxy triglycerides must be solely lesquerolic-erucic estolides. The estolides made from the trihydroxy triglycerides are a mixture of ricinoleic-erucic and lesquerolic-erucic linkages. These longer chain multiacyl triglycerides derived from *Lesquerella* were found to separate in a manner similar to the multiacyl triglycerides derived from *Linum* and *Ricinus*. However, each class was retained proportionately longer on the reverse phase HPLC column due to the longer chain lengths.

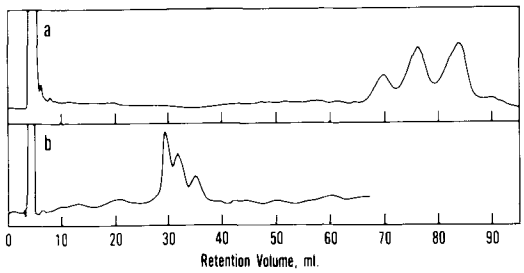

FIG. 2. Pentaacyl triglyceride separation. (a) Reverse-phase HPLC on a triglyceride column (Waters Assoc.) eluted with 75% acetonitrile/tetrahydrofuran at 2 ml/min. (b) Normal phase HPLC on μ-Porasil eluted with isooctane/ether/acetic acid (98:2:1) at 1 ml/min.

Separations between and within classes on the Waters triglyceride column were similar to those obtained on the μ-Bondapak C_{18} column. On the μ-Porasil column, separations between multiglyceride classes were again similar, with the compounds having larger numbers of acyl groups retained longer. Within each class, however, separations on the μ-Porasil column were the reverse of those found on the μ-Bondapak C_{18} and Waters triglycerides columns. When components containing the same number of acyl groups were analyzed, those with longer chain lengths and less unsaturation were eluted earlier.

Figure 2 shows the chromatograms of the pentaglycerides prepared from *L.globosa* oil on the triglyceride column (Figure 2a) and on the μ-Porasil column (Fig. 2b). The component eluting first on the triglyceride column (Fig. 2a) is the component eluting last on the μ-Porasil column (Fig. 2b). This effect of chain length and degree of unsaturation in conventional phase HPLC of triglycerides has been examined in detail and will be discussed in a separate paper.

Table I gives retention volume ranges for the synthetic estolides on the three columns.

TABLE I

Retention Volume (ml)

Multiglycerides	Column		
	μ-Bondapak C-18[a]	Waters triglyceride[b]	μ-Porasil[c]
70 Carbons 4 Acyl groups	28-40	29-38	18-25
96 Carbons 5 Acyl groups	43-65	44-53	26.35
112 Carbons 6 Acyl groups	80-85	60-70	37-49
82 Carbons 4 Acyl groups	43-73	34-52	16-22
104 Carbons 5 Acyl groups	95-150	65-90	27-37
126 Carbons 6 Acyl groups	150-180	132-154	34-45

[a]Acetone/acetonitrile (3:1).
[b]Acetonitrile/tetrahydrofuran (3:1).
[c]Isooctane/ether/acetic acid (98:2:1).

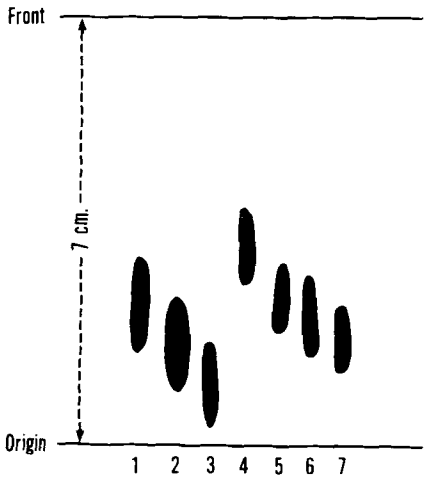

FIG. 3. Triple development TLC of estolide triglycerides on silica gel with benzene. 1-3 Triglycerides containing one, two, and three ricinoleic-palmitic estolides, respectively. 4 Soybean oil. 5-7 Triglycerides containing one, two and three lesquerolic-erucic estolides, respectively.

TLC migration characteristics of the synthetic multiacyl triglycerides were examined on silica gel by multidevelopment with benzene (Figure 3). Examination of the multiacyl triglycerides derived from *Linum* and castor oils and containing only ricinoleic-palmitic estolides shows that the tetraacyl triglyceride migrated furthest, followed by the pentaacyl triglycerides and then the hexaacyl triglycerides. Multiacyl triglycerides containing lesquerolie-erucic estolides migrated slightly further than the corresponding ones with ricinoleic-palmitic estolides. As expected, migration on the silica TLC plate is similar to the elution from the μ-Porasil HPLC column. An increased number of acyl groups retards migration, whereas increasing the chain length enhances migration. Therefore, conventional phase chromatograhy by HPLC or by TLC is not a reliable indicator of the degree of acylation in estolides unless all of the component acid chain lengths are similar.

CONCLUSION

In recent years, an increasing number of oils containing estolides have been found, making reliable analytical methods necessary for their detection and quantitation. The HPLC techniques described here provide the means for establishing the presence of simple estolides in oils. HPLC on a micro-preparative scale can yield enough purified estolide triglyceride for conventional analytical methods such as hydrolysis followed by GLC and TLC. In this way, conclusive identification of the structures of estolides can be obtained. HPLC techniques still need to be developed for more complex estolides containing free hydroxyl functions such as those reported in *Cardamine* (10), *Chamaepeuce* (2), and *Lesquerella auriculata* (1).

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Phospholipid Exchange between Subcellular Organelles of Rabbit Lung

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ABSTRACT

A soluble protein fraction (PLEP) prepared from rabbit lung can catalyze the exchange of phospholipids between subcellular organelles of the lung and between these subcellular organelles and synthetic liposomes. Phospholipid exchange between microsomes and synthetic liposomes and between mitochondria and synthetic liposomes was stimulated 8-fold and 2.5-fold, respectively, in the presence of the protein fraction. Lung exchange protein could also catalyze phospholipid exchange between subcellular organelles of the liver and synthetic liposomes. Phospholipid transfer between microsomes and lamellar bodies of the lung was stimulated 2-fold by the exchange protein. Both radiolabeled phosphatidylcholine (PC) and phosphatidylinositol (PI) were transferred from ^{32}P-labeled microsomes to lamellar bodies, but the exchange protein exhibited no transfer activity for phosphatidylglycerol (PG) and that for phosphatidylethanolamine (PE) was insignificant compared to the transfer activity for phosphatidylcholine and phosphatidylinositol. While the physiological role of the phospholipid exchange proteins in the lung is unknown, it is possible that they participate in the distribution of the newly synthesized phospholipids from the site of synthesis to lamellar bodies and other membrane compartments of cells.

INTRODUCTION

Considerable attention has been focused on phospholipid metabolism in the lung, especially since the phospholipids of pulmonary surfactant play a vital role in maintaining the physiological and structural integrity of the terminal airways and alveoli of the lungs (1-3). A good characterization of the various phospholipid classes that are distributed among lung subcellular organelles and pulmonary surfactant has been achieved (4-7); however, despite intensive investigation (for a recent review, see ref. 3), the current understanding of pulmonary phospholipid biosynthesis remains incomplete. Much less attention has been directed towards phospholipid transport mechanisms which may be involved in intracellular transport of newly synthesized phospholipids and subsequent incorporation into subcellular organelles and membranes.

Proteins which can exchange or transfer phospholipids between subcellular organelles and between synthetic membranes and subcellular organelles have been demonstrated in nonpulmonary tissues (8-11). Several of these proteins exhibit a marked preference for the exchange of a single class of phospholipid (9-11). Intracellularly, these proteins may transport phospholipids from the site of biosynthesis in the endoplasmic reticulum to subcellular organelles unable to synthesize phospholipids.

We have previously reported the presence of such phospholipid transfer proteins in the lung (12,13), and our observations have been confirmed and extended recently by two other laboratories (14,15). In this report some of the properties of a phospholipid exchange protein preparation obtained from the rabbit lung are characterized.

MATERIALS AND METHODS

Preparation of Lung Exchange Protein Fraction

New Zealand rabbits (2 kgm) were sacrificed by injection of pentobarbital (150 mg) in the marginal ear vein. Lungs were removed intact and subjected to pulmonary lavage with 3 successive washes of 0.9% (w/v) saline, 20 C. Lungs freed of extraneous tissue were minced with an Arbor Tissue Press (Model 142, Harvard Apparatus Co., Dover, MA), and a 15% homogenate (w/v) was prepared in 0.25 M sucrose, 1 mM EDTA, pH 7.6 according to the method of Hook et al. (16). All subsequent steps were conducted at 0-4 C. The procedures followed for preparing the pH 5.1 supernatant exchange protein (PLEP) were essentially according to the method of Wirtz and Zilversmit (8). Both male and female rabbits were used, but for any particular preparation of PLEP or labeled subcellular organelles, pooled tissues were from rabbits of the same sex.

Cell debris was removed by centrifugation at 600 x g, 15 min, and the supernatant was centrifuged at 10,000 x g, 15 min (Sorvall, SS-34 anglehead rotor). The 140,000 x g postmicrosomal supernatant (Beckman L3-50 centrifuge, anglehead 60 Ti rotor) was adjusted

to pH 5.1 with 0.5N HCl and the precipitate allowed to form for 1 hr. The precipitate was removed by sedimentation and the clear supernatant brought to pH 7.4 by dialysis in SET buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) at a ratio of 1:20. The PLEP fraction was stored at -20 C, and it retained exchange activity for up to 3 months.

Preparation of Liver Exchange Protein Fraction

Rabbit liver PLEP was prepared from 15% liver homogenates in the same manner as above. Because of their size, rabbit livers were minced in a meat grinder prior to homogenization.

Preparation of ^{14}C-Choline-Phosphatidylcholine-Labeled Subcellular Fractions

A saline solution of ^{14}C-methyl-choline chloride (^{14}C-choline) 40-50 MC/mM (New England Nuclear Boston, MA) was administered I.V. at 250 μc per animal. One or two hours after isotope incorporation, the animals were sacrificed as above, and 15% homogenates of lung or liver tissue in 0.25 M sucrose and 1 mM EDTA, pH 7.6, were prepared as described above. Supernatants obtained after the removal of cell debris at 600 x g, 15 min, were further sedimented at 10,000 x g, 15 min. The mitochondrial pellet was washed several times in SET buffer and a final suspension of mitochondria in SET was stored at -20 C. The 10,000 x g supernatant was centrifuged at 140,000 x g, 60 min, and the microsomal pellet was washed free of loose debris and resuspended via homogenization in SET and stored at -20 C. Mitochondrial and microsomal fractions from unlabeled animals were isolated in a similar manner from lung and liver tissue, during the preparation of PLEP.

Preparation of ^{32}P-Labeled Phospholipids in Lung Subcellular Organelles

Three to five millicuries of ^{32}P-orthophosphate (New England Nuclear, carrier-free in H_2O) in 0.5 ml saline were administered I.V. to each rabbit. Food was withheld and incorporation of isotope was permitted for 14-16 hr. The ^{32}P-labeled lung and liver subcellular organelles were prepared as previously described.

Preparation of Liposomes

Liposomes were prepared by a modification of the method described by Johnson and Zilversmit (17). Egg phosphatidylcholine (Grand Island Biological Co., Grand Island, NY) with 10% butylated-hydroxytoluene was suspended in SET and the vesicles allowed to swell overnight under N_2. The suspension was enclosed in an ice bath and given 12-15 30-second bursts of sonication at 80 watts with a Branson microprobe sonicator. The appearance of the final preparation was as a clear opalescent solution. The liposomes were stored under N_2 and used within 4-5 hr of sonication.

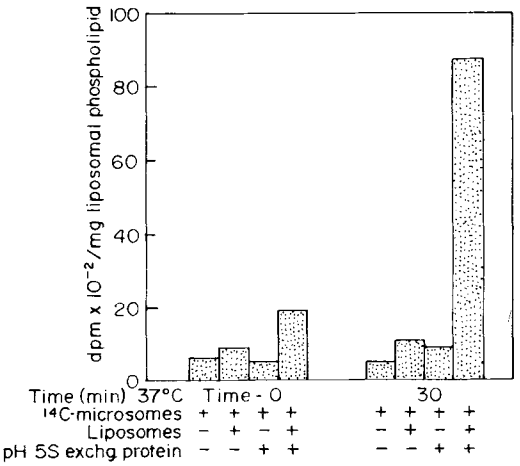

FIG. 1. Stimulation of phosphatidylcholine transfer by rabbit lung phospholipid exchange protein. Transfer of ^{14}C-phosphatidylcholine was from lung microsomes to liposomes comprised of egg yolk phosphatidylcholine and 10% BHT. The complete incubation mixture consisted of 1.43 mg microsomal protein (0.94 μmoles phospholipid); 6.1 mg PLEP; and 1.0 mg liposomal phosphatidylcholine (1.27 μmoles phospholipid) in a total volume of 2.5 ml of SET buffer (0.25 M sucrose, 0.001 M EDTA, 0.01 M Tris-HCl, pH 7.4). Incubation was at 37 C for 30 min. Phospholipid exchange activity at 30 min was 1.02 nmoles phospholipid phosphorus (P)/min/mg PLEP protein.

Assay for the Phospholipid Exchange Reaction

The transfer of radiolabeled phospholipid from either microsomes or mitochondria to synthetic liposomes was employed as a model system to measure the phospholipid exchange activity of the PLEP preparations. All reaction components were added to the incubation mixture as SET buffer suspensions. Frozen microsomal or mitochondrial fractions were washed and suspended in appropriate volumes of SET by gentle homogenization prior to their addition to the incubation mixture. The final volume of the incubation suspension varied from 2.5 to 5.0 ml. Incubations were performed in a shaking water bath and conducted for intervals up to 40 min at 37 C. The exchange reaction was terminated by immediate immersion of the incubation vials in an ice bath. Radiolabeled mitochondria were separated from liposomes by sedimentation at 10,000 x g, 5 min (Sorvall rotors, SS-34 or SS-24). Liposomes remained suspended in the

supernatant fraction. Radiolabeled microsomes were selectively separated from liposomes by acid precipitation at pH 5.1 according to the method described by Kamp et al. (9).

Assay for Exchange Activity

The amount of phospholipid exchange was measured by recovery of phospholipid from the acceptor organelle by (a) direct solvent extraction or by (b) acid precipitation.

Solvent extraction. The phospholipid in liposomes, lamellar bodies and donor organelle fractions was extracted quantitatively by the method of Folch et al. (18). Phospholipid phosphorous determination was performed by the method of Bartlett (19). Appropriate aliquots of the recovered phospholipid extracts were assayed for radioactivity.

Acid precipitation. The phospholipid in liposomes or labeled donor organelles was precipitated with 0.5 N perchloric acid (PCA). The presence of the exchange protein fraction in the liposomal supernatant facilitated the precipitation and recovery of the liposomal phospholipid. The contribution of any radiolabeled phospholipid that was associated with the exchange protein fraction was small (Fig. 1). The acid precipitates were recovered by sedimentation and the pellets which contained phospholipid were washed three times with 0.5 N PCA. Then the pellets were dispersed in scintillation fluid for radioisotope analysis. Ninety to ninety-five per cent of the liposomal phospholipid could be recovered by this method. The recovery of radiolabeled liposomal phospholipid by acid precipitation was in good agreement with the phospholipid recovery obtained by solvent extraction.

Phospholipid Exchange between Lung Subcellular Organelles and Lamellar Bodies

Lamellar bodies were isolated from the lungs of rabbits using the method of Spalding, Gilmore and Hook (unpublished procedure). A 20% homogenate (w/v) in 0.25 M sucrose was prepared from the lungs of 3-5 rabbits. All procedures were performed at 0-4 C. Cell debris was removed by centrifugation at 1100 x g, 10 min (Sorvall, SS-34 rotor). The supernatant from a subsequent centrifugation at 4300 x g, 10 min was centrifuged at 22,000 x g, 20 min. The sediment was suspended in 0.25 M sucrose and centrifuged again at 22,000 x g, 20 min. The sediment was resuspended in 0.25 M sucrose and layered on 30 ml of 0.60 M sucrose. The discontinuous gradient was centrifuged at 82,500 x g, 60 min (Beckman, L3-50 ultracentrifuge, SW 27 rotor). The lamellar bodies which collected at the 0.25 M – 0.60 M sucrose interphase were recovered, pooled and the suspension diluted with distilled water to a final sucrose concentration of 0.25 M. The lamellar bodies were sedimented at 176,000 x g, 30 min (Beckman, rotor 60 Ti). Then the lamellar bodies were suspended in 0.25 M sucrose, layered over 0.60 M sucrose and again processed as described above. The purified lamellar bodies were finally suspended in SET buffer. The purity of the lamellar bodies was determined using several criteria including: phospholipid/protein ratio (8.5:1); succinate cytochrome *c* reductase as an indicator of mitochondrial contamination (undetectable); NADPH-cytochrome *c* reductase as an indicator of microsomal contamination (less than 2% phospholipid due to endoplasmic reticulum); and by electron microscopy (the lamellar bodies retained excellent integrity with perilamellar membranes and internal content generally undisturbed). These data will be published elsewhere. ^{32}P-labeled mitochondria and microsomes suspended in 0.25 M sucrose were layered over 0.60M sucrose and centrifuged at 82,500 x g, 60 min in the same way as the lamellar bodies. Both microsomes and mitochondria were collected as pellets in the bottom of the tube. Contaminating lamellar bodies were collected at the interface and discarded. After the phospholipid transfer reaction was terminated, the lamellar bodies were isolated from the other incubation components by layering the incubation mixture over 0.60 M sucrose and centrifuging in the manner described above. Lamellar body phospholipids were recovered by solvent extraction.

Analysis of Phospholipid Extracts by Thin Layer Chromatography (TLC)

Phospholipid extracts of donor organelles, liposomes, and lamellar bodies were resolved in a two-dimensional solvent system (20) on Silica Gel H, magnesium acetate-impregnated TLC plates (Analtech Newark, DE). Phospholipids were located by iodine vapor and identified by comparison with appropriate standards. Phosphorous analysis (19) was performed in the same tubes in which acid hydrolysis of phospholipid phosphate from recovered gel spots had occurred. Appropriate areas of blank gel served as controls. Four replicate plates were run for each extract sample, and replicate pairs of gel spots were analyzed for either lipid-phosphorous or radioactivity. Recovered gels were added directly to 15 ml of scintillation fluid.

Other Methods

Protein analysis was performed by the

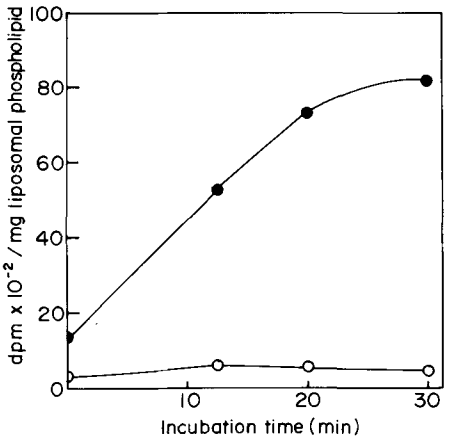

FIG. 2. Transfer of phosphatidylcholine from rabbit lung microsomes to liposomes as a function of incubation time. The incubation system was the same as that described for Fig. 1. Incubations were in the presence ●——● or absence ○——○ of rabbit lung PLEP. Phospholipid exchange activity at 20 min was 1.33 nmoles phospholipid P/min/mg PLEP protein.

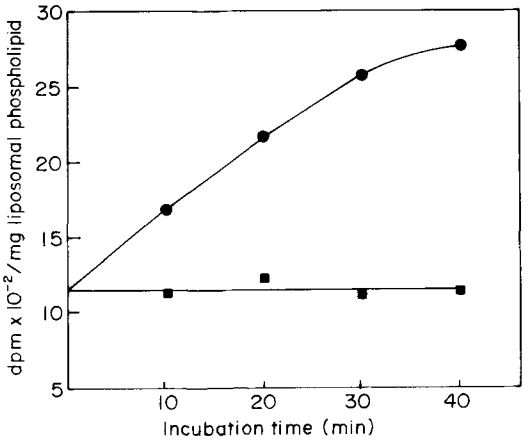

FIG. 3. Transfer of phosphatidylcholine from rabbit lung mitochondria to liposomes as a function of incubation time. Transfer of ^{14}C-phosphatidylcholine was from lung mitochondria labeled with ^{14}C-choline-phosphatidylcholine to liposomes comprised of egg yolk phosphatidylcholine and 10% BHT. The complete incubation mixture ● consisted of 0.9 mg mitochondrial protein, 1.25 mg of liposomal phosphatidylcholine (1.27 μmoles phospholipid) with 10% BHT, and 3.6 mg lung PLEP in a total volume of 3.0 ml SET buffer. Liposomes were omitted in control incubations ■. Incubation was at 37 C.

method of Lowry et al. (21). Samples for radioisotope analysis were combined with 1 ml of water and 15 ml of either Aquasol or Biofluor (New England Nuclear). Radioisotope analysis was performed in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3380-544. ^{14}-C-radioisotope activity is expressed as dpm; ^{32}-P-radioactivity is expressed as cpm.

RESULTS

Transfer of Labeled Phosphatidylcholine between Lung Subcellular Organelles and Synthetic Liposomes

Rabbit lung PLEP readily stimulates the transfer of ^{14}C-choline-labeled phospholipid from lung microsomes to synthetic liposomes (Fig. 1). After 30 min, there was an 8-fold increase in phosphatidylcholine (PC) transfer compared to the control without PLEP. The transfer of labeled PC to liposomes represented 16% of the total acid-insoluble PC present in the labeled microsomes. The addition of either liposomes or PLEP to microsomes in the incubation mixture did not significantly increase the amount of acid-insoluble radioactivity contributed by the donor microsomal fraction alone (Fig. 1). The background exchange activity in control incubations was low and was probably due to the release of acid-insoluble phospholipid from the labeled microsomes, the binding of small microsomal vesicles to liposomes, or exchange of labeled PC with the phospholipid component of PLEP.

The transfer of labeled PC from microsomes to liposomes was linear with incubation time up to 20 min (Fig. 2). After this period, the rate of exchange of PC between donor and acceptor organelles began to fall. When ^{14}C-choline was employed as the labeled precursor for in vivo phospholipid synthesis, 85-90% of the acid-insoluble radioactivity in microsomes, mitochondria, or synthetic liposomes could be recovered by solvent extraction. Chromatographic analysis of the phospholipid extracts by TLC revealed that all of the radioactivity was associated with phosphatidylcholine.

Lung mitochondria labeled with ^{14}C-choline also exchanged PC with liposomes in the presence of lung PLEP. Transfer of PC to liposomes was linear with time up to 30 min (Fig. 3). The net transfer of labeled PC from mitochondria to liposomes after 40 min incubation time represented 15% of the total acid-insoluble labeled phospholipid present in the mitochondrial fraction. Incubation mixtures in which liposomes were omitted several as controls. Background activity was low and remained constant throughout the incubation period. The background activity probably represented the exchange of labeled PC between mitochondria and the very small amount of phospholipid that is associated with lung PLEP. This exchange apparently reached an

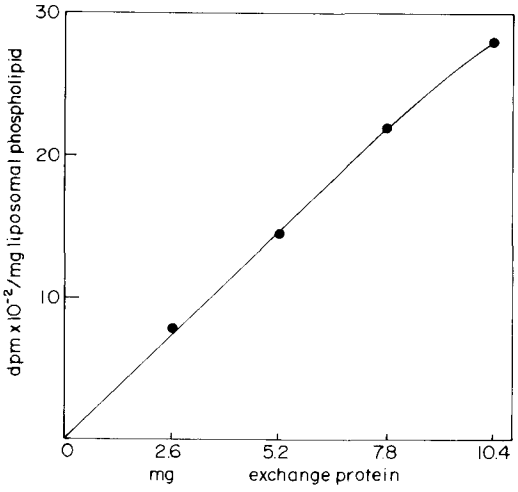

FIG. 4. The transfer of phosphatidylcholine from liver mitochondria to liposomes as a function of lung phospholipid exchange protein concentration. The incubation mixture contained ^{14}C-phosphatidylcholine-labeled rabbit liver mitochondria (1.64 mg protein), liposomes (1.6 μmoles egg phosphatidylcholine with 10% BHT), and rabbit lung PLEP at the concentrations indicated, in 3.0 ml of SET buffer. Incubation time was 20 min at 37 C.

equilibrium rapidly and remained constant throughout the incubation interval.

The transfer of labeled PC from donor organelles to liposomes was proportional to the amount of lung PLEP present in the incubation mixture (Fig. 4). The concentration of PLEP was the rate-limiting component in the exchange reaction, since increasing the concentrations of either donor or acceptor organelles did not result in a proportional increase in the rate at which labeled PC was transferred (results not shown). Also, the phospholipid exchange activities of different preparations of rabbit lung PLEP proved to be markedly similar when their activity was compared on the basis of mg protein/ml PLEP preparation.

Both rabbit lung and liver PLEP preparations exhibited similar activities when ^{14}C-choline-labeled mitochondria from either lung or liver were employed as the donor organelles (Table I). However, when the donor organelles were either ^{14}C-choline-labeled lung or liver microsomes, the phospholipid transfer activity of lung PLEP was greater than that of the liver preparation. When lung microsomes were the donor of labeled PC, the lung PLEP had twice the activity of the liver PLEP. Surprisingly, the same lung preparation was six times more active, 99 vs. 16%, than the liver PLEP when liver microsomes were the donor of phospholipid (Table I).

Exchange of Phospholipid between Lung Subcellular Organelles and Isolated Lamellar Bodies

There is good evidence that the lamellar bodies of pulmonary type II cells are the storage sites for pulmonary surfactant (2,22-24). It was of interest to determine whether or not lung PLEP could participate in the transfer of phospholipid from lung subcellular organelles to lamellar bodies from rabbit lung; therefore, lamellar bodies were compared with synthetic liposomes as acceptor organelles in the phospholipid exchange reaction. Lung microsomal and mitochondrial fractions containing phospholipids labeled with $^{32}PO_4$ were employed as the donor organelles.

The phospholipid extracts from lamellar bodies and liposomes were resolved by TLC and the distribution of ^{32}P in the different phospholipid fractions was compared. Similar phospholipid analyses were performed on the extracts of lung microsomes and mitochondria. In these experiments, the specific activities of both the total phospholipids and the PC fraction of the donor organelles were approximately the same (results not shown). The specific activities of the ^{32}P-labeled phospholipids transferred to the lamellar bodies were also similar (Table II), which indicates there was no difference in the ability of microsomes and mitochondria to serve as donor organelles. The stimulation of ^{32}P-labeled phospholipid transfer to lamellar bodies by lung PLEP was 2- and 3-fold, respectively, when lung microsomes and mitochondria were the donor organelles; and when the microsomal fraction was the donor of labeled phospholipid, lamellar bodies proved to be nearly as efficient phospholipid acceptors as liposomes (Table II).

Phosphatidylcholine was the major phospholipid recovered from acceptor organelles after their isolation from both the control incubations and from those containing lung PLEP. The relative increase in specific activities of PC due to the stimulation of PC transfer by PLEP was in agreement with the increases observed in the specific activities of the total phospholipid extracted from the donor organelles (Table II).

Lamellar bodies contain small amounts of phosphatidylinositol (PI) and phosphatidylethanolamine (PE) (6,7,24) while in both lung microsomes and mitochondria, less than 10% and between 20-25% of the total phospholipids are comprised of PI and PE respectively, (4,6,7). The TLC analysis of lamellar body extracts from control incubations revealed only faint spots, < 0.1 μg P, corresponding to PI and PE, and the level of radioactivity recovered in these spots was negligible. In phos-

TABLE I

Comparison of Rabbit Lung and Liver PLEP Activities: Transfer of Phospholipid Between Lung and Liver Subcellular Organelles and Synthetic Liposomes[a]

	Lung[b]			Liver[c]	
	Liposomes	DPM		Liposomes	DPM
^{14}C-Microsomes	Net DPM exchgd/mg PLEP protein	Liver/lung x 100	^{14}C-Microsomes	Net DPM exchgd/mg PLEP protein	Liver/lung x 100
Lung PLEP	265	---	Lung PLEP	167	---
Liver PLEP	124	47%	Liver PLEP	26	16%
^{14}C-Mitochondria			^{14}C-Mitochondria		
Lung PLEP	126	---	Lung PLEP	112	---
Liver PLEP	115	91%	Liver PLEP	111	99%

[a]Other incubation components added where indicated were: 1 mg liposomal phosphatidylcholine-10% BHT; rabbit lung PLEP (5.0 mg protein); rabbit liver PLEP (6.0 mg protein). All components were added in a final volume of 3.0 ml SET buffer. Incubation was for 20 min, 37 C. Liposomes were omitted in controls.

[b]Donor organelles were rabbit lung ^{14}C-methyl-choline labeled microsomes (1.6 mg protein) and mitochondria (0.7 mg protein).

[c]Donor organelles were rabbit liver ^{14}C-methyl-choline labeled microsomes (3.3 mg protein) and mitochondria (2.4 mg protein).

TABLE II

Stimulation of Phospholipid Exchange Between Lung Subcellular Organelles and Lamellar Bodies and Liposomes

^{32}P-Microsomes[a]	CPM/μG P	% Control	CPM/μG P PC	% Control
	Lamellar bodies[b]			
Control	45	---	38	---
Lung PLEP	102	226	80	210
	Liposomes[b]			
Control	28	---	12	---
Lung PLEP	131	467	86	716
^{32}P-Mitochondria[a]	CPM/μG P	% Control	CPM/μG P PC	% Control
	Lamellar bodies[b]			
Control	38	---	37	---
Lung PLEP	112	295	86	232
	Liposomes[b]			
Control	137	---	78	---
Lung PLEP	213	155	139	178

[a]Donor organelles labeled with ^{32}P-orthophosphate were either rabbit lung microsomes (39 μg phospholipid phosphorous [P]) or rabbit lung mitochondria (55 μg phospholipid P).

[b]Acceptor organelles were rabbit lung lamellar bodies (48 μg phospholipid P) or synthetic liposomes (140 μg phospholipid P) comprised of egg phosphatidylcholine with 10% BHT. Rabbit lung PLEP (15.5 mg protein) was omitted from control incubations. Incubations were for 30 min, at 37 C, in a final volume of 6.0 ml SET buffer. Radioactivity is expressed as cpm/μg of phospholipid phosphorous [P].

pholipid extracts of lamellar bodies from complete incubations however, PI was readily visible and the level of radioactivity recoverd in PI represented 20% of the total PI radioactivity present in the donor microsomes and mitochondria (Table III). In contrast, while more total radioactivity was recovered in the PC of lamellar bodies, the amount recovered represented only 4.6 to 6.6% of the total PC radioactivity present in the donor organelles. There was no corresponding increase in the radioactivity associated with PE. If the high level of radioactivity associated with the PI from lamellar bodies was due to contamination by the donor organelles, then a corresponding increase in the activity associated with PE

TABLE III

Transfer of Phospholipids from ^{32}P-Labeled Lung Subcellular Organelles to Lamellar Bodies by Lung PLEP[a]

	Lamellar Bodies[b]		
	Percent		
	PC	PI	PE
^{32}P-Microsomes[c]	4.6	20.5	1.1
^{32}P-Mitochondria[c]	6.6	19.5	0.6

[a]Incubation conditions were the same as those described in Table II.

[b]Results are expressed as percent cpm transferred from donor organelles to lamellar bodies. The data have been corrected for control values.

[c]Donor organelles: The respective activities (cpm) of the microsomal phospholipids were: PC, 20,340; PI, 2610; PE, 3760. For mitochondrial phospholipids, they were: PC, 21,810; PI, 2990; PE, 9780.

would also have been observed; but this was not the case. In other experiments, where synthetic liposomes comprised of egg phosphatidylcholine were the acceptor organelles, lung PLEP was also able to facilitate the net transfer of PI from microsomes to liposomes. These data indicate that the net transfer of PI is dependent on the properties of the lung PLEP rather than on those of the acceptor organelle.

DISCUSSION

In this study we have shown that a protein fraction (PLEP) from rabbit lung can enhance the exchange of phospholipids between microsomes, mitochondria, lamellar bodies, and synthetic liposomes. In addition, we have made a direct comparison between the phospholipid exchange activities of lung PLEP and a similarly prepared rabbit liver fraction.

While this work on the rabbit PLEP system was in progress, two reports appeared which described phospholipid exchange proteins prepared from the lungs of rats, mice (14), and sheep (15). Some direct comparisons can be made, and our results indicate that the rabbit PLEP system is similar to those present in these other species: for instance, the phospholipid exchange proteins obtained from sheep (15) and rabbit lungs can catalyze the exchange of phospholipids between microsomes and synthetic liposomes; also, both the rat (14) and rabbit lung PLEP can stimulate phospholipid exchange between microsomes and lamellar bodies.

The properties of the lung phospholipid exchange protein preparations described in this and other studies (12-14) appear basically similar to those obtained from other organs that readily exchange phosphatidylcholine and other phospholipids between mitochondria, microsomes, and synthetic liposomes. These include the liver (8,9,25), heart (10), and brain (11,26). However, a number of minor differences exists which indicate that the systems are not identical in all organs. While rabbit lung microsomal and mitochondrial fractions could be employed interchangeably with comparable rabbit liver subcellular fractions as donor organelles for the phospholipid exchange reaction, the pulmonary PLEP appeared more effective than the hepatic PLEP in exchanging phospholipids between microsomes and liposomes. Such differences were not apparent when mitochondria were the donor organelles (Table I).

As indicated above, reports have appeared from two other laboratories on the presence of phospholipid transfer proteins in the lung (14,15). Our data agree with these reports and confirm the transfer of phosphatidylcholine from microsomes to liposomes (15) and lamellar bodies (14). In addition, we would like to emphasize several of our new observations: 1) the transfer of phosphatidylinositol from donor organelles to lamellar bodies, 2) the absence of phosphatidylglycerol and phosphatidylethanolamine transfer to lamellar bodies, and 3) the transfer of phospholipids from mitochondria to lamellar bodies.

Phospholipid exchange proteins from nonpulmonary tissues are characterized by the variability of their specificity for the transfer of different phospholipid species. PLEP preparations from both beef (9,27) and rat liver (25) stimulate the transfer of phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) from microsomes and mitochondria to liposomes, with the relative transfer activities being in the order of PC > PI > PE. It is likely that these exchange protein fractions contain several different transfer proteins that are highly specific for a single phospholipid species; e.g., a purified protein derived from beef liver was found to stimulate exclusively the transfer of PC (9). Three distinct proteins were isolated from rat liver (25); one protein fraction was specific for PC exchange, while the other two proteins preferentially catalyzed the transfer of PI and to a lesser extent PC. Since the phospholipid exchange protein preparations from the lung probably exhibit the exchange of activities representative of the many different cell types in the lung, it is likely that a number of PLEP proteins with different exchange properties and phospholipid specificities are present. Indeed, while the partially purified preparations of PLEP from rat

and rabbit lung exchange PC and PI, the highly purified proteins from sheep lung (15) show a high degree of specificity for PC and very little ability to catalyze the exchange of PI and PE.

In the recent reports which have described the characteristics of rat lung (14) and sheep lung (15) phospholipid exchange proteins, the ability of these proteins to catalyze phosphatidylglycerol exchange was apparently not examined. In our studies we did not detect any transfer of PG from labeled lung subcellular organelles to lamellar bodies. This observation is especially noteworthy, since PG is the second most abundant component of these phospholipid storage organelles.

Although the presence of phospholipid exchange proteins has been demonstrated in the lungs of several species, their precise role within the cell is not clear. Our major concern with these exchange proteins has stemmed from an interest in the lamellar bodies of the pulmonary type II cell. These storage sites of surface-active phospholipids are the focus of intensive research acitivity at present, and many attempts have been made to explain their peculiar phospholipid composition, especially their very high content (50-60%) of dipalmitoylphosphatidylcholine (4,6,7). Several mechanisms have been proposed to account for their composition including (1) the ability to synthesize their own phospholipids, and (2) the ability to modify the structure of unsaturated forms of PC. At the present time, there is no unequivocal evidence to support either of these proposals. Isolated lamellar bodies appear to be deficient in the enzymes required for de novo phospholipid synthesis (4,28,29), and more recent evidence indicates that lamellar bodies also lack the enzymes necessary for the structual modification of unsaturated precursor forms of dipalmitoylphosphatidylcholine (29).

In the absence of de novo synthetic processes, the unique composition of lamellar bodies must be accounted for by another mechanism, perhaps one involving phospholipid transfer proteins. If the specificity for the transfer of a phospholipid subclass resides with a particular phospholipid transfer protein, then it is tempting to speculate that the transfer of a specific phospholipid species from the site of its synthesis or modification might be directed to the lamellar body. For example, phospholipids such as dipalmitoylphosphatidylcholine synthesized in the endoplasmic reticulum could be transferred to lamellar bodies by specific transfer proteins. These proteins having specific receptors on the lamellar bodies could account for and regulate the biosynthesis of the lamellar bodies.

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The Effect of Clofibrate on Heart and Plasma Lipids in Rats Fed a Diet Containing Rapeseed Oil

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ABSTRACT

The effect of clofibrate on heart and plasma lipids in rats fed a diet containing 30% of the calories as peanut oil (PO) or rapeseed oil (RSO) (42.7% erucic acid and 0.5% eicosenoic acid) was studied. A decrease of erucic acid content to one-third and concomitant increase in the content of 18:1, 16:1 and 16:0 fatty acids in plasma triacylglycerols were observed after administration of clofibrate to rats fed the RSO-diet. It is suggested that these changes reflect the increased capacity of the liver to chain-shorten very long chain length fatty acids. The extent of lipidosis in the heart of rats fed the RSO-diet was decreased by 50% by clofibrate. However, the concentration of erucic acid in heart triacylglycerols decreased much less (30%) than the concentration of all other fatty acids (50-65%). It is concluded that the clofibrate administration increased the oxidative capacity of the heart mitochondria and that the heart cell does not have an efficient system to handle very long chain length monounsaturated fatty acids as does the liver.

INTRODUCTION

The myocardial lipidosis produced in young rats by feeding diets containing high levels of rapeseed oil, rich in erucic acid (22:1ω9), has been documented by many laboratories (1-3). The accumulation of triacylglycerols containing erucic acid in the heart has been attributed to the very slow oxidation of this fatty acid and to its inhibitory effect on the oxidation of other dietary fatty acids (4,5). Erucic acid is also very slowly metabolized in liver cells isolated from rats fed usual laboratory diet (6-8).

We have recently reported that the administration of clofibrate to the rats resulted in a 3-5 fold increase in the oxidation rate of erucate in isolated hepatocytes which is probably the consequence of an increased capacity of a chain-shortening system (6,7). It was of interest to investigate whether an increased capacity of the liver to metabolize erucate influences the composition of the lipids exported by the liver to the blood in vivo and how changes in blood lipids effect the extent of lipidosis in the heart of rats given erucate in the diet. The studies on the effect of clofibrate on plasma and heart lipids of rats fed a diet containing rapeseed oil and peanut oil (control rats) were also undertaken to investigate a possible direct effect of clofibrate on the heart metabolism in vivo.

MATERIALS AND METHODS

Male Wistar rats weighing ca. 60 g were fed the usual laboratory diet with or without 0.3% (w/w) clofibrate for 8 days and subsequently the semisynthetic diet containing 30% of the calories as either peanut oil or rapeseed oil with or without 0.3% clofibrate for 3 days. The exact composition of the semisynthetic diet and fatty acid composition of the oils used are described elsewhere (9). Table I shows that the addition of clofibrate to the diet did not change either the food consumption or the weight gain of the rats. Only in the group of rats fed the peanut oil-containing diet a very small increase in the weight gain due to clofibrate was observed.

Before the experiment, the animals were fasted 7 hr (from 6 am til 1 pm). Blood was collected from lower aorta into a syringe wetted with 0.1 M EDTA pH 7.0. After spinning down the erythrocytes (5 min at 600 Hg), the plasma was extracted for total lipids according to Christie (10). The hearts were removed, weighed, minced, homogenized and extracted for total lipids (10).

Lipid extracts of heart and plasma were separated by thin layer chromatography on silica gel (Stahl H) using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as a solvent system. Fractions of free fatty acids and triacylglycerols were extracted from the gel with $CHCL_3$-CH_3OH (2:1, v/v) and phospholipids with $CHCL_3$-CH_3OH-acetic acid-H_2O (65:25:2:2, v/v/v/v). An internal standard (nonadecanoic acid) was added to lipid eluates which were subsequently transmethylated according to Metcalfe and Schmitz (11). The methyl esters were separated isothermically at 200 C in a Carl Erba gas chromatograph model Fractovap 2101 Ac equipped with a wall-coated open tubular glass column SE-30 (Chrompack, Middelburg, The Netherlands).

The determination of total fatty acids was

TABLE I

The Effect of Clofibrate on the Food Consumption and Weight Gain of Rats Fed the Laboratory Pelleted Chow for 8 Days and Subsequently High-Fat Diet for 3 Days*

Diet	Gram per day per one rat			
	- clofibrate		+ clofibrate	
	Food Consumption	Gain of body weight	Food consumption	Gain of body weight
Pelleted chow	15.1 ± 0.2 (10)	6.3 ± 0.2 (10)	15.1 ± 0.5[a] (10)	6.1 ± 0.2[a] (10)
Rapeseed oil diet	15.4 ± 0.8 (6)	5.8 ± 0.3 (6)	15.0 ± 0.9[a] (6)	6.4 ± 0.3[a] (6)
Peanut oil diet	15.9 ± 0.1 (4)	6.0 ± 0.1 (4)	15.0 ± 0.4[a] (4)	6.6 ± 0.2[b] (4)

*The results are expressed as mean ± S.E.M. Number of animals is given in brackets. Different from rats fed the clofibrate-free diet with: [a]$p > 0.05$, N.S., [b]$p < 0.05$.

carried out using the hydroxamate colorimetric reaction (12,13) after transmethylation of fatty acids in total lipid extracts of the heart. Phospholipids were determined according to Zilversmith (14) and protein by the method of Lowry et al. (15). Triacylglycerol glycerol was determined after the alkaline hydrolysis as described by Eggstein and Kuhlmann (16) after the phospholipids had been removed from lipid extracts. The activated mixture (12 hr at 110 C) of zeolite-$CuSO_4$-$Ca(OH)_2$-Fuller's earth (20:1:2:2, w/w/w/w) was used for adsorption of phospholipids.

The total free fatty acids in the plasma were determined using ^{57}Co as a tracer (17).

The enzymes were purchased from Sigma Chemical Co., St. Louis, MO. All the chemicals used were of analytical grade.

The Student's t-test was used for the statistical treatment of the results.

RESULTS

Heart Lipids

The fatty infiltration of the heart and skeletal muscles occurs after one day of the RSO feeding and becomes most severe after 3-6 days (1). In the present experiments, feeding period of 3 days was chosen to assure the maximal effect of the diet. Table II shows that the rapeseed oil-containing diet caused a 13-fold rise in triacylglycerols and a 3-fold rise in total fatty acids in the heart compared to the diet containing the same per cent of peanut oil. The concentration of heart phospholipids was not influenced by the diet. As the result, the ratio triacylglycerols/phospholipids increased from 0.15 in the PO-fed group to almost 2 in the RSO-fed group of rats which is in accordance with earlier observations (3). The increase in triacylglycerol fraction caused by the RSO-diet could almost completely account for the increase in the total fatty acids. The clofibrate administration reduced the accumulation of triacylglycerols in the heart of rats fed the RSO-diet to about half. The content of heart lipids in the group of rats fed the PO-diet was not significantly influenced by clofibrate.

Fatty Acids in Heart Triacylglycerols

The fatty acid analysis of the heart triacylglycerols (Table III) showed a big increase in all fatty acids in rats fed the RSO-containing diet and a high incorporation of erucic acid into this fraction (ca. 30% of the total). Surprisingly, the administration of clofibrate caused 50-65% decrease in the content of all fatty acids except for erucate which decreased significantly but only by a factor of 1.4. As a result, a relative content of erucate was, in fact, increased from 30 to 44%.

Clofibrate did not have any pronounced effect on the fatty acid content of the heart triacylglycerol fraction in rats fed the PO-containing diet. Only a small increase, due to clofibrate, was observed in 20:1 fatty acid in this group.

Fatty acid analysis of the phospholipid fraction revealed no changes due to clofibrate administration neither in heart of rats fed the RSO- or the PO-containing diet. The incorporation of erucate into phospholipids was only ca. 4% of total fatty acids when erucate was supplied in the diet (not shown). This indicates that, also in the heart, erucate is more easily incorporated into the neutral lipid fraction than into the phospholipids as it was reported for the liver (7,18).

Plasma Lipids

The concentration and composition of fatty acids in heart lipids are apparently reflections

TABLE II

The Effect of Clofibrate on the Heart Lipids of the Rats Fed Diets Containing Peanut Oil and Rapeseed oil*

Lipid fraction	Peanut oil	Peanut oil + clofibrate	Rapeseed oil	Rapeseed oil + clofibrate
Triacylglycerol glycerol	31.0 ± 0.8 (4)	28.9 ± 2.5[b] (4)	396.4 ± 34.2 (10)	191.3 ± 18.8[a] (10)
Total fatty acids	521.0 ± 19.4 (4)	536.5 ± 45.8[b] (4)	1560.0 ± 108.5 (10)	931.0 ± 67.0[a] (10)
Phospholipids	211.9 ± 15.7 (4)	192.8 ± 0.7[b] (4)	200.3 ± 7.2 (4)	188.3 ± 6.9[b] (4)

*All the determinations were made as described in Material and Methods. The results are presented in nmol x mg^{-1} protein, as mean ± S.E.M. The number of experimental animals is given in brackets. Different from the results obtained with rats fed clofibrate-free diet with: [a]$p < 0.001$, [b]$p < 0.05$, N.S.

TABLE III

The Effect of Clofibrate on the Content of Fatty Acids in the Heart Triacylglycerols of Rats Fed Diets Containing Peanut Oil and Rapeseed Oil*

Fatty acid[a]	Peanut oil (4)	Peanut oil + clofibrate (3)	Rapeseed oil (9)	Rapeseed oil + clofibrate (10)
14:1	1.8 ± 0.3	1.2 ± 0.1[e]	7.9 ± 0.5	2.9 ± 0.3[b]
16:0	21.5 ± 1.8	20.0 ± 1.7[e]	124.4 ± 5.7	48.3 ± 2.5[b]
16:1	2.6 ± 0.5	2.2 ± 0.1[e]	14.7 ± 1.1	6.3 ± 0.3[b]
18:0	6.7 ± 0.4	6.8 ± 0.2[e]	44.4 ± 2.8	19.6 ± 1.2[b]
18:1	33.7 ± 3.1	30.0 ± 3.2[e]	331.3 ± 9.1	123.5 ± 8.9[b]
18:2	20.1 ± 0.9	17.3 ± 1.4[e]	139.5 ± 17.8	46.2 ± 7.7[b]
20:0	1.3 ± 0.2	1.3 ± 0.1[e]	8.7 ± 0.7	4.4 ± 0.3[b]
20:1	1.4 ± 0.3	3.3 ± 0.5[d]	122.4 ± 7.2	49.0 ± 4.0[b]
20:4	1.7 ± 0.1	1.6 ± 0.1[e]	10.5 ± 2.0	5.1 ± 0.8[d]
22:1	0	0	349.4 ± 25.0	250.0 ± 19.5[c]
22:5	0.6 ± 0.1	0.8 ± 0.1[e]	13.2 ± 1.4	6.9 ± 0.3[b]
22:6	1.2 ± 0.4	2.1 ± 0.2[e]	22.8 ± 2.3	11.4 ± 0.9[b]

*The results are presented in nmol x mg $protein^{-1}$, as mean ± S.E.M. The data on triacylglycerol glycerol (nmol of glycerol x 3, Table I) were used to recalculate the data from weight % to nmol of fatty acids. Number of animals is given in brackets.

[a]Indicates number of carbon atoms/number of double bonds. Different from the results obtained with the rats fed clofibrate-free diet with: [b]$p < 0.001$, [c]$p < 0.01$, [d]$p < 0.05$, [e]$p > 0.05$, N.S.

of the oxidative capacity of heart mitochondria and the rate of uptake of fatty acids from the blood. Rat hearts can take up free fatty acids from the blood directly. Blood triacylglycerols can be taken up after previous hydrolysis by heart lipoprotein lipase. Jensen et al. (19) have reported that there is no change in lipase activity of rat heart between the groups fed the RSO- and the olive oil-containing diet for 3 days. This means that, under the conditions of the present experiments, the fatty acid uptake by the heart is probably dependent only upon the concentration of free fatty acids and triacylglycerols in the plasma.

Table IV shows that the concentration of triacylglycerols in the plasma was the same with the two diets. As expected (20), clofibrate decreased the concentration of plasma triacylglycerols in rats fed the PO-containing diet, but surprisingly no effect of clofibrate was observed in rats fed the RSO-containing diet. The concentration of free fatty acids in the plasma was not significantly changed by clofibrate administration (Table IV). The decrease in the heart lipidosis due to clofibrate in the RSO-fed group of rats is apparently not due to the effect of clofibrate on the total amounts of plasma lipids.

Fatty Acids in Plasma Triacylglycerol and Free Fatty Acid Fractions

Analysis of fatty acid composition of plasma triacylglycerols (Table V) shows a decrease in the content of very long chain length fatty acids (C_{20}-C_{22}) due to clofibrate treatment, which was compensated for mainly by the increase in the concentration of 18:1, 16:1 and 16:0 fatty acids. A decrease to one-third in the

TABLE IV

The Effect of Clofibrate on Plasma Lipids in Rats Fed Diets Containing Rapeseed Oil and Peanut Oil*

Lipid fraction	(mM)			
	Peanut oil	Peanut oil + clofibrate	Rapeseed oil	Rapeseed oil + clofibrate
Triacylglycerol glycerol	0.83 ± 0.09 (5)	0.46 ± 0.05[a] (5)	0.81 ± 0.10 (5)	0.93 ± 0.19[b] (5)
Free fatty acids	0.88 ± 0.05 (3)	0.90 ± 0.02[b] (3)	0.92 ± 0.04 (4)	0.80 ± 0.03[b] (3)

*The results are presented as mean ± S.E.M. The number of experimental animals is given in brackets. Different from the results obtained with rats fed clofibrate-free diet with:
[a]$p < 0.01$, [b]$p > 0.05$, N.S.

TABLE V

The Effect of Clofibrate on the Composition of Fatty Acids in Plasma Triacylglycerol and Free Fatty Acid Fractions in Rats Fed the Diet Containing Rapeseed Oil*

Fatty acid	Weight % of total fatty acids			
	Triacylglycerol		Free fatty acids	
	Rapeseed oil	Rapeseed oil + clofibrate	Rapeseed oil	Rapeseed oil + clofibrate
14:1	0.6 ± 0.1	0.5 ± 0.1[e]	1.1 ± 0.3	3.4 ± 0.4[b]
16:0	14.8 ± 0.9	23.6 ± 1.2[a]	21.8 ± 1.2	33.5 ± 1.1[a]
16:1	2.3 ± 0.3	3.5 ± 0.2[b]	3.4 ± 0.5	5.1 ± 0.4[d]
18:0	2.5 ± 0.3	3.0 ± 0.2[e]	8.4 ± 0.8	7.9 ± 0.4[e]
18:1	40.9 ± 1.5	50.9 ± 1.1[a]	25.2 ± 0.3	28.6 ± 1.2[d]
18:2	15.4 ± 1.1	9.7 ± 0.7[b]	11.1 ± 0.6	11.5 ± 0.4[e]
20:1	5.1 ± 0.4	1.7 ± 0.1[a]	5.6 ± 0.2	2.8 ± 0.2[a]
20:4	3.7 ± 0.8	1.7 ± 0.2[d]	1.9 ± 0.1	1.6 ± 0.1[d]
22:1	12.4 ± 0.6	4.3 ± 0.7[a]	21.0 ± 1.4	6.1 ± 0.9[a]
22:5	0.8 ± 0.1	0.3 ± 0.1[c]	0	0
22:6	1.6 ± 0.2	0.6 ± 0.1[c]	traces	traces

*The results are presented as mean ± S.E.M. from 5 animals in each group. Different from the results obtained with rats fed clofibrate-free diet with:
[a]$p < 0.001$, [b]$p < 0.005$, [c]$p < 0.01$, [d]$p < 0.05$, [e]$p > 0.05$, N.S.

erucate content was observed. Changes in the composition of the free fatty acid fraction of the plasma follow generally the same pattern as in triacylglycerol fraction.

DISCUSSION

The observed changes in the plasma fatty acids, caused by clofibrate administration to the rats fed the RSO-diet, seem to reflect the increased capacity of the liver to chain-shorten erucic acid and most probably also other very long chain length fatty acids. At low concentration of erucate in the medium, oleic and palmitoleic acids were the main products of chain-shortening of [14-^{14}C]erucate in isolated hepatocytes (6,7). The decrease in erucic acid and concomitant increase in oleic and palmitoleic acids content of the plasma triacylglycerols agree with what we could predict from experiments with isolated hepatocytes. Chain-shortened fatty acids formed extramitochondrially from erucate are probably competing for the mitochondrial β-oxidation with other endogenous fatty acids in the liver cell. The increase in palmitate concentration in plasma triacylglycerol and free fatty acid fractions, due to clofibrate administration, might be the result of "saving" of endogenous palmitate by the chain-shortened fatty acids derived from very long chain length fatty acids under the conditions of an increased capacity of the chain-shortening system in the liver. It is interesting that, in rats fed the RSO-diet, the fatty acid composition of plasma triacylglycerols (Table V) is almost identical with the composition of fatty acids in plasma very low density lipoproteins (VLDL) triacylglycerols as reported by Thomassen et al. (9). This means that the plasma triacylglycerols of rats fasted for 7 hr are mainly accounted for by VLDL-triacylglycerols. Further, the changes in the fatty acids content of plasma triacylglycerols caused by clofibrate, although much more pronounced,

correspond to the changes caused by prolonged feeding of rats (3 weeks) with the RSO-containing diet. It seems then that the continuous supply of erucate to the liver induces the same system (chain-shortening system?) in the liver as does clofibrate.

It seems peculiar that even though the supply of erucate in the plasma is significantly reduced, due to clofibrate administration, the relative content of erucate in heart triacylglycerols is increased. Since the total concentration of plasma lipids is not decreased by clofibrate in rats fed the RSO-diet, the decreased lipidosis in the heart must be due to the increase in the oxidative capacity of the heart. It has been recently found that clofibrate administration to the rats fed usual laboratory diet increases the oxidation rate of erucate ca. 2-fold in isolated perfused rat heart (J. Norseth and B.O. Christophersen, unpublished data).

However, in the in vivo situation, erucate reaches the heart in the blood stream together with the excess of shorter, readily oxidizable fatty acids, which apparently compete very efficiently for the oxidation with erucic acid. Vasdev and Kako (21) have reported that palmitate and erucate are taken up by the perfused heart at the same rate, but erucate is preferentially incorporated into heart lipids, especially when palmitate is added to the perfusion medium.

The heart has apparently the system to chain-shorten very long chain length monounsaturated fatty acids (22,23). The capacity of this system is only slightly, if at all, stimulated by the clofibrate administration to the rats (J. Norseth and B.O. Christophersen, unpublished data).

The presented results suggest, however, that the capacity of the chain-shortening system of the heart is very low, since erucate poorly competes for oxidation with other, shorter fatty acids supplied to the heart in the blood, and is mainly incorporated into triacylglycerols.

Thus, it seems that the properties of heart and liver with respect to metabolism of very long chain length monounsaturated fatty acids are significantly different.

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15,18,21,24-Triacontatetraenoic and 15,18,21,24,27-Triacontapentaenoic Acids: New C_{30} Fatty Acids from the Marine Sponge *Cliona celata*

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ABSTRACT

The marine sponse *Cliona celata* contains 3.5% 30:4ω6 and 7.0% 30:5ω3 in its total fatty acids. These C_{30} polyunsaturated structures are unknown in other living organisms. Both acids occur mainly as phosphatidylserine esters.

INTRODUCTION

Marine sponges of the class Demospongiae contain high levels (34-79%) of characteristic C_{24}-C_{30} fatty acids (1). Recent studies have shown that many of these "demospongic acids" possess new fatty acid structures not found in other organisms. We have identified 26:2Δ5,9 and 26:3Δ5,9,19 in *Microciona prolifera* (2); 28:3Δ5,9,19 in *Xestospongia halichondroides* (3); and 30:3Δ5,9,23 in *Chondrilla nucula* (4).

Our survey of fatty acid chain lengths in sponges (1) revealed that the yellow boring sponge *Cliona celata* contains 11% C_{30} chain lengths in its total fatty acids. We have now characterized the structures of the C_{30} chains and have identified two new polyunsaturated fatty acids.

EXPERIMENTAL PROCEDURES

Materials

Cliona celata sponge colonies collected near Woods Hole, Massachusetts were purchased from the Northeast Marine Specimens Co. (Woods Hole, MA) in October 1974 and June 1978. The live sponges were packed in seawater and shipped by air to our laboratory. Upon arrival, they were washed in seawater, carefully cleaned of all visible algae and debris, and cut into small cubes. Immediate extraction with 2:1 chloroform/methanol (5) yielded 2.2% (dry weight) total lipids. BHT was added to this sample and to all further lipid isolates to retard autoxidation. All quantitative data reported in this paper are for the June 1978 sponge sample.

Methyl esters were prepared from total *Cliona* lipids by refluxing in 98:2 methanol/conc. H_2SO_4 for 3 hr or using KOH-catalyzed methanolysis (6). The esters were purified by preparative thin layer chromatography (TLC) on 1.0 mm thick silicic acid layers developed in 95:5 hexane/diethyl ether. The yield of methyl esters was ~60% of the total lipids reacted.

The following standards were purchased from reliable sources and used in the identification of fatty acids and of their ozonolysis products by gas liquid chromatography (GLC): 14:0, 16:0, 18:0, 18:1ω9, 18:2ω6, 20:0, 22:0, 24:0, 28:0 and 30:0. Methyl esters of linseed oil, cod liver oil, and *Tropaeolum speciosum* seed fat [containing 22:1ω9, 24:1ω9, 26:1ω9 (7)] were prepared by KOH-catalyzed methanolysis (6). Pure phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, lysophosphatidylcholine, and sphingomyelin TLC standards were purchased from Supelco Inc. (Bellefonte, PA).

Methods

GLC analyses of methyl esters were run isothermally at temperatures between 175 C and 200 C using either a 1.80 m x 2.4 mm ID column packed with 10% Silar-10C on 100/120 mesh Gas Chrom Q or a 1.21 m x 2.4 mm ID column packed with 15% OV-101 on 100/120 mesh Gas Chrom Q. Peaks were identified using cochromatography, by comparison of retention times with known standards, and by graphic correlation of equivalent chain length (ECL) values in semilog plots (8-10).

Methyl esters were separated according to degree of unsaturation by preparative TLC on 1.0 mm thick $AgNO_3$-impregnated silicic acid (12% wt/wt) developed in diethyl ether. Bands were located under UV light after spraying with 2′,7′-dichlorofluorescein, and the methyl esters were recovered by the extraction method of Hill et al. (11).

Methyl esters were hydrogenated in methanol solution using a PtO_2 catalyst (12).

Infrared analyses were carried out in CCl_4 solution using a Perkin-Elmer 700 spectrophotometer. Ultraviolet spectra were run in hexane solution on a Beckman DB spectrophotometer. The 60 MHz proton magnetic resonance (PMR) spectra were obtained with a

Varian T-60 instrument using 17-21 mg samples dissolved in CCl_4 with $Si(CH_3)_4$ as a reference marker. Mass spectrometric analyses were performed on a Biospect quadrupole mass spectrometer using CH_4 chemical ionization conditions and direct insertion of the sample probe at 220 C.

Reductive ozonolyses of 10-50 μg methyl ester samples were carried out in purified hexane (3) at -10 C using the method of Stein and Nicolaides (13) with a Supelco micro-ozonizer. Aldehydic products were analyzed by GLC as described above using column temperatures at 65 C and 180 C. Peaks were identified by cochromatography with known standards and by semilog retention time plots for homologous series.

Lipid class separations utilized column chromatography followed by preparative TLC. Total *Cliona* lipids (290 mg) were first separated on a 20 x 50 mm column of silicic acid (Silica Gel G60, E. Merck, Darmstadt, Germany) into four fractions by successive elution with 2 column volumes of $CHCl_3$, 2 column volumes of acetone, 5 column volumes of 1:1 $CHCl_3/CH_3OH$, and 2 column volumes of CH_3OH. The $CHCl_3$ fraction was further separated by preparative TLC on Whatman K5 silica gel plates (Whatman Inc., Clifton, NJ) developed with 97:3 hexane/diethyl ether. Bands were visualized with rhodamine 6G, identified by comparison with the R_f values of known standards, and then scraped off the plates. Lipids were recovered with diethyl ether. The acetone fraction was treated similarly, using 70:30 hexane/acetone to develop the TLC plate.

The $CHCl_3/CH_3OH$ and CH_3OH column fractions were combined and then separated on Whatman K5 TLC plates using a 65:32:3 $CHCl_3/CH_3OH/NH_4OH$ developing solvent. Bands were located with iodine vapor and identified by comparison with the R_f values of authentic lipid standards. The identity of the phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine bands was further confirmed using Dragendorff and ninhydrin spray reagent tests (14). For preparative TLC separations, bands were located by placing a glass cover over most of the plate and exposing just one edge to iodine vapor. Each band (where it had not been exposed to iodine) was then scraped off and the lipids recovered by extraction with 45:45:10 $CHCl_3/CH_3OH/H_2O$.

The amount of 30:4ω6 and 30:5ω3 in each lipid class was determined by GLC of the derived methyl esters using methyl triacontanoate (30:0) as an internal standard. To avoid interfering peaks, the 30:4ω6 and 30:5ω3 esters were purified by Ag^+-TLC before GLC analysis.

RESULTS

Purification of Methyl Esters

GLC analysis of *Cliona celata* total methyl esters on a Silar-10C column at 200 C revealed two unknown peaks having ECL values of 33.28 and 34.47. These peaks constituted 3.5 wt. % and 7.0%, respectively, of the total methyl esters.

To purify these two unknowns for further study, we first separated *Cliona* total methyl esters by TLC into two bands. The upper band containing the two unknowns was recovered and then further fractionated using Ag^+-TLC. Development with diethyl ether resolved two distinct bands both having $18:3 > R_f > 20:5$. Each band was recovered for GLC analysis. The faster moving one contained 91% of the ECL 33.28 unknown with no other component ≥ 2%. The slower moving one contained 96% of the ECL 34.47 unknown.

Identification of 30:4

Hydrogenation of the ECL 33.28 methyl ester produced an n-30:0 peak when characterized by GLC. Mass spectrometric analysis gave an $(M + 1)^+$ ion at 459. Thus an n-30:4 structure was indicated.

The unknown showed no absorption bands in the 220-300 nm region, proving the absence of conjugated double bonds (15). Its infrared spectrum exhibited the usual 3050-2820 cm^{-1} hydrocarbon and 1712 cm^{-1} ester bands and had no prominent absorption at 980-968 cm^{-1}, indicating *cis* rather than *trans* unsaturation (16). The PMR spectrum corresponded to that of a polyunsaturated fatty acid methyl ester. Integration of the CH_3O-, -HC=CH-, and =C-CH_2-C= signals gave a 3.0:8.3:6.0 ratio, pointing to a methylene-interrupted tetraene structure of the type found in arachidonic acid (17).

Reductive ozonolysis produced two major GLC peaks, which were identified as a C_{15} aldehyde ester and a C_6 aldehyde. Hence, the first and last double bonds in the chain were at the 15- and 24-positions.

We conclude that the unknown compound was the methyl ester of *cis*-15,*cis*-18,*cis*-21,*cis*-24-triacontatetraenoic acid or 30:4ω6.

Identification of 30:5

The ECL 34.47 methyl ester was identified in the same manner as the 30:4ω6. Hydrogenation of the unknown produced an n-30:0 peak

on GLC. Mass spectrometry gave an $(M + 1)^+$ ion at 457. Hence, an n-30:5 structure seemed probable.

The unknown ester had no UV absorption between 220 and 300 nm, and its infrared spectrum was identical with that of the 30:4ω6. PMR analysis yielded a normal polyunsaturated methyl ester spectrum with a 3.0:10.0:7.9 ratio for the CH_3O-, -HC=CH-, and =C-CH_2-C= signals. This indicated a methylene-interrupted pentaene structure. Ozonolysis of the 30:5 ester produced only one major GLC peak, a C_{15} aldehyde ester, showing that the first double bond in the chain was at the 15-position.

Thus, the unknown compound was the methyl ester of *cis*-15,*cis*-18,*cis*-21,*cis*-24,*cis*-27-triacontapentaenoic acid or 30:5ω3.

Location in *Cliona celata* Lipids

To determine how the unusual 30:4ω6 and 30:5ω3 acids were distributed among the various lipids of the sponge, the five major classes of *Cliona* ester lipids (Table I) were isolated by consecutive column and thin layer chromatography. A sixth sample containing all the other lipids (extracted from the remaining areas of the TLC plates on which the lipid classes were separated) was also recovered. Methyl esters were prepared from each sample and purified by TLC. Then the combined 30:4 and 30:5 bands were isolated by Ag^+-TLC, and a known amount of methyl triacontanoate (30:0) was added. GLC analysis of this mixture permitted direct calculation of the weight of 30:4ω6 and 30:5ω3 in each lipid class.

Results (Table I) show that the 30:4ω6 and 30:5ω3 chains are mainly located in phosphatidylserine molecules. One-fifth of the 30:5ω3 is esterified in the triglycerides, but this is not true for 30:4ω6. Only minor amounts ($\leqslant 0.10$) of the 30:4ω6 and 30:5ω3 chains are present in the other lipid classes.

TABLE I

Distribution of 30:4ω6 and 30:5ω3 in *Cliona celata* Lipid Classes

Lipid class	30:4ω6	30:5ω3
Phosphatidylserine	0.74	0.62
Phosphatidylcholine	0.10	0.09
Phosphatidylethanolamine	0.05	0.05
Triglyceride	0.06	0.19
Sterol ester	0.04	0.04
Other[a]	0.01	0.01
Total lipids	1.00	1.00

[a]Includes all remaining areas of the TLC plates on which lipid classes were separated.

DISCUSSION

The 30:4ω6 and 30:5ω3 fatty acids identified in *Cliona celata* have not, to our knowledge, been previously found in other living organisms. These acids contain the familiar ω6 and ω3 types of polyunsaturation but have longer hydrocarbon chains than previously encountered.

The 30:4ω6 and 30:5ω3 structures probably originate via the chain elongation pathway from 20:4ω6 (arachidonate) and 20:5ω3 precursors, both of which are commonly found in marine organisms. Since 30:4ω6 and 30:5ω3 are unknown in other organisms, it seems likely that they are synthesized by the sponge itself. Morales and Litchfield (18) have shown that the marine sponge *Microciona prolifera* possesses a very active chain elongation system that produces C_{24}-C_{28} fatty acids from normal chain length precursors. However, the comparable *Cliona* chain elongation system must have a distinctive specificity, since C_{24}-C_{30} tetraenes, pentaenes, and hexaenes were not found in *Microciona* even though the probable C_{18}-C_{22} precursors were present (19).

Both 30:4ω6 and 30:5ω3 are mainly esterified in the phosphatidylserine molecules of *Cliona* (Table I). This finding implies that the C_{30} acyl chains may participate in the membrane lipid bilayers of the sponge. If true, the extra bulk of their longer chains would probably make that bilayer thicker and more rigid than normal, a possibility that has been discussed in detail elsewhere (20).

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The Metabolic Fate of Cholesterol-5α,6α-Epoxide in vivo

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ABSTRACT

[^{14}C]Cholesterol-5α,6α-epoxide, administered to mice by either gastric intubation or skin painting, was rapidly and primarily excreted in the feces. Residual amounts of the epoxide and its metabolites were found in a wide variety of organs, and persisted for at least 72 hr. At some sites (principally the liver, the small intestinal contents and the combined stomach/duodenum and their contents), the labeled compound existed in a water-soluble form which could not be extracted with chloroform/methanol. Treatment of the small intestinal contents with a preparation of β-glucuronidase/sulfatase produced a marked increase in the amount of organic-solvent-extractable cholesterol-α-epoxide and other polar metabolites. Unchanged epoxide was found mainly in the feces and the skin at the site of application. On the basis of these results, stool specimens, and not blood samples, should be analyzed to detect the presence of this compound and/or its metabolites in vivo.

INTRODUCTION

Black and coworkers have implicated cholesterol-5α,6α-epoxide (α-CE) as a possible skin carcinogen which is formed by the action of ultraviolet-irradiation (1-4). However, their studies were unable to identify α-CE, or a derivative, as the agent directly responsible for the tumorigenic effects of UV irradiation. Bischoff (5) reported that α-CE was capable of producing local sarcomas in mice when it was administered by intradermal injection.

Besides these studies, there have been only a few reports concerning the metabolic fate of α-CE. Chan and Black (4) reported that α-CE, a "polar metabolite" generated in vitro in mouse epidermis by UV-irradiation, could diffuse quite rapidly into the dermis and eventually into their agar support media. They also demonstrated the capacity of mouse skin and liver to catalyze the hydration of α-CE to cholestan-3β,5α,6β,triol (triol). Aringer and Eneroth (6) and Martin and Nicholas (7) also demonstrated this hydrase activity in rat liver and brain, respectively. Bischoff and Bryson (8) reported that the loss of intradermally injected α-CE from the site of its injection (7-22 μg/day) was roughly equivalent to the extent of formation by UV light (16 μg/mouse), and therefore, they suggested that it may be a systemic carcinogen. Fioriti et al. (9) failed to detect α-CE in epididymal fat pads, liver, kidney, serum or spleen after feeding the test compound to rats in the diet (1.5%) for 90 days. However, they could account for 50% of the ingested α-CE in the fecal sterols. These results imply that the α-CE is either poorly absorbed or rapidly excreted in the feces with only 50% conversion to other products. A second report from this group (10) demonstrated that the ingested α-CE was converted to its corresponding triol derivative when the contents of the entire gastrointestinal tract were examined. This work was performed with unlabeled material and since then no other studies have centered on the fate of α-CE in vivo.

Other workers have followed up this work with detailed studies on the metabolic fate of the triol (11-13). In summary, they demonstrated that the triol undergoes three primary reactions in vivo: a) oxidation to cholestan-3β, 5α-diol-6-one; b) oxidation at the side chain to trihydroxy bile acid, and c) esterification at the 3β-position with long chain fatty acids.

A determination of the in vivo fate of α-CE would allow a better pharmacological analysis of its action and could provide a rational basis for carcinogenicity experiments. In that respect, this report deals with the in vivo fate of α-CE after administration by gastric intubation or skin painting.

MATERIALS AND METHODS

[4-^{14}C]Cholesterol (53.7 μCi/mmole) and PCS scintillatin cocktail were purchased from Amersham Searle Corp., Arlington Heights, IL. Two samples of [4-^{14}C]cholesterol-5α,6α-epoxide and unlabeled palmitoyl cholesterol-5α,6α-epoxide were synthesized according to a procedure described by Fieser and Fieser (14). The final specific activities of the ^{14}C-labeled α-CE preparations were 50 and 110 μCi/mmole. The purity of all these synthesized preparations was greater than 98-99% on the basis of mobility on thin layer chromatography (TLC) and gas liquid chromatography (GLC). β-Glucuronidase, containing 92,000 and 10,000 units per ml of glucuronidase and sulfatase activities respectively, was obtained from Sigma Chemical Co. (St. Louis, MO). The standard reference compounds: cholesterol, cholesterol

TABLE I

Distribution of Radioactivity in the Organs and Excreta of Mice after Gastric Intubation with [4-^{14}C] Cholesterol-α-epoxide[a]

Tissue/speciment analysed	% Distribution of recovered [^{14}C]label
Feces	91.5
Urine	0.5
Blood	1.3
Liver	1.4
Small intestine	2.4
Large intestine	0.4
Kidney	0.5
Spleen	0.2
Adrenal	0.1
Testes	0.3
Epididymal fat pads	0.3
Heart	0.2
Lung	0.2
Brain	0.2
Skin	0.5

[a]The distribution of radioactivity in the pooled organs and excreta of two 20 g B6C3F mice was determined 18 hr after they received 0.36 μCi (1.5 mg) [4-^{14}C] cholesterol-α-epoxide by gastric intubation. Radioactivity measurements were performed on aliquots of 25% homogenates which had been solubilized with NCS solution. The contents of the gastrointestinal tract were removed and not analyzed. Approximately 30% of the theoretically administered dose was recovered in the tissues that were examined. The percent distribution of this recovered activity is presented here.

palmitate, cholesterol-5α,6α-epoxide, cholestan-3β,5α,6β-triol, 5α-cholestan-3-one, 5α-cholestan-3,6-dione, 5α-cholestan-3β-ol-6-one, cholestan-3β,5α-diol-6-one, and 5α-cholestan-3β,6β-diol were obtained from Steroloids, Inc. (Wilton, NH). ITLC-SA glass fiber sheets for TLC were purchased from Gelman Instrument Co (Ann Arbor, MI). Analtech Silica Gel G plates were obtained from Fisher Scientific (Silver Spring, MD). NCS tissue solubilizer was obtained from New England Nuclear (Boston, MA). All solvents were obtained from Burdick and Jackson (Muskegon, MI), and other reagents used were of the highest purity commercially available.

Animal Treatment and Tissue Preparation

The mice used in these experiments were 20 g males, strain $B_6C_3F_1$, from the Animal Breeding facilities at the Frederick Cancer Research Center. Three mg of [4-^{14}C]-α-CE (0.36 μCi) dissolved in 0.25 ml corn oil were introduced into the stomachs of two mice by intubation. Skin painting experiments were performed on pairs of mice whose dorsal hair had been removed with electric clippers. The [^{14}C]-α-CE (1.5 mg; 0.4 μCi) dissolved in 25 μl acetone, was applied to the shaved skin using an Eppendorf pipette. In the second experiment, the solution was diluted (1:4) and 25 μl aliquots were applied on 4 consecutive days.

The animals were kept in individual metabolism cages where urine and feces could be collected separately for the entire time of the experimental period, i.e., immediately after treatment until the animals were sacrificed. In the gastric intubation and single dose skin painting experiments, the organs were removed and analyzed 18 hr after treatment. In the multiple dose skin painting experiment, the animals were killed by decapitation 3 days after the last dose was administered and bled into a test tube containing 1 ml 3% heparin. The animals were eviscerated and the individual organs pooled and weighed. Twenty-five percent homogenates of the various organs in 0.1 M Tris buffer, pH 7.5, were prepared in a Waring Blender. Aliquots were taken for lipid extraction or digestion with NCS for direct quantitation of radioactivity.

Lipid Extraction and Analysis

All lipid-containing materials were extracted using 10 volumes of chloroform/methanol (2:1, v/v). The mixture was vortexed vigorously and then centrifuged to separate the phases. The aqueous phase was removed and the chloroform layer washed with 1 volume 0.9% NaCl. The solvent layer was evaporated to dryness under nitrogen, redissolved in a small volume of chloroform/methanol (1:1, v/v) and chromatographed as described below.

TLC systems utilizing Gelman ITLC-SA glass fiber sheets were used for resolving mixtures of α-CE and its metabolites from total lipid

extracts. The ITLC-SA sheets were developed with isooctane/methyl ethyl ketone (2:1, v/v). All chromatography was in unlined tanks and generally was completed in less than 30 min. The identity of the metabolites was determined by chromatographing standard reference compounds in parallel with the unknown samples. Areas on the sheet containing radioactive compounds were located by scanning with a Berthold TLC scanner. Standard compounds were visualized by spraying with H_2SO_4/ethanol/water (2:1:1, v/v/v) followed by heating at 110 C for 10 min. The sheets were cut into sections according to the distribution of the standards and the radioactivity of each section quantitated. In most cases, further characterization was not made because only small quantities of compounds were isolated.

Because of the uncertainty of identification of minor metabolites by relative mobility on TLC, the designation of these compounds in this report as cholesterol triol, etc., does not exclude the possible presence of other compounds which chromatograph with R_f values similar to these standards. In some instances, larger amounts of metabolites were separated by TLC on 0.25 mm Silica Gel G plates using a solvent system consisting of benzene/ethyl acetate (1:2, v/v). The areas containing the compounds of interest were eluted with acetone, converted to their trimethylsilyl (TMS) derivatives (15) and qualitatively identified by radio GLC or GLC/MS as described below.

Gas Liquid Chromatography/Mass Spectrometry (GLC-MS)

The GLC/MS system used was a Varian Aerography GLC (Varian Associates, Palo Alto, CA) equipped with 6 foot column of 1% SE-30 maintained at 240 C with helium as the carrier gas at a flow rate of 20 ml/min. The GLC was interfaced with a Finnigan Model 1015 mass spectrometer (Finnigan Corp., Sunnyvale, CA), and the effluent was ionized at 70 eV in an electron impact source. A Finnigan 6000 Data System was used for data acquisition.

Radioactivity Measurements

All quantitative measurements of radioactivity were performed in an ISOCAP 300 liquid scintillation counter (Searle-Analytic, Chicago, IL) using PCS cocktail. Qualitative distributions of [^{14}C]labeled compounds on TLC were performed using a Berthold LB-2760 TLC scanner (obtained through Shandon Inst., McKeesport, PA). Radio GLC identification of [^{14}C]labeled compounds was accomplished by using a Packard Model 427 GLC (Packard Inst., Co., Downers Grove, IL) equipped with a 6 foot 1% SE-30 column run with a temperature program from 220 - 260 C at 5 C/min. Helium was the carrier gas at a flow rate of 42 ml/min. This chromatograph was connected by a nickel conduit to a Packard Model 894 gas proportional counter.

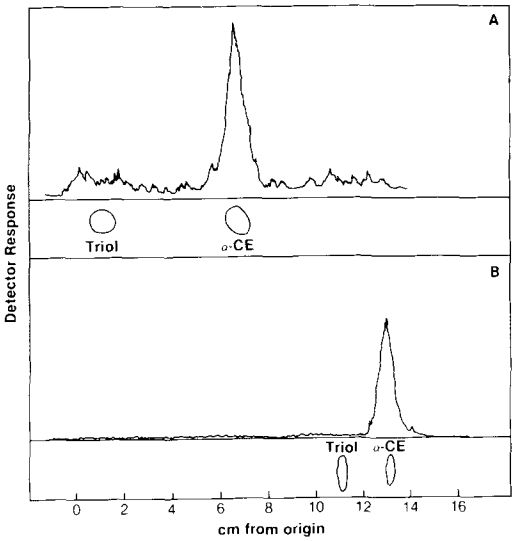

FIG. 1. a. Radio TLC scan of total lipid extract from mice fecal pellets of mice 18 hr after skin painting [^{14}C]-α-CE. The sample was applied to 0.25 mm Analtech Silica Gel G plate and developed with benzene/ethyl acetate (1:2). b. Regions corresponding to α-CE in Panel A were eluted from the plates and chromatographed separately using a solvent system composed of ethyl acetate/benzene/methanol (4:2:1).

RESULTS

Fate of α-CE after Gastric Intubation

When mice were intubated with α-CE in corn oil, ca. 92% of the recovered label was found as free epoxide in the feces after 18 hr. Most of the remaining label was in the urine, blood, liver, small intestine, large intestine, and kidneys (Table I). Smaller amounts of label were found in the epididymal fat pads, testes, and lungs. TLC-separation of lipids from tissues extracted with chloroform/methanol showed that the recovered label was present in forms having polarity similar to that of the triol and its oxidation product, cholestan-3β,5α-diol-6-one (12). Some material migrated in regions less polar than α-CE and cholesterol. These metabolites could be fatty acid esters of the α-CE or other metabolites (12). No attempts were made to identify these compounds because of the small quantities of material recovered.

TABLE II

Recovery of [^{14}C]-Labeled Compounds after Painting Dorsal Skin of Mice with [4-^{14}C]Cholesterol-α-epoxide

Tissue/specimen	% Distribution of recovered [^{14}C]labeled compounds			
	Experiment I[a]			Experiment II[b]
	Total radioactivity[c]		Water-soluble[d] radioactivity	Total radioactivity
Dorsal skin	9.6	(27.2)[e]	0.8	79.6
Ventral skin	0.1	(0.3)	22.9	4.2
Liver	2.5	(7.2)	44.1	2.1
Stomach/duodenum and contents	2.8	(7.9)	45.5	f
Small intestine	3.2	(9.2)	22.0	6.0
Large intestine	1.2	(3.3)	15.0	7.3
Kidney	0.1	(0.3)	15.3	0.1
Feces	64.2	(——)	11.1	g
Contents of Sm. intestine	6.0	(17.2)	72.0	h
Contents of Lg. intestine	9.7	(27.6)	18.2	h
Urine	6.2	(——)	28.8	g

[a]In experiment I, the organs were analyzed 18 hr after the animals were painted with a single dose of the [4-^{14}C]-α-CE (0.4 μCi). Total recovery of the theoretically administered dose was 65%.

[b]In experiment II, the organs were analyzed 72 hr after the animals had received 4 consecutive daily dose of [4-^{14}C]-α-CE (0.1 μCi/dose). Total recovery of the [^{14}C]labeled compounds in the organs was 13% of the administered dose.

[c]Percent distribution of recovered [^{14}C]labeled material in various tissues/specimens.

[d]The fraction of label in these tissues that was not extractable with chloroform/methanol.

[e]The values in parentheses represent the distribution of label in these tissues excluding that present in the excreta.

[f]The stomach was not analyzed, but the duodenum was removed with the remainder of the small intestine.

[g]Feces and urine were not collected.

[h]Contents of the small and large intestines were not removed prior to analysis.

Fate of α-CE after Skin Painting with Single Dose (Experiment I)

When a solution of α-CE was painted on the shaved dorsal skin of mice in a single dose, 65% of the recovered label (apparently as unmetabolized α-CE) was excreted in the feces within 18 hr. This compound was present in relatively high proportions and had mobility on TLC corresponding to that of α-CE in two sequential solvent systems (Fig. 1a and 1b). The TMS-ether derivative had a mass spectrum identical to that of the corresponding standard. Analysis of this same sample by radio GLC indicated that a [^{14}C]labeled silylated compound had the same retention time as silylated-[^{14}C]-α-CE. No other [^{14}C]labeled peaks were observed on this chromatogram even though several other components were observed when the column effluent was monitored with a flame ionization detector. These same components had retention times corresponding to those obtained from extracts of feces from untreated mice.

The compound isolated from the TLC with an R_f value similar to that of the triol was also silylated and analyzed by GLC/MS. None of the retention times of the components agreed with those of a triol standard and concurrently neither did any of the mass spectra. Radio TLC analysis of this fraction in a very polar solvent system [ethyl acetate/benzene/methanol 4:2:1 v/v/v] indicated the presence of several components. Therefore, this sample may not contain any triol. Although the identity of these components is unknown, they cannot be one of the following on the basis of their TLC and GLC/MS characteristics: 5α-cholestan-3,6-dione, 5α-cholestan-3β-ol-6-one, cholestan-3β, 5α-diol-6-one nor 5α-cholestan-3β,6β-diol.

In this same experiment, smaller amounts of label were also found in the skin, kidneys, small intestine, large intestine, combined stomach/

duodenum and the contents of the large and small intestines. The distribution is shown as experiment I in Table II. Very little α-CE was found in these organs or any of the other organs examined, which included the spleen, adrenals, testes, epidiymal fat pads, heart, brain, bladder, and pancreas. In each of these organs, the [^{14}C] labeled compounds migrated on TLC as triol or its 6-keto oxidation product.

Most of the label found in the contents of the small intestine was water-soluble. A significant amount of water-soluble material was also present in the liver, and the combined stomach/duodenum homogenate. Table II also lists the percent total radioactivity as water-soluble [^{14}C] labeled derivatives contained in these tissues. Note the large difference between the amounts of water soluble [^{14}C] in the contents of the small and large intestines (72% and 18%, respectively, of the total [^{14}C] in these homogenates).

To test for conjugated α-CE metabolites (glucuronides or sulfates), aliquots of homogenate of the small intestinal contents were diluted with an equal volume of 1 M sodium acetate buffer, pH 5.0, and incubated at 37 C for 18 hr with 4,600 and 500 units of β-glucuronidase and sulfatase, respectively. The reaction mixtures were extracted and analyzed as usual. The homogenate was also run through the procedure without added enzyme preparation. The treated sample showed a significant increase in solvent-extractable [^{14}C] label when compared with its unhydrolyzed control (Fig. 2). The labeled compounds had TLC mobilities generally more polar than that of the epoxide and similar to the triol and cholestan-3β,5α-diol-6-one.

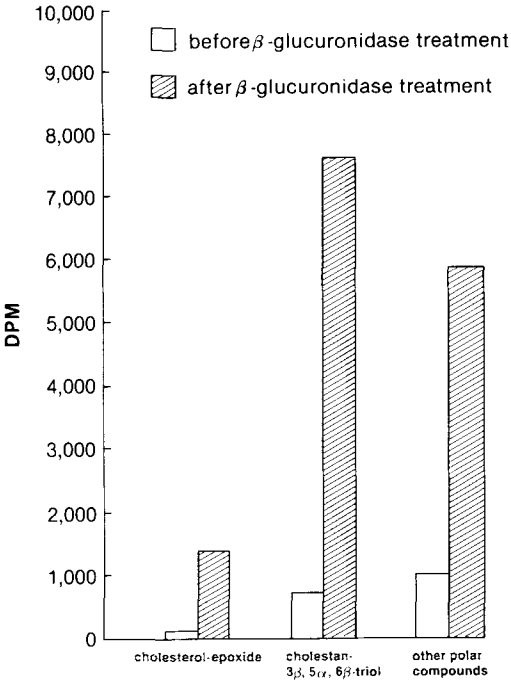

FIG. 2. The release of chloroform/methanol soluble [^{14}C] labeled derivatives by the action of β-glucuronidase/sulfatase on the small intestine contents of mice painted on the skin with acetone solutions of [4-^{14}C] cholesterol-α-epoxide.

Fate of α-CE after Administration in Multiple Doses (Experiment II)

One-quarter of the dose of the α-CE used previously was applied to the skin of mice on each of 4 consecutive days and the animals were killed 72 hr after the last application. Table II-Experiment II lists the distribution of [^{14}C] labeled compounds among the organs containing the highest amount of labeled material. In comparison with the former experiment, the results obtained indicated that higher levels of radioactivity were found in the dorsal skin at the site of application after treatment with multiple doses of α-CE. These levels (10.5% of the administered dose) were considerably greater than those found in the same site only 18 hr after animals received a single treatment (6.2% of the administered dose) with an equivalent total dose of the test compound. Most of the radioactivity in the dorsal skin was apparently in the form of the unmetabolized epoxide (data not shown). This is in contrast to the wide distribution of very low quantities of metabolites of varied polarity isolated from the ventral skin of the same animals.

DISCUSSION

α-CE has been found in normal mouse skin (1,2) and liver (16). It is also present in sera of patients with high blood pressure, peptic ulcers and hypercholesterolemia (17) as well as in the organs of patients with Wolman's disease (18). Surprisingly, its levels were barely detectable in sera from normal healthy humans (17).

Apart from its reported carcinogenic activity (19), α-CE and other auto-oxidation products of cholesterol (20) have been reported to exhibit toxic effects both in vivo (10,21) and in vitro (22,23). Recently, Kelsey and Pienta (24) demonstrated that α-CE was capable of transforming hamster embryo cells.

Despite the potential toxic and carcinogenic hazards associated with this compound, its fate in the whole animal has never been completely examined. The purpose of the present studies

was to examine the distribution and transformation of α-CE in vivo since it is so easily formed from cholesterol.

When cholesterol epoxide was administered to mice, whether by gastric intubation or skin painting, it was rapidly excreted in the feces. These results were in general agreement with studies using Sprague-Dawley rats reported by Fioriti and coworkers (9,10). In the 18 hr skin painting experiment, [^{14}C]labeled material in the gastrointestinal tract was only partially extractable with organic solvents. That this nonextractable label was present in the form of glucuronides or sulfates was confirmed by treatment of the contents of the small intestine with a β-glucuronidase/sulfatase preparation. This treatment released the majority of [^{14}C]-labeled steroids from the water-soluble label allowing their extraction into chloroform/methanol.

Since a large proportion (64%) of the recovered cholesterol epoxide appeared in the feces, it is likely that the compound was largely unmetabolized during the early stages of absorption and was rapidly excreted via the gastrointestinal tract. A smaller portion was metabolized to cholestane triol and other derivatives, many of which were conjugated to water-soluble forms. The fact that a greater amount of conjugated [^{14}C]labeled compound was found in the contents of the small intestine rather than in the large intestine, or the excreted feces, suggests that the conjugates were either hydrolyzed by the flora of the large intestine or conjugation occurred only during the later stages of absorption.

With the possible exception of the production of α-CE from cholesteryl palmitate by rat brain homogenates (7), there is little or no evidence that significant amounts of cholesterol epoxides are produced by direct enzymatic epoxidation of cholesterol. According to Smith and Kulig (20), α-CE is a secondary product arising from the interaction of cholesterol with hydroperoxides. Cholesterol hydroperoxides are the primary products of the oxidation of cholesterol by UV light or lipid peroxidation (25,26). The fact that α-CE was found as a normal constituent in the liver and skin of hairless mice may verify the observations of Aringer and Eneroth (6) and Mitton et al. (27), who demonstrated that cholesterol epoxides were generated during lipid peroxidation in liver microsomes in vitro. One could infer then, that lipid peroxidation in the liver and skin of mice is a normal occurrence and as a result of this, α-CE would be formed as a natural by-product of these reactions.

Based on the results reported here, one would not expect to find significant levels of α-CE in the blood since it is rapidly excreted in the feces. Analysis of stool specimens for the presence of α-CE or its metabolites could be employed as a screening procedure for diseases giving rise to increased levels of lipid peroxidation. The work of Reddy and coworkers has shown that the triol, the hydration product of cholesterol epoxide, exists in significantly higher quantity in the feces of patients with ulcerative colitis (28) and colon cancer (29). No attempt was made during these studies to determine the levels of α-CE. Although we were unable to demonstrate the hydration of cholesterol epoxide by mice fecal flora, Hwang and Kelsey (30) have clearly demonstrated the presence of hydrase activity in feces of normal adult humans.

The following conclusions can be drawn from these studies: 1) regardless of the source of α-CE in the mouse, (via UV irradiation with subsequent formation in the skin, ingestion of α-CE produced from cholesterol by peroxidized lipids in foods, or its nonspecific formation by microsomal hydroxylases), it is rapidly excreted, primarily in the feces, with only a small percentage of the residual compound remaining in the organs or at the site of its formation in, or application to, the skin; 2) as the skin painting studies indicated, multiple doses over a 4-day-period resulted in a higher level of residual α-CE at the site of painting than an equivalent total amount administered in one large dose which would indicate that small repeated exposures to this compound at a single site would have a greater effect than one large acute exposure; 3) there are two possible target sites which may be implicated in carcinogenesis in animals exposed to α-CE via the skin, the large intestine, and the skin at the site of application because these two tissues contain the highest levels of free α-CE, and one would expect tumor formation to be evident here.

Whether or not α-CE is a direct acting carcinogen remains to be proven. Further works, including appropriate carcinogenicity experiments with this and related compounds, are in progress.

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Varietal Differences in Peanut Triacylglycerol Structure[1]

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ABSTRACT

Stereospecific analysis of triacylglycerols from six peanut varieties showed diversity in percent fatty acid placement. Distribution of the fatty acids among the *sn*-1, -2 and -3 positions was clearly nonrandom. The percentages of palmitic and stearic acids, generally very low at the *sn*-2 position, were more predominant at the *sn*-1 than the *sn*-3 position. Long chain fatty acids were located almost exclusively at the *sn*-3 position. The *sn*-2 position of all varieties was high in unsaturated fatty acids. Triacylglycerols were sufficiently different to suggest that concentrations of specific triacylglycerol species may vary with variety.

INTRODUCTION

Stereospecific analyses of animal (1-5) and plant (4,6-9) triacylglycerols indicate that the distribution of fatty acids is not random, but that each position has a characteristic fatty acid pattern. Usually, the fatty acid compositions of *sn*-1 and -3 positions are similar, but none of the fats examined has exhibited a completely symmetrical distribution. Triacylglycerol composition and structure are important from the standpoint of nutrition (10), oil stability (11) and possible physiological effects (12).

Early studies of plant triacylglycerols were conducted on refined oil unidentified as to specific variety and probably representing genetically heterogeneous source material. Analyses of maize (7,8) and soybean (9) triacylglycerols from several specific varieties indicate that structure is not constant but is variable among varieties. Biochemical studies on heterogeneous material may be of little value especially if one of the goals of the study is to effect some eventual genetic change in the source material should the conclusions so indicate. The variation in fatty acid composition of oil from various peanut varieties is well documented (13) and is an indication of the variability found in the triacylglycerol fraction which routinely accounts for more than 90% of the total composition. de la Roche et al. (8) reported that the fatty acids at each position of corn oil triacylglycerols were influenced by the fatty acid concentration in the total triacylglycerol except for the saturates in the *sn*-2 position. This indicates that triacylglycerols with diversity in fatty acid composition might reasonably be expected to have diverse structures.

Stereospecific analysis of peanut oil has been reported by Brockerhoff and Yurkowski (6). Myher et al. (14) compared the triacylglycerol structures of native, rearranged, and simulated peanut oils and found that native oil, the most atherogenic in laboratory animals, contained a significantly greater proportion of certain triacylglycerol structures than the synthetic oils. Only dietary testing will determine whether those triacylglycerol structures are indeed associated with increased atherogenic potency (14). Hokes (15) determined the fatty acids attached to the *sn*-2 position of triacylglycerols from several peanut cultivars, but did not differentiate between fatty acids at the *sn*-1 and *sn*-3 positions.

This paper describes the variability that exists in the stereospecific structure of triacylglycerols from six peanut varieties.

MATERIALS AND METHODS

All of the peanut varieties were grown, harvested and cured using conventional methods in Headland, AL as part of the 1976 National Peanut Performance Trials. Peanuts were shipped to this laboratory where they were shelled. Seed riding a 0.635 x 1.905 cm shaker screen were sealed in plastic bags and stored at 4 C.

For lipid extraction, random 10 g samples of sound, mature, intact peanuts of each variety

TABLE I

Mean Differences between *sn*-3 Position Fatty Acid Percentages of Six Peanut Varieties Calculated by Two Methods

Fatty Acid	Difference
16:0	2.6 ± 1.2
18:0	0.9 ± 0.6
18:1	1.1 ± 1.2
18:2	3.1 ± 0.8
20:0	0.2 ± 0.1
20:1	0.5 ± 0.3
22:0	0.3 ± 0.1
24:0	0.3 ± 0.2

[1]Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

TABLE II

Stereospecific Analyses of Triacylglycerols from Six Peanut Varieties

Variety	Compound or position	Fatty acid distribution, mole %[a]							
		16:0	18:0	18:1	18:2	20:0	20:1	22:0	24:0
Florigiant	TG	10.8	2.9	53.1	27.3	1.8	1.0	1.9	1.1
	1	20.1	4.9	50.7	22.6	0.5	0.7	0.4	0.3
	2	2.2	0.7	51.5	45.3	0.1	0.3	0.1	—
	3	10.3	3.2	57.2	14.0	4.8	2.0	5.3	3.0
Early Bunch	TG	13.3	2.6	42.0	36.3	1.5	0.9	2.4	1.1
	1	24.7	4.4	38.3	31.2	0.3	0.4	0.5	0.2
	2	3.5	1.5	37.2	57.4	0.1	0.2	—	—
	3	11.8	1.9	50.6	20.3	4.0	1.9	6.5	2.9
Florunner	TG	11.4	2.1	50.9	29.1	1.6	1.1	2.4	1.3
	1	20.7	3.5	49.5	24.4	0.3	0.7	0.5	0.5
	2	2.1	0.6	47.8	48.8	0.1	0.4	0.1	0.1
	3	11.4	2.3	55.5	14.1	4.4	2.3	6.5	3.4
Tifrun	TG	12.6	2.4	42.4	36.8	1.5	0.9	2.6	0.8
	1	22.7	4.2	38.8	32.7	0.3	0.4	0.6	0.2
	2	3.2	1.2	36.1	58.8	0.2	0.2	0.1	—
	3	11.9	1.7	52.2	18.9	4.0	2.0	7.2	2.2
Starr	TG	14.2	3.3	43.3	33.0	1.8	1.1	2.7	0.7
	1	24.2	4.9	40.4	28.4	0.4	0.6	0.7	0.3
	2	2.4	0.8	39.5	56.9	0.1	0.2	0.1	—
	3	16.0	4.2	50.0	13.8	4.8	2.4	7.3	1.8
Spancross	TG	13.5	2.9	44.1	33.4	1.9	1.1	2.2	0.9
	1	24.2	5.2	40.3	28.4	0.3	0.5	0.6	0.4
	2	2.6	0.9	40.2	56.2	0.1	—	—	—
	3	13.5	2.8	51.8	15.6	5.2	2.5	6.5	2.3

[a]Each value is the mean of 3 replications.

were blended in 100 ml petroleum ether (B.P. 35-60 C). The homogenates were filtered and the solvent was removed on a rotary evaporator. Triacylglycerols were separated by thin layer chromatography (TLC) on Silica Gel G (.05 mm)(Brinkmann) with a developing solvent of petroleum ether/diethyl ether/acetic acid (80:20:1). Careful attention was given to prevention of autoxidation. All thin layer plates were developed in a nitrogen atmosphere and sprayed with 0.02% butylated hydroxytoluene (BHT) in petroleum ether. BHT (ca. 10 μl of 0.1% solution) was added in each step of the isolation procedure and stereospecific analysis.

Methyl esters were prepared with boron trifluoride-methanol (14% w/v, Applied Science Laboratories, Inc.) according to a modified Morrison and Smith (16) procedure. Benzene was replaced with toluene in the methylation mixture of methyl alcohol/benzene/boron trifluoride-methanol (11:4:5). Lipids were transmethylated in 9 ml vials sealed with teflon cap liner and tape. The vials were heated in an oven at 100 C for 20-30 min, depending on the lipid type. Water (1 ml) was added to the cooled mixture, and the methyl esters were extracted with two 3 ml portions of hexane; then they were analyzed by GLC. The gas chromatograph, equipped with a FID and a 6.35 mm x 1.83 m stainless steel column that was packed with 10% EGSS-X on 100/120 Gas-Chrom P (Applied Science Laboratories, Inc.), was operated isothermally at 210 C. Carrier gas was helium at 100 ml/min. Fatty acid percentages were determined by digital integration and normalization of peak areas. Accuracy of the system was verified by analysis of National Heart Institute-type fatty acid standard KD. Fatty acids were identified by comparison with known standards.

Stereospecific analysis was conducted essentially according to Brockerhoff (17) as modified by Weber et al. (7). The method for preparing of phosphatidyl phenols was modified such that the diacylglycerols in no more than 0.5 ml diethyl ether were added slowly with shaking to 2 ml pyridine (spectrophotometric grade, Aldrich Chemical Company) and 0.12 ml phenyl dichlorophosphate (Aldrich Chemical Company) to prevent precipitate formation.

RESULTS AND DISCUSSION

Two determinations provide an indication of the accuracy of stereospecific analysis (4,7).

TABLE III

Linear Regression Analyses and Correlation Coefficients for the Relationship of Fatty Acids in Total Triacylglycerols and Fatty Acids at Each Position

Fatty acid	Position	Slope	y Intercept	r[a]
16:0	1	1.43	4.68	0.95[b]
	2	—	—	0.43[d]
	3	1.32	-4.23	0.86[c]
18:0	1	1.28	1.03	0.88[c]
	2	—	—	0.09[d]
	3	1.82	-2.23	0.83[c]
18:1	1	1.16	-10.33	0.99[b]
	2	1.28	-16.87	0.99[b]
	3	0.56	26.84	0.95[b]
18:2	1	1.01	-5.07	0.99[b]
	2	1.38	8.83	0.95[b]
	3	0.60	-3.75	0.82[c]
20:0	3	2.78	-0.15	0.99[b]
20:1	3	2.31	-0.16	0.91[c]
22:0	3	2.39	0.88	0.96[b]
24:0	3	2.65	-0.01	0.99[b]

[a]r = Correlation coefficient.
[b]Significant at 1% level.
[c]Significant at 5% level.
[d]Not significant.

The mixed 1,2(2,3)-diacylglycerols used to make the phosphatidyl phenols must be representative of the triacylglycerols; therefore, they must agree in composition to that calculated for diacylglycerols. Differences in calculated and analyzed diacylglycerols of the six varieties were less than 2% for any fatty acid. Data obtained by the two methods of determining the fatty acid composition of the *sn*-3 position should always be compared. Analyses with minor differences (<5%) for major components are generally considered acceptable (7) although agreement should be closer. The mean differences between the *sn*-3 fatty acid percentages calculated by two methods for each fatty acid of all varieties are presented in Table I. Composition calculated by subtracting the *sn*-1 and *sn*-2 position fatty acid percentage from the percentage composition of the whole triacylglycerol is regarded as more accurate; however, agreement of the two methods indicates overall accuracy.

The results shown in Table II indicate a nonrandom distribution of fatty acids among the *sn*-1, -2 and -3 positions of the triacylglycerols. The percentages of palmitic and stearic acids were generally very low for the *sn*-2 position, higher for *sn*-3, and highest for *sn*-1. The long chain (20-24) fatty acids were located almost exclusively at the *sn*-3 position. The *sn*-2 position of triacylglycerols from all the varieties was high in unsaturated fatty acids. The general patterns of fatty acids found at the *sn*-1 and *sn*-3 positions were similar for all varieties, although the mole percentages of each acid at the two positions frequently differed widely. Mole percentages of palmitic, stearic and linoleic acids were always higher for the *sn*-1 than for the *sn*-3 position, while those of oleic acid were consistently higher for the *sn*-3 position. The patterns of fatty acid distribution at *sn*-2 differed not only from those at *sn*-1 and -3, but with variety as well. On the *sn*-2 position, the percentage of oleic acid was higher than that of linoleic acid in Florigiant, but the percentages were about the same in Florunner. In the other four varieties, there was more linoleic acid esterified at the *sn*-2 position than oleic acid. Florigiant and Florunner triacylglycerols contained more oleic acid and less linoleic acid than the other varieties examined; and the concentration effect, as reported by de la Roche et al. (8), probably was reflected by the fatty acid placement in the molecule.

The stereospecific analyses previously reported (6,14) are similar to the assay of Florigiant triacylglycerol (Table II), which showed more oleic acid than linoleic acid at the *sn*-2 position. The general fatty acid patterns at *sn*-1 and *sn*-3 positions were similar in all analyses. The stereospecific analyses of two commercially available peanut oils (data not presented) were very similar to the analysis of Florigiant triacylglycerols shown here.

Linear regression equations and correlation coefficients were calculated for the plots of the percentage of a fatty acid in the total triacylglycerol vs. the percentage of that fatty acid at one of the positions of the triacylglycerol (Table III). Significant correlations indicate that the total fatty acid present influenced placement of that fatty acid on the triacylglycerol (8). de la Roche et al. (8) found that major saturated, monoene and diene fatty acids of corn triacylglycerols exhibited a concentration effect in all cases except for saturated acids in the *sn*-2 position. Peanut triacylglycerols exhibited this same pattern, and the low concentrations of the long chain fatty acids in the triacylglycerol were significantly correlated with percentages found at the *sn*-3 position only. This may be due to the general restriction of the saturated acids (16:0 and 18:0) from the *sn*-2 position and the long chain acids from the *sn*-1 and *sn*-2 positions. Fatemi and Hammond (9) attribute any substantial deviation from the regression line to a change in the mechanism of fatty acid distribution and suggest genetic control of the deviation. No substantial deviation of any fatty acid from the regression lines was detected in the six peanut varieties examined. Although concentration effects were similar for the varieties, the variation in percentage of a fatty acid at any position is sufficient to indicate possible concentration differences in the various triacylglycerol species found in the total triacylglycerol fraction.

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Epoxides as Products of Lipid Autoxidation in Rat Lungs

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ABSTRACT

The nature and content of lipid epoxides in rat lung were examined in air-breathing control rats and those exposed to nitrogen dioxide. Exposure to 6.5 ppm NO_2 for 24 hr resulted in significantly greater epoxide content in a number of lipid classes. It is proposed that lipid autoxidation in lung tissues may contribute to the levels of epoxide-containing lipids. Furthermore, the processes involved in epoxide formation may be predicted from autoxidation studies utilizing a system of unsaturated fatty acid monolayers on silica gel which serves as a model for biomembranes. The findings indicate that exposure to oxidizing gases can lead to an accumulation of lipid epoxides in both lung parenchymal tissue and on the alveolar surface.

INTRODUCTION

Lipid autoxidation in biological systems is often associated with processes leading to cell or tissue damage. These processes are particularly apparent following exposure to oxidants and free radical-generating systems. Numerous studies have been concerned with the effects of inhaled oxidants on lungs, the effects on lipids in pulmonary membranes and the effects on lipid metabolism. Studies of this nature have often been hampered by the enormous complexity of cells and natural membranes such that an understanding of the basic mechanisms involved in membrane lipid autoxidation is far from complete. A common problem encountered is the difficulty in measuring the extent of lipid peroxidation in tissue, and the identification and measurement of products.

An approach used by a number of investigators has been the devising of model systems which attempt to simplify features present in natural membranes while maintaining as much of their basic nature as possible. A number of recent studies involving autoxidation of membrane lipids have used adsorbed unsaturated fatty acid monolayers (1-3), a system particularly useful in observing the effect of a single parameter in a particular process. This model possesses a number of features which are analogous to one-half of a biomembrane lipid bilayer. The feature most similar to natural membranes is shown in the ordered arrangement of the fatty acids when a monolayer consisting only of fatty acid at a ratio of ca. 200 mg/g of silica gel is prepared. The result is a system which is pictured as having fatty acid chains extending at roughly right angles to the silica gel surface and arranged at an interchain distance of ca. 5 angstroms (1). The silica gel in this system would represent the polar regions of the membrane.

Figure 1 shows the two major products of linoleic acid autoxidation formed in such a monolayer. These products were identified as 12,13-epoxy-9-octadecenoic acid and 9,10-epoxy-12-octadecenoic acid in equal amounts. The samples on the left side of the chromatogram were formed from bulk phase autoxidation of linoleic acid. Important features that distinguish these two samples are the absence of epoxides in the bulk phase autoxidation process and the very small amounts of linoleic acid hydroperoxide in the monolayer samples (indicated by the arrow) (2).

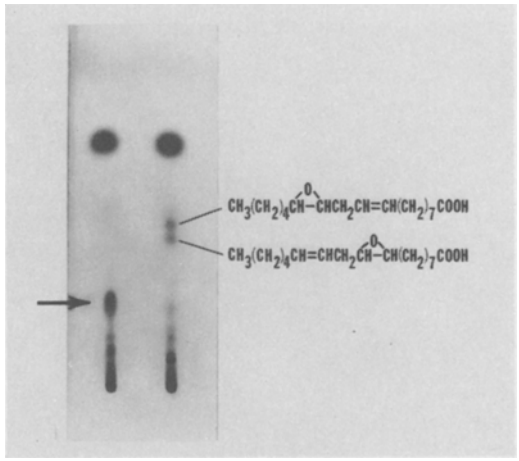

FIG. 1. Thin layer chromatogram of the products of bulk phase autoxidation of linoleic acid (left) and monolayer autoxidation of linoleic acid (right). Indicated are the isomeric epoxides of linoleic acid, being 12,13-epoxy-9-octadecenoic acid and 9,10-epoxy-12-octadecenoic acid. The spot corresponding to linoleic acid hydroperoxide is indicated by an arrow.

The diagram in Figure 2 is a representation of how the formation of epoxides was rationalized. It appears that the arrangement of the fatty acids in the monolayers favors addition of the originally formed peroxy radical to a neighboring unsaturated fatty acid over abstraction of an allylic hydrogen. The transient addition product formed could then decompose, probably to an alkoxy radical and the epoxide. Such a process apparently occurs with other olefins in the presence of autoxidizing linoleic acid. We have found that monolayers consisting of a mixture of linoleic and oleic acids or linoleic acid and cholesterol (unpublished observation) form the epoxides of these lipids as major isolable products (4).

It was also observed that with monolayers of linoleic acid, in particular preparations with incomplete coverage of the silica gel, a major product was a mixture of isomeric hydroxy-epoxy-octadecenoic acids, the yield of which increased as the ratio of linoleic acid to silica gel was reduced (4). Such products appear to arise from the hydroperoxide.

A study of lipid oxidation in the rat lung was undertaken as an extension of the studies using the monolayer system, and isolation of lipid epoxides similar to those found in the monolayer was initially sought. Analysis of lung tissue and, in particular, alveolar lavage, which primarily recovers surface-active material lining the alveoli, seemed appropriate for the following reasons:

1) Surface active material and the tissues of the terminal respiratory units come in direct contact with inspired oxidants. The lipids of surfactant, because of their location, could constitute an in vivo model for the oxidation of noncellular lipids and for the formation of products.

2) Surfactant lipids of the lung act as a monolayer spread over an extracellular lining which is derived from the alveolar epithelium consisting largely of Type I and Type II pneumocytes. The lipids produced in the epithelial cells, and then released from them, enter into a reserve pool in this extracellular fluid. This reserve of lipids may be specificially adsorbed at the air-water interface, forming a surface film during the respiratory cycle. Lipids reaching the surface spread as a monolayer, with their polar regions immersed in the aqueous phase phase and their hydrophobic regions extending out into the airspaces. The surfactant lipids are made up of a discrete mixture of phospholipids (particularly phosphatidylcholine), fatty acyl glycerols, cholesterol and other minor lipids. The conceptual arrangement of these lipids resembles that of the silica gel monolayer and provides an interesting in vivo counterpart which might permit similar autoxidative processes.

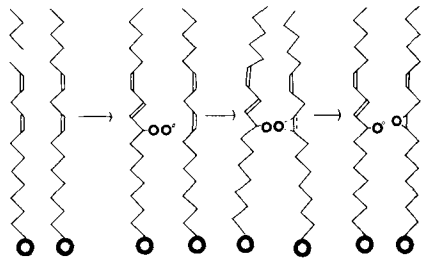

FIG. 2. A scheme by which formation of epoxy octadecenoic acid may occur using linoleic acid monolayers. From left to right are shown: 1) neighboring linoleic acids; 2) formation of a peroxy radical as an initial autoxidative step; 3) an intermediate addition product formed between the peroxy radical and neighboring linoleic acid; 4) decomposition to an alkoxy radical and epoxy octadecenoic acid.

METHODS

Exposure Protocol and Preparation of Tissues

Male Sprague-Dawley rats, littermates raised in a specific pathogen-free colony (Hilltop Labs) and weighing 135-155 g, were used in all experiments.

Exposure to nitrogen dioxide gas was carried out in stainless steel chambers identical to those used by Hinners et al. (5). Rats were exposed to 6.5 ± 0.5 ppm NO_2 for 24 or 48 hr intervals. A total of 10 control rats were allowed to breathe filtered air and were paired with 10 NO_2-exposed rats for each exposure time interval studied. All rats were maintained on Purina rat chow and water during the exposure interval. Following the exposure to gases, the rats were sacrificed using sodium pentobarbital, I.P., and the lungs excised intact along with the heart and major airways.

Alveolar washings were obtained by first cannulating the trachea and immersing the lungs in a saline bath at 37 C. The airways were then slowly washed with Krebs-Ringer buffer containing 1% bovine serum albumin (lipid-free).

The washings were centrifuged at 800 x g for 10 min, the supernatant removed, lyophilized and stored at -70 C until analysis. The lung tissue was trimmed free of nonpulmonary tissues, vessels and airways, and lyophilized and stored along with the lavage samples.

Analysis of Tissue and Lavage Lipids

Lipid extraction was accomplished using a modified Bligh and Dyer procedure (6) fol-

TABLE I

Major Fatty Acids and Fatty Acid Epoxide Composition of Triglycerides from Lung Tissue of Rats

		% Of total fatty acid content				
	MG/lung	16:0	16:1	18:1	18:2	20:4
Control	5.57 ± 0.651	25 ± 2.9	6 ± 0.6	27 ± 3.0	25 ± 2.5	1.4 ± 0.2
NO_2	4.95 ± 0.227	28 ± 1.8	7 ± 0.6	26 ± 2.1	20 ± 4.3	2.2 ± 1.0

		% Of total fatty acid epoxides		
	μg/lung	16 Carbon	18 Carbon	20 Carbon
Control	71.7 ± 1.26	12 ± 1.1	85 ± 5.4	3 ± 0.2
NO_2	72.4 ± 4.00	23 ± 2.6[a]	71 ± 8.0	6 ± 0.8[a]

[a]Significant, $P<0.05$.

lowed by ether-chloroform solvent extraction to facilitate recovery of cholesterol. Samples of lung tissue from 6 control and 6 NO_2-exposed rats were extracted in their dry state with 10 volumes of 2:1 chloroform/methanol (v/v) per gram of tissue or 15 ml lyophilized lavage solution, using a polytron homogenizer. To the homogenate was added ca. 80,000 dpm of ^{14}C-cholesterol epoxide, synthesized from 4-^{14}C cholesterol as described by Chakravorty and Levin (7). This provided a basis for estimating procedural losses. The extracts were recovered following centrifugation at 500 x g for 10 min and filtered through Whatman No. 42 paper. The tissue residue was re-extracted with 3:1 ether/chloroform and the extract passed through the same filter.

Total lipid conjugated diene content was measured on an aliquot of the washed lipids using a Cary Model 14 scanning spectrophotometer (E_{max} at 233 nm in ethanol = 3.2 10^4).

Lung tissue and lavage neutral lipids and phospholipids were fractionated by means of silicic acid column chromography (Biorad-325 mesh). Neutral lipids were eluted with 10 void volumes of chloroform (or until all cholesterol epoxide radioactivity was recovered). Phospholipids were then recovered with 5 volumes of methanol. Phospholipid content was measured by the Bartlet method (8).

The neutral lipids were fractionated by thin layer chromatography (TLC on silica gel 60 plates in a solvent system consisting of petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v), TLC System I. The areas corresponding to cholesteryl esters, triglycerides, free fatty acids, mono and diglycerides and steroids were recovered. The steroid fraction was further developed by thin layer chromatography using benzene/ethyl acetate (3:2, v/v), TLC System II, which separated cholesterol rf = 0.60 and cholesterol epoxide rf = 0.47. Preparation of fatty acid methyl esters derived from phospholipids, monoacyl-, diacyl- and triacylglycerols involved the hydrolysis of these lipids in a two-phase system consisting of 20% KOH in methanol and diethyl ether. Hydrolysis was continued from 4-6 hr with gentle stirring at room temperature (22 C) using a magnetic mixer. The mixture was neutralized with 20% acetic acid and extracted twice with water. The ether phase was freed from solvent under nitrogen and treated with ethereal diazomethane containing 2% methanol to yield the fatty acid methyl esters. This method proved to be effective in quantitatively recovering total and epoxidized fatty acids.

Fatty acid methyl ester composition for each lipid class was determined on either a Beckman model GC-M or Hewlett-Packard model 5830-A gas chromatograph equipped with a 6 foot silar 10 C column. Epoxide fatty acid methyl esters were isolated from the total fatty acid methyl esters by thin layer chromatography in TLC System I. Methyl 9,10-epoxystearate was used as a standard, and the recovered epoxide fatty acids were analyzed and their content estimated after derivatization (2) by means of gas chromatography mass spectrometry (TC-MS) using a Finnigan quadrapole mass spectrometer coupled with a Varian model 2100 gas chromatograph equipped with a 5 ft 3% OV-1 stainless steel column.

The isomeric forms of cholesterol epoxide were determined and their proportions measured following reduction using $LiAlH_4$ in ether (9) or by reaction with BF_3-methanol under nitrogen at 22 C. The products of each reaction were isolated and quantitated using either thin layer chromatography with TLC Solvent System II followed by densitometric measurement of the charred plates using a Kontes K-495000 Densitrometer, or by gas

TABLE II

Fatty Acids and Fatty Acid Epoxide Composition of Triglycerides from Lung Lavage of Rats

		% Of total fatty acid content				
	μg/lung	16:0	16:1	18:1	18:2	20:4
Control	32.2 ± 2.47	37 ± 2.8	10 ± 0.7	16 ± 1.0	7 ± 0.5	1.2 ± 0.08
NO_2	26.6 ± 0.64	44 ± 1.0	3 ± 0.6[a]	12 ± 0.4[a]	3 ± 0.7[a]	1.3 ± 0.04

		% Of total fatty acid epoxides		
	μg/lung	16 Carbon	18 Carbon	20 Carbon
Control	0.13 ± 0.028	b	54 ± 5.9	46 ± 5.0
NO_2	0.56 ± 0.028	b	86 ± 4.3[a]	14 ± 1.0[a]

[a]Significant, $P<0.05$.

[b]Not measured.

chromatography on a 3% OV101 column at 265 C.

Analysis of Cholesterol Epoxide Hydrase Activity in Lung Homogenates

Approximately 5 g of lung tissue from 3 control or 3 NO_2-exposed rats were homogenized in 40 ml of 0.25 M sucrose for 30 sec with a polytron homogenizer. The homogenate was then centrifuged at 500 x g for 10 min and the supernatant recovered and centrifuged at 18,000 x g for 15 min. The supernatant fraction was used in the enzyme analysis following the methods of Aringer and Eneroth (10). The protein concentrations for the control and NO_2-exposed preparations were equalized and routinely adjusted to ca. 0.1 mg/ml buffer 0.1 M phosphate pH 7.4, using the Lowry method (11). ^{14}C-Cholestan-5α,6α-epoxy-3β-ol (spec. act. 3.18 μCi/μmole) was added in 25 μl acetone to 8 ml of the incubation medium while mixing vigorously with an omnimixer. This preparation was kept under nitrogen at 37 C during the mixing and for a 3 min pre-incubation interval following mixing.

Flasks were filled with 95% O_2 + 5% CO_2 and incubated for 30 and 60 min intervals at 37 C. Blank determinations were made with an enzyme preparation which was boiled for 15 min.

Following incubation, the flasks were chilled on ice and extracted as described in Section II of the Methods. Cholesterol epoxide and cholestane triol were isolated using TLC System II and the silica gel scrapings counted in a Beckman LS 8100 scintillation counter. Hydration of cholesterol epoxide was determined by measurement of cholestane triol radioactivity, and calculations were based on the assumption that the specific activities for the product and substrate were the same. Total losses of the epoxide were determined by subtraction of the labeled substrate recovered from the amount of radioactivity originally present. All measurements were performed in duplicate.

Studies on the Formation of Cholesterol Epoxides from Cholesterol Using Lung Homogenate Preparations

Lung tissue obtained from control rats and rats exposed to NO_2 for 24 hr was prepared as described above. These experiments were performed identically to those described for cholesterol epoxide hydrase except that 4-^{14}C-cholesterol (spec. act. = 10.5 μCi/μmole, 80,000 cpm/sample) was used as a substrate, and the pH of the phosphate buffer was adjusted to 7.4. The incubation medium also contained an NADPH regenerating system as described by Aringer and Eneroth (10), and 100 nmoles of cyclohexene oxide. The products were isolated using TLC system II, and the amounts of labeled cholesterol converted to cholesterol epoxide measured as described above.

The data from all analyses are presented as means plus or minus one standard deviation derived from three separate experiments for the 24 hr NO_2 exposure interval and one experiment at 48 hr. Statistical computations were made using the Student's t-test and linear regression analysis at the 95% confidence level.

RESULTS

The amounts of lipid epoxides recovered from rats exposed to NO_2 for 24 hr and from control rats breathing filtered air are presented in Tables I-V. Since triglycerides, cholesterol and phospholipids represented the only lipid fractions containing appreciable amounts of epoxides, only the data for these lipids are considered.

TABLE III

Major Fatty Acids and Fatty Acid Epoxide Composition of Phospholipids from Lung Tissue of Rats

		% Of total fatty acid content					
	mg/lung	16:0	16:1	18:1	18:2	20:4	22:6
Control	15.2 ± 1.09	27 ± 1.9	6 ± 0.4	13 ± 0.8	8 ± 0.6	16 ± 1.3	3 ± 0.3
NO_2	17.2 ± 0.24	27 ± 0.4	7 ± 0.5	14 ± 0.5	9 ± 0.5	15 ± 0.9	3 ± 0.3

		% Of total fatty acid epoxides		
	μg/lung	16 Carbon	18 Carbon	20 Carbon
Control	1.86 ± 0.125	25 ± 1.5	49 ± 3.2	27 ± 2.1
NO_2	6.27 ± 0.761	22 ± 3.0	41 ± 4.9	37 ± 4.1[a]

[a]Significant, $P<0.05$.

Table I shows the composition and total amounts of fatty acids and fatty acid epoxides obtained from triglycerides of lung tissue. No significant differences were found between the controls and NO_2-exposed groups for the content of total triglycerides, the proportions of fatty acids, or the amounts of fatty acid epoxides. There were only minor variations in the proportions of epoxides from the 16-, 18-, and 20-carbon epoxide species. The 18-carbon unsaturated fatty acids make up ca. 50% of the total unsaturated fatty acids in these triglycerides and also constitute the major component of the epoxy fatty acids isolated.

As shown in Table II, the situation for triglycerides from lung lavage is quite different. In this case, the NO_2-exposed group had less recoverable triglyceride and decreased amounts of the 16- and 18-carbon unsaturated fatty acids. Accompanying these changes was a significant increase in epoxy-fatty acid content, made up primarily of the 18-carbon species. In these triglycerides, the content of epoxide fatty acids in the NO_2-exposed group represented over 2% of the total fatty acids.

The 18-carbon epoxides consisted of a mixture of 9,10-epoxystearic acid, and 12,13-epoxy-9- and 9,10-epoxy-12-octadecenoic acids in approximately equal amounts. These epoxides were derived presumably from oleic and linoleic acids. The 16-carbon epoxides appeared to be formed from palmitoleic acid, and the 20-carbon epoxides appeared to be a mixture of positional isomers of arachidonic acid. The increases found for epoxides in all the lipids analyzed were not accompanied by increases in the content of conjugated dienes.

Table III presents the results for the composition and amounts of the fatty acids from phospholipids of lung tissue. There are no significant differences between the two groups in the content of phospholipids, nor are there any differences in the proportions of the unsaturated fatty acids. The 18-carbon fatty acids make up ca. 25% of all the fatty acids, and in this case, there is substantially more arachidonic acid than was found in triglycerides. The proportions of unsaturated fatty acids present in a given lipid type appear to influence the proportions of the fatty acid epoxides that are formed. Thus, in the triglycerides presented in Table II and in the phospholipids presented in Table III, the unsaturated fatty acids in the largest proportions are those which seem to be converted to epoxides in the greatest amounts.

The results for the phospholipids from lung lavage, shown in Table IV, are unlike those for triglycerides recovered from lavage (Table II). Specifically, there are no differences in either the amounts of phospholipids recovered from control and NO_2-exposed rats, nor are there any differences in the proportions of unsaturated fatty acids and epoxide fatty acids in these phospholipids. Instead, these data are similar to the findings for triglycerides recovered from lung tissue.

The content of cholesterol in lung tissue and in the lavaged material, as well as the amounts of cholesterol epoxide recovered from these two fractions, are shown in Table V. Significant increases were found in the amounts of cholesterol epoxide in both lung tissue and lavage after NO_2 exposure. The differences between the control and NO_2-exposed group are also significant when calculated as a percent of the total cholesterol content (data not shown).

The proportions of the 5α- and 5β-isomers of cholesterol epoxide were determined using two methods. Reduction using $LiAlH_4$ (9) to cholestane diols resulted in a single product obtained from the α-epoxide and identified as 5α-hydroxycholesterol. The β-epoxide was converted primarily to 6β-hydroxycholesterol

TABLE IV

Fatty Acids and Fatty Acid Epoxide Content of Phospholipids from Lung Lavage of Rats

		% Of total fatty acid content				
	mg/lung	16:0	16:1	18:1	18:2	20:4
Control	0.67 ± 0.156	47 ± 5.3	18 ± 2.1	9 ± 1.0	9 ± 1.2	3 ± 0.4
NO_2	0.69 ± 0.170	46 ± 7.1	21 ± 2.6	10 ± 1.2	9 ± 1.2	3 ± 0.3

		% Of total fatty acid epoxides		
	μg/lung	16 Carbon	18 Carbon	20 Carbon
Control	1.06± 0.177	a	59 ± 5.0	41 ± 3.5
NO_2	0.89 ± 0.189	a	47 ± 5.0[b]	54 ± 5.8[b]

[a]Not measured.

[b]Significant, $P<0.05$.

along with smaller quantities of 6α- and 5β-hydroxycholesterol. The extent of reduction was variable but never complete following treatment of standard samples at various concentrations. Furthermore, reduction of α-epoxide was considerably more efficient such that mixtures of the isomers invariably yielded the 5α-hydroxycholesterol as the major product. Analysis of cholesterol epoxide isolated from lungs revealed a ratio for the α and β isomers of ca. 8:1.

BF_3 methanolysis of cholesterol epoxide appeared to be a more desirable technique. Reactions were compelete by 30 min, yielding different and discrete products for each isomer. An example, shown in Figure 3, demonstrates resolution of the derivatized isomers on a thin layer chromatogram (TLC System II). Gas chromatographic analysis of lung samples indicated approximately equal proportions of the α- and β-isomers.

A comparison of the amounts of phospholipid, triglyceride and cholesterol epoxides following 24 and 48 hr of exposure to nitrogen dioxide is presented in Figures 4a and 4b. The data from lung tissue (Fig. 4a) indicate no significant changes after an additional 24 hr of exposure. The same trend is seen for the epoxides isolated from lung lavage (Fig. 4b).

Results for the analysis of cholesterol epoxide hydrase activity in lung tissue preparations are shown in Fig. 5a. Utilization of the 18,000 x g supernatant fraction of lung homogenate was selected, since this fraction contains the microsomes, and, as reported by Aringer and Eneroth (10), possesses essentially all the epoxide hydrase activity of the cell and is easily prepared. The activity of the hydrase in these preparations was found to remain essentially linear over the 60 min time course of incubation. Both the control lungs and those exposed to NO_2 for 24 hr displayed the same level of activity over 60 min, and the respective rates were found to be 0.021 ± 0.005 nmoles/mg protein/min for the control prepartions vs. 0.023 ± 0.008 nmoles/mg protein/min for the NO_2-exposed preparations.

The effect on epoxide hydrase activity of a 48 hr exposure of rats to NO_2 was examined, and the results are shown in Figure 5b. Rates were computed as being 0.022 ± 0.003 and 0.025 ± 0.007 nmoles/mg protein/min for the control, and 48 hr NO_2-exposed preparations, respectively. These rates are not significantly different from those measured for the 24 hr NO_2-exposed group. The activity of lung epoxide hydrase was assayed using preparations in which the protein concentrations ranged from 0.1 to 1.5 mg/ml. The activity of the enzyme preparation remained consistent throughout this concentration range.

The major enzyme product isolated was identified as cholestane triol, which we presume was the 3β,5α,6β-triol isomer. This product typically represented 35-50% of the total labeled cholesterol epoxide that was metabolized or otherwise lost.

The extent of enzymatic conversion of cholesterol to cholesterol epoxide by what is presumed to be a mixed-function oxidase

TABLE V

Content of Cholesterol and Cholesterol Epoxide in Rat Lung Tissue and Lung Lavage

	Tissue	Lavage
	mg/lung	μg/lung
Cholesterol		
Control	3.95 ± 0.200	118 ± 11.3
NO_2	4.84 ± 0.187	121 ± 7.1
Cholesterol epoxide		
Control	6.58 ± 0.663	0.033 ± 0.0042
NO_2	10.05 ± 1.230	0.062 ± 0.0021

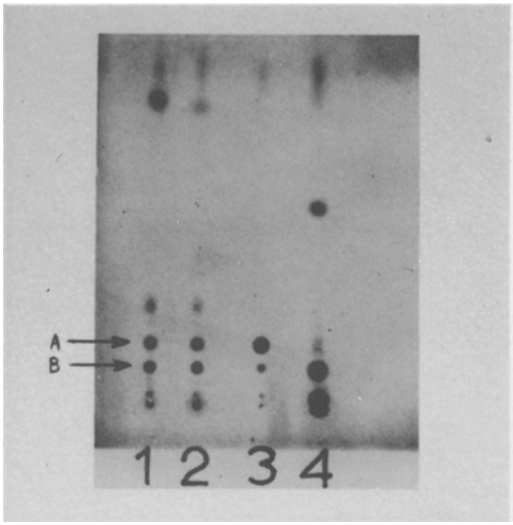

FIG. 3. A thin layer chromatogram of the products formed following BF_3 methanolysis of cholesterol epoxide.

A) Methanolysis product of cholestan-5α,6α-epoxy-3β-ol.

B) Methanolysis product of cholestan-5β,6β-epoxy-3β-ol.

1) Cholesterol epoxides isolated from control rat lung.

2) Cholesterol epoxides isolated from NO_2-exposed rat lung.

3) Cholestan-5α,6α-epoxy-3β-ol standard.

4) Cholestan-5β,6β-epoxy-3β-ol standard.

system was analyzed in the presence of cyclohexene oxide, an inhibitor of epoxide hydrase. Cyclohexene oxide present at concentrations of 10-15 nmoles/ml buffer reduced epoxide hydrase activity by 85-90%. The resulting rates for cholesterol epoxide formation were thus measured as being 0.30 ± 0.07 and 0.23 ± 0.07 pmoles/mg protein/min in control and NO_2-exposed lung preparations, respectively. The amounts of cholesterol epoxide formed from cholesterol represented ca. 1% of the total cholesterol metabolized during the incubation interval (based on loss of labeled cholesterol in the medium).

DISCUSSION

The presence of epoxides derived from natural substances such as lipids has been recognized in animal tissues (12,13). These lipid epoxides are thought to represent products of monooxygenases. The results from this investigation suggest that lipid epoxides may also be formed nonenzymatically and, in the tissues of the lung, may also originate through autoxidative processes. A number of observations indicate that lipid autoxidation in lung tissue may be, at least in part, the origin of lipid epoxides. One indication is the apparent randomness of their formation, i.e., the types of epoxides formed are largely dependent on the proportions of the fatty acids from which they would presumably be derived. The formation of lipid epoxides in rat lungs is markedly increased following exposure to NO_2 gas. Under similar analytical conditions, there were no observed changes in enzymatic cholesterol "epoxidation" nor in epoxide hydrase following NO_2 exposure. Furthermore, the rate of enzymatic cholesterol epoxidation appears to be insufficient to account for the increases that are found in the NO_2-exposed lungs.

Of the various lipids analyzed, only the phospholipids, triglycerides and cholesterol contained measurable levels of epoxides. There were indications of epoxides in cholesteryl esters, both in the cholesterol and fatty acid moieties; however, their presence and amounts were highly variable. Variability may have resulted from the methods of preparation and analysis of these esters, which required much longer periods of hydrolysis in methanolic KOH-ether, and which could thereby have hydrolyzed some epoxide.

Regardless of their source, these lipid epoxides appear to be major isolable products representing lipid peroxidation in tissue and are present in far greater amounts than lipid-conjugated dienes. The detection of lipid epoxides may, therefore, be a more sensitive and representative reflection of lipid peroxidation in animal tissues than the measurement of conjugated diene produced in vivo.

Formation of phospholipids and cholesterol epoxides can be predicted from the results of the monolayer studies. According to this scheme, autoxidation would result in the accumulation of epoxide rather than hydroperoxide. An explanation for the presence of epoxide fatty acids in triglycerides of lung tissue is less obvious. Triglycerides are not considered as membrane lipids but rather serve as storage or metabolic lipids. The data provide no means of distinguishing whether epoxidation occurred directly in triglycerides, or whether these epoxides were derived from another lipid class, e.g., phospholipids, by an acyl transfer process.

The triglycerides and phospholipids obtained from lung lavage samples are uniquely enriched in saturated fatty acids, particularly palmitic acid. Based on this feature, it is intriguing that the epoxide content following NO_2 exposure is greater in triglycerides but not in phospholipids. A possible explanation for this

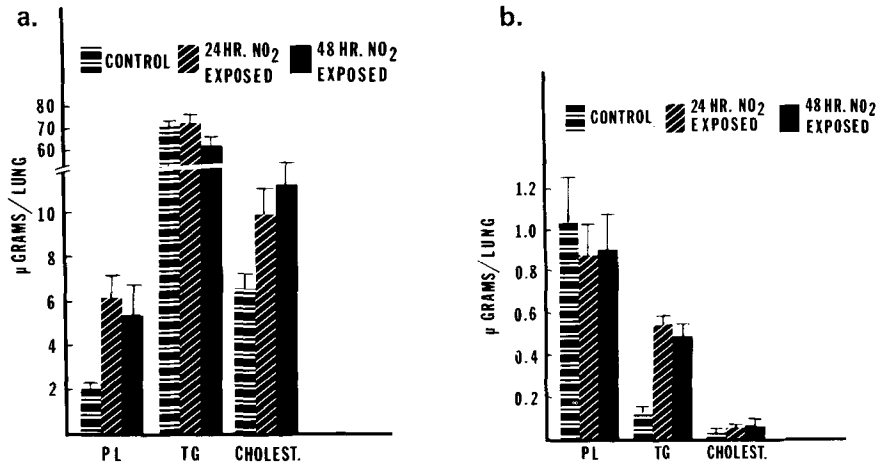

FIG. 4. a. Content of epoxidized fatty acids in phospholipids (PL), triglycerides (TG) and cholesterol epoxide in lung tissue following 24 and 48 hr of exposure to 6.5 ppm NO_2 and a comparison to amounts present in control lungs from rats breathing filtered air. b. Content of epoxidized fatty acids in phospholipids (PL), triglycerides (TG) and cholesterol epoxide in lung lavage following 24 and 48 hr of exposure to 6.5 ppm NO_2 and a comparison to amounts present for control lung lavage from rats breathing filtered air.

phenomenon may be that the arrangement of fatty acids in the triglycerides differs from that in phospholipids. Such differences in the positional distribution of fatty acids may create domains of concentrated and/or adjacent olefinic centers which could facilitate autoxidation and epoxidation on the alveolar surface.

This situation may be further realized since it is estimated that as much as 40% of the surface-active material consists of dipalmitoylphosphatidylcholine (14). This lipid is believed to be largely responisible for reducing the surface forces at the alveolus by forming a continuous monolayer at the air-water interface (14). A monolayer enriched in adjacent saturated fatty acids would not only be expected to reduce the rate of lipid peroxidation, but could also prevent epoxide formation in a manner similar to the situation when silica gel monolayers with incomplete coverage are prepared. A condition such as this can be duplicated by the inclusion of palmitic acid into linoleic acid monolayers, resulting in a reduced rate of peroxidation and decreased amount of epoxide formation (4). It is, therefore, possible that large proportions of saturated fatty acids alter the spatial relationships between lipid components in such a manner as to prevent epoxide formation.

The data in Figures 4a and 4b indicate that increases in lipid epoxides take place largely during the first 24 hr of NO_2 exposure. The subsequent plateau may represent the attainment of a new homeostatic level for lipid epoxide or general lipid turnover. If one were to assume that there is no increase in lipid epoxide hydrase in general, just as there is no observed increase in cholesterol epoxide hydrase activity following oxidant exposure, then an attainment of a plateau may be the result of a limiting diffusion-controlled or steady-state process. A comparison of the data in Figures 4a and 4b suggests that such a homeostasis, if it does exist, is maintained in the turnover between tissue and alveolar lipids as well.

The results for cholesterol epoxide hydrase indicate that increases in the amounts of cholesterol epoxide in tissues may not be effective in activating this enzyme. It must, therefore, be concluded that the activity of epoxide hydrase in itself is not a primary factor in limiting the levels of lipid epoxides and that increases in lipid epoxides may not be controlled in the same manner as that reported for xenobiotic epoxides such as benzo(a)pyrene. Additional studies are required to test these possibilities since prolonged exposures to oxidants can alter lipid metabolism in general and thus preclude any specific autoxidative or enzymatic processes. Furthermore, the preparations used for the assay of epoxide hydrase activity contained both microsomal, soluble and possibly mitochondrial epoxide hydrase. The soluble epoxide hydrase, recently reported by Mumby et al. (15), may be present in this system along with glutathione S-epoxide transferase, also believed to be a soluble enzyme (16). The presence of all these enzymes could contribute to the total elimination of

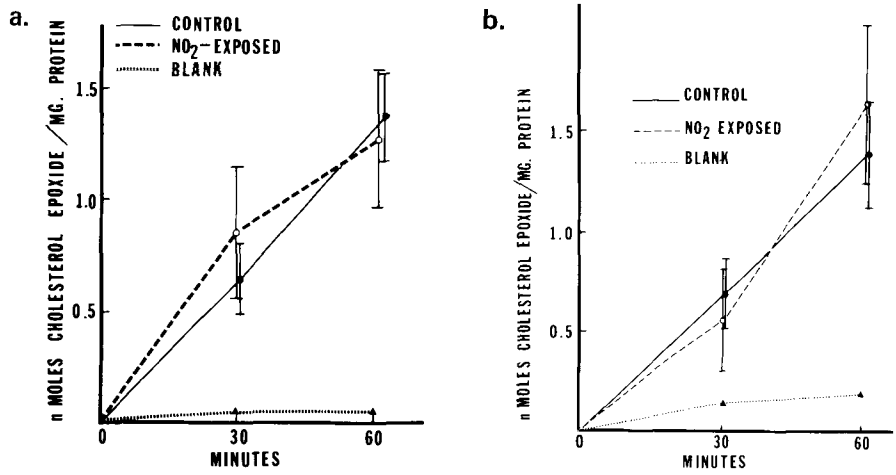

FIG. 5. a. Measurements of cholesterol epoxide hydrase activity in rat lung tissue homogenates following exposure to NO_2 gas at 6.5 ppm for 24 hr. b. Cholesterol epoxide hydrase activity in rat lung tissue following exposure to NO_2 gas at 6.5 ppm for 48 hrs. Values are expressed as mean plus or minus one standard deviation. Blank represents activity of a boiled tissue preparation. (see text for details).

cholesterol epoxide. Grover (17) has reported that glutathione S-epoxide transferase is a particularly active enzyme in rat lung and may therefore be an important enzyme in limiting lipid epoxides. We are currently examining the role of this enzyme in lung lipid epoxide metabolism in relation to oxidant stress.

The occurrence of lipid epoxides in lung tissues and the greater content in the tissues and airspaces of oxidant breathing rats supports the hypothesis derived from the monolayer experiments. The results to date indicate that autoxidative processes within ordered arrangements of lipids, as in biomembranes or in natural monolayer systems, such as pulmonary surfactant, result in the formation of products unlike those formed from lipid autoxidation in a bulk phase.

The conversion of lipids into epoxides, which are electrophilic compounds and capable alkylating agents, creates an undesirable situation for the cell. One of these epoxides (Cholestan-5α,6α-epoxy-3β-ol) is thought to be a carcinogen (12), but the carcinogenic potential for any of the other forms of lipid epoxides found in lung tissue is a possibility that deserves further study. Carcinogenicity or mutagenicity of lipid epoxides would come about through covalent binding with enzymes or macromolecules much like the situation described for polycyclic aromatic hydrocarbons (16,18). Unlike many of the polycyclic aromatic hydrocarbons, however, lipid epoxides may not be effective activators of epoxide hydrase, which would be expected to limit their concentrations or residence time. The formation of carcinogenic or mutagenic compounds in membranes as the result of nonenzymatic peroxidation and the unchecked accumulation of such compounds may be a contributing factor to malignancy or cellular demise.

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Autoxidation of Fatty Acid Monolayers Adsorbed on Silica Gel. IV. Effects of Antioxidants[1]

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ABSTRACT

Inclusion of α- or γ-tocopherol in linoleic acid monolayers deposited on a silica gel surface introduced a definite induction period before the initiation of rapid autoxidation, as measured by the disappearance of linoleic acid. The length of the induction period was found to be proportional to the amounts of tocopherol incorporated at the concentration range tested, and the protection was generally efficient with little loss of linoleic acid to autoxidation. At the onset of the rapid autoxidation, approximately 12% of the tocopherol remained, and most of this was destroyed after an additional short period. γ-Tocohperol, at the same concentration, was found to be 1.4 times as effective as the α-isomer. 3-(ω-Carboxynonyl)-4-methoxy-5-pentylphenol, the synthesis of which is described here, did not introduce a detectable induction period, but rather reduced the overall rate of autoxidation throughout a very long period.

INTRODUCTION

Studies of membrane-related phenomena, including peroxidation, are often hampered by the complexity of the living systems used. To overcome this difficulty, artificial mono- and bilayers are frequently used as admittedly oversimplified models for such systems but aid in rational approaches to their understanding.

Several aspects of membrane autoxidation have recently been studied using lipid monolayers adsorbed on a silica gel surface. The extent of the surface coverage has been correlated to the rate of autoxidation and the combined effects of tocopherol, metals and acid synergists have been examined (3). The effective adsorption sites on the silica gel were shown to be the isolated, nonhydrogen-bonded hydroxyl groups on the surface (4). The autoxidation of linoleic acid monolayers has been found to be entirely different from that of bulk phase both in the rate and the mechanism of the major reaction (5,6). Other important membrane constituents, such as saturated fatty acids and cholesterol, have been incorporated into the linoleic acid monolayers, and their effects on the rates and products of autoxidation have been investigated (7).

The effects of antioxidants on monolayer autoxidation have also been investigated by others (6,8,9). By measuring the disappearance of oxygen in the headspace, Porter et al. showed that autoxidation of silica gel-supported linoleic acid monolayers containing 0.05 mole % of α-tocopherol was preceded by an induction period of ca. 80 min, which could be lengthened by the removal of iron from the silica gel (3).

As part of our continuing effort to clarify various important aspects of membrane autoxidation by using the unsaturated fatty acid-silica gel system, we studied the effect of α- and γ-tocopherol as well as a synthetic antioxidant, 3-(ω-carboxynonyl)-4-methoxy-5-pentylphenol, on the rate of autoxidation of linoleic acid monolayers. The relationship between the length of the induction period and the concentration of the antioxidants was established, and the disappearance of the tocopherols during the induction period was measured. The results and our interpretations are presented in this paper.

EXPERIMENTAL PROCEDURES

Materials and Methods

Linoleic and palmitic acids (99+ % pure), purchased from Applied Science Laboratories, Inc., were used directly after a purity check with thin layer chromatography (TLC) and gas liquid chromatography (GLC). Both d-α-tocopherol (Eastman Organic Chemicals) and dl-γ-tocopherol (a gift from Dr. H.S. Olcott, University of California, Davis) were found to be pure on TLC and gave UV absorption maxima at 292 nm (ε 3,430) and 297 nm (ε 4,570), respectively. Bathophenanthroline purchased from Sigma Chemical Company and analytical grade ferric chloride from Mallinckrodt Chemical Works were used directly. 3-(ω-Carboxynonyl)-4-methoxy-5-pentylphenol was synthesized in this laboratory (see below). The pertinent physical characteristics of the Silica Gel G used have been reported (4).

GLC was carried out using either a Varian

[1]Preliminary reports of some of this material have been published as parts of symposia (1,2).

Aerograph Model 2100 coupled with an electronic integrator (Infotronics Corp., Model CRS-11 HBS) or a Hewlett Packard Gas Chromatograph Model 5830 A. The GLC columns used were an 0.20 x 183 cm glass U-tube containing 3% OV-101 on 100/120 mesh Gas Chrom Q, and an 0.20 x 300 cm metal coiled column packed with 10% Silar 10 C on 100/120 mesh Gas Chrom Q. TLC was carried out using precoated Silica Gel G plates (0.25 mm thick, Analtech, Inc.) with the solvent system – petroleum ether/diethyl ether/acetic acid, 80:20:1 – and precoated alumina sheets (Brinkmann Instruments, Inc). with the solvent system – benzene/diethyl ether, 50:50. Visualization of spots in both cases was carried out by spraying with 3% cupric acetate solution in 8.5% phosphoric acid with subsequent charring at 140 C. Ultraviolet and visible spectra were recorded using 1 cm path quartz cells in a Cary Model 14 Spectrophotometer. Elemental analysis was performed at Elek Microanalytical Laboratories, Torrance, CA.

Preparation and Autoxidation of Tocopherol-Containing Monolayers

A detailed description of our preparation and autoxidation of monolayers has been published (5). In a typical preparation, a solution of 0.522 g (1.86 mmole) of linoleic acid and 5 ml of α-tocopherol stock solution (1.35×10^{-4}M in hexane) in a total of 44 ml of hexane was stirred under nitrogen at room temperature with 2.013 g of Silica Gel G for 1 hr. After the adsorption, the supernatant was withdrawn and freed from solvent to give 0.141 g of linoleic acid and 58.2 μg of α-tocopherol. The amount of α-tocopherol remaining in the supernatant was determined by the bathophenanthroline-ferric chloride color reaction (see below). It was noted that α-tocopherol was adsorbed preferentially from the hexane solution. The ratio of α-tocopherol to linoleic acid in the initial solution before adsorption was 0.036 mole % and the ratio adsorbed was 0.040 mole%. The dry, coated silica, ca. 0.220 g portions, was incubated at 60 C for the desired length of time and then extracted with methanol. The unchanged acid was methylated and quantitated by GLC as previously described. The α-tocopherol in the control and incubated samples was extracted with acetone and assayed by the bathophenanthrolin-ferric chloride reaction.

Monolayers coated with α-tocopherol alone were prepared using the same proportions as in the above run but omitting the linoleic acid.

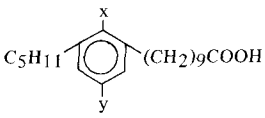
For the preparation of linoleic acid-palmitic acid-α-tocopherol monolayers, the procedure previously described was adapted (7), but α-tocopherol was included in the coating solution to give an adsorbed concentration close to 0.04 mole % of the linoleic acid.

The γ-tocopherol-linoleic acid monolayers were prepared in a manner similar to that for the α-tocophoerol-containing monolayers. The partition coefficient for γ-tocopherol between silica gel and hexane was also slightly higher than for linoleic acid, so that from a solution of 0.018 mole % of γ-tocopherol, the final adsorbed concentration was 0.020 mole %.

Determination of Tocopherols Using Bathophenanthroline

The method used is basically that of Emmerie and Engel except that bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) was used in place of 2,2′-bipyridine to improve the sensitivity (10). The tocopherol-containing solution was usually freed from solvent and was made up to 3 ml with ethanol. To this solution, 0.5 ml of bathophenanthroline (0.09% in ethanol) and 0.5 ml of ferric chloride (0.03% in ethanol) were added. The absorbance of the solution at 534 nm (ε 42,950) was measured with a Cary Model 14 spectrophotometer.

Preparation of 3-(ω-Carboxynonyl)-4-Methoxy-5-Pentylphenol



I x = OH, y = NO_2
II x = OCH_3, y = NO_2
III x = OCH_3, y = NH_3Cl
IV x = OCH_3, y = OH

To a solution of 10 g (26 mmole) 2-(ω-carboxynonyl)-6-pentyl-4-nitrophenol (I) (11) in 50 ml acetone was added 11.0 g (80 mmole) anhydrous potassium carbonate and 9.5 g (75 mmole) dimethyl sulfate dissolved in 25 ml acetone. The reaction vessel was fitted with a reflux condenser protected by a drying tube and a magnetic stirrer. The mixture was refluxed and stirred for 10 hr. At the conclusion of the reaction, the product was poured into 200 ml water and extracted with ether, and the extract washed well with water. The ether was evaporated, and the residue was dissolved in 150 ml methanol and then mixed with 8 g (0.2 mole) sodium hydroxide in 50 ml water. The mixture was warmed until complete solution was achieved and then allowed to stand at room temperature over night. A solution of 50 ml concentrated hydrochloric acid in 200 ml water was added to the alkaline mixutre. The oil which separated was extracted with ether, the extract washed well with water and dried over $MgSO_4$. Removal of the ether left an oil that

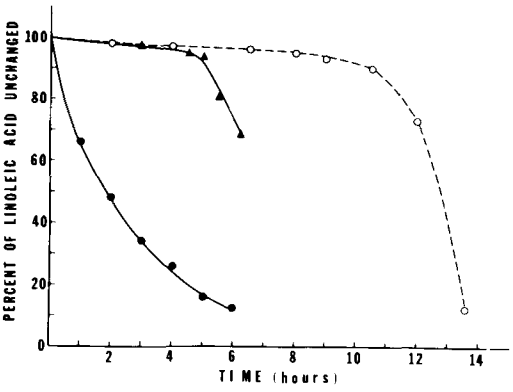


FIG. 1. Effect of α-tocopherol on autoxidation of linoleic acid monolayers at 60 C. —●—●—, Disappearance of linoleic acid in pure linoleic acid monolayers; —▲—▲—, disappearance of linoleic acid in monolayers containing 0.04 mole % of α-tocopherol; – –○–○– –, disappearance of linoleic acid in monolayers containing 0.074 mole % of α-tocopherol.

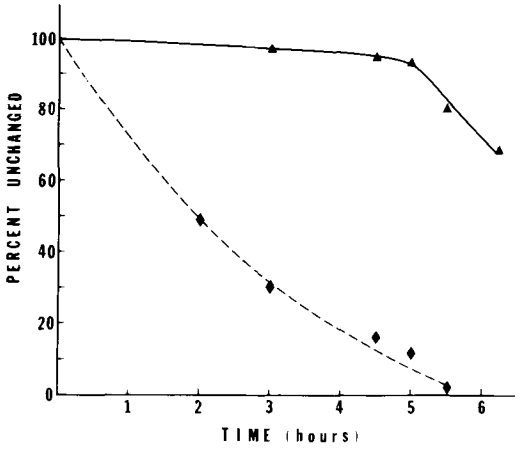


FIG. 2. Rate of autoxidation of linoleic acid and α-tocopherol in linoleic acid monolayers containing 0.04 mole % of α-tocopherol. —▲—▲—, Disappearance of linoleic acid; – –◆–◆– –, disappearance of α-tocopherol.

crystallized slowly after standing at room temperature several days. Yield of crude II was 9.7 g (25 mmole) mp 50-52 C. A suitable solvent was not found for recrystallization.

The nitroanisole derivative, II, was reduced to the aminoanisole hydrochloride, III, by stannous chloride. To a solution of 8.7 g (22 mmole) 11 in 80 ml warm acetic acid was added 15 g (66 mmole) $SnCl_2.2H_2O$ in 15 ml concentrated HCl. The mixture was heated on a steam bath for 1 hr (complete solution was achieved in ca. 10 min) and allowed to cool to room temperature. After addition of 200 ml water and cooling in the refrigerator, the precipitated material was collected in a Buchner funnel, washed with water and dried in vacuo. The dried material was slurried with ether to remove any neutral material and was again collected in a Buchner funnel and dried in vacuo. Yield of III was 8.5 g (21 mmole), mp 128-132 C.

The replacement of the amino by a hydroxyl group was accomplished by diazotizing III and allowing the diazonium compound to decompose in aqueous solution (12). A solution of 6.1 g (15 mmole) III in 50 ml acetic acid was diluted by 50 ml water, cooled in an ice bath and stirred with a magnetic stirrer. A solution of 1.76 g (25 mmole) $NaNO_2$ in 5 ml water was cooled in ice and added over a period of 5 min to the bottom of the solution of III. Stirring was continued for 30 min and 0,60 g (10 mmole) urea crystals were added all at once. The red-orange solution was allowed to warm to room temperature and let stand for 24 hr. The product was extracted with ether (emulsification occurred readily) and was shown by infrared spectrometry to be about ¼ the acetate ester of IV. To hydrolyze the acetyl group, the product was dissolved in 50 ml methanol to which 2 g NaOH in 20 ml water was added. After standing at room temperature overnight, the solution was acidified by addition of dilute HCl, and the product extracted with ether, washed with water and dried ($MgSO_4$). The reddish-brown oil isolated after evaporation of the ether weighed 5.0 g. A preliminary purification was achieved by dissolving the oil in 20 ml ether and adding 80 ml hexane. An insoluble oil separated at the bottom of the flask and the upper layer, which contained IV, was decanted and evaporated. Yield of crude IV was 3.4 g (9.3 mmole). Although the material crystallized in several days at room temperature, a suitable solvent for recrystallization was not found. A pure sample was prepared by chromatography through a solvent-activated silica gel column, the methoxyphenol, IV, being eluted by 30% ether in pentane, but not by 20%, mp 69-70 C, λ_{max} 95% EtOH 283 nm (ε 2600).

Anal. Calcd for $C_{22}H_{36}O_4$:C, 72.49; H, 9.96. Found: C, 72.68; H, 9.72.

Preparation of 3-(ω-Carboxynonyl)-4-Methoxy-5-Pentylphenol)-Containing Linoleic Acid Monolayers

Linoleic acid, 0.530 g (1.89 mmole) was dissolved in 73 ml of methoxyphenol solution (7.88×10^{-5}M in hexane). The solution was poured on 2.006 g of activated silica gel. After stirring for 1 hr, the supernatant was removed. The amount of methoxyphenol left in the supernatant was found to be only a trace

quantity by its absorption at 283 nm (ε 2,600). The concentration adsorbed was calculated to be 0.45 mole % of the linoleic acid. The bathophenanthroline color reaction was not suitable for this quantitation.

Control Experiments

The disappearance of linoleic acid from pure linoleic acid monolayers, which served as the controls for all antioxidant experiments, was determined three times and the results shown in Figures 1, 3 and 4 are means of these runs. In all three runs, in which the amounts of linoleic acid unchanged were expressed as % of the original, the average deviation from the mean values were found to be less than 1%. The rate measurements involving 0.04 mole % of α-tocopherol and 0.02 mole % of γ-tocopherol are means of duplicate experiments. Other rate measurements including the disappearance of α- and γ-tocopherol are results of single experiments. However, for each series of experiments, at least three points on the curve were repeated to assure the reproducibility of a particular experiment. The repeated values were usually found to conform to the variation of ± 1% from mean value as stated above.

RESULTS

As is evident from Figure 1, the autoxidation of linoleic acid monolayers at 60 C, without added α-tocopherol, commences without any detectable induction period (3,5). The rate of disappearance of linoleic acid follows apparent first order kinetics. The incorporation of 0.04 mole % of α-tocopherol introduces an induction period of close to 5 hr. The end of the induction period was chosen as the point at which the tangent to the early slow oxidation curve intercepted the tangent drawn to the succeeding rapid oxidation curve. During the entire tocopherol-dependent induction period, the loss of linoleic acid to autoxidation is small and proportional to the length of the induction period (Fig. 2). For example, with an induction period of 5 hr, the loss of linoleic acid amounted to 6% at the end of the induction period, whereas after a 10 hr induction period, the loss increased to 10%. Once the induction period terminates, rapid autoxidation occurs. When the concentration of α-tocopherol is doubled, the induction period produced is also approximately doubled. γ-Tocopherol at a concentration of 0.04 mole % introduced an induction period ca. 1.4 times that from 0.04 mole % of α-tocopherol (Fig. 3). When the concentration of γ-tocopherol was increased from 0.02 to 0.04 mole %, the length of the induction period was slightly more than doubled.

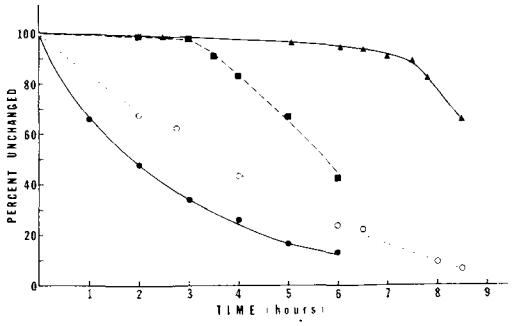


FIG. 3. Rate of autoxidation of linoleic acid and γ-tocopherol in linoleic acid monolayers containing γ-tocopherol. —●—●—, Disappearance of linoleic acid in pure linoleic acid monolayers; – –■–■– –, disappearance of linoleic acid in monolayers containing 0.02 mole % of γ-tocopherol; —▲—▲—, disappearance of linoleic acid in monolayers containing 0.04 mole % of γ-tocopherol; —○—○—, disappearance of γ-tocopherol in monolayers containing 0.04 mole % of γ-tocopherol.

Concomitantly with the determination of the disappearance of linoleic acid, the consumption of α-tocopherol at various stages of the induction period was also measured. Several routine methods for assaying tocopherols were found to be unsuitable in quantitating small quantities of α-tocopherol in a mass of autoxidized products without prior isolation. The ultraviolet absorption of α-tocopherol at 291 nm (ε 3,430) is not intense enough for this purpose; high pressure liquid chromatography gave peaks from autoxidized mixtures which were not resolved from those of α-tocopherol, and gas liquid chromatography requires removal of massive amounts of linoleic acid and its autoxidized products prior to injection.

The Emmerie and Engel reaction using bathophenanthroline (10) was successfully adapted in assaying tocopherols that remained in the equilibrium solution (in hexane) during the preparation of linoleic acid-tocopherol monolayers. The method, however, is not applicable for assaying tocopherols extracted from silica gel with methanol, a solvent used routinely for efficient removal of all organic materials from silica gel. Methanol apparently also extracts some inorganic materials that are capable of reducing ferric ion slowly during the assay. Acetone extraction, although less effective in removing tocopherols from silica gel (90% recovery), was found to give satisfactory results in the bathophenanthroline reaction. To check the possibility of interference by reducing agents, such as aldehyde, produced

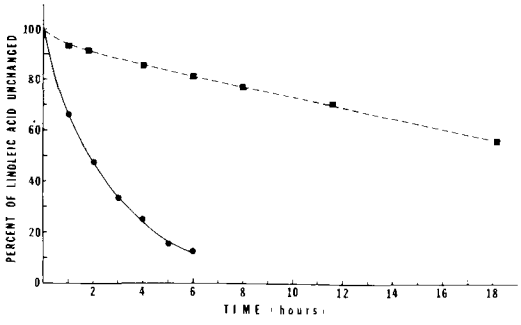


FIG. 4. Effect of 3-(ω-carboxynonyl)-4-methoxy-5-pentylphenol on autoxidation of linoleic acid monolayers at 60 C. —●—●—, Disappearance of linoleic acid in pure linoleic acid monolayers; – –■–■– –, disappearance of linoleic acid in monolayers containing 0.45 mole % of 3-(ω-carboxynonyl)-4-methoxy-5-pentylphenol.

during autoxidation, α-tocopherol in acetone extracts of autoxidized mixture was isolated from the total mixture at several stages of the induction period by preparative TLC using an alumina sheet (13) prior to the bathophenanthroline reaction. The isolation procedure further reduces the recovery of α-tocopherol to only ca. 70%. The loss of α-tocopherol during the induction period, as revealed by the isolation procedure, is similar to that obtained using the total autoxidation mixture for the color reaction. Therefore, it appears that the accumulation of autoxidized products does not significantly affect the assay of tocopherols, at least during the induction period.

As shown in Figure 2 for 0.04 mole % of α-tocopherol and also in Figure 3 for 0.04 mole % of γ-tocopherol, the amount of tocopherol diminished gradually throughout the induction period. In both cases, at the end of the induction period, the amount of tocopherol remaining was ca. 12% of the original quantity. The total disappearance of α-tocopherol came about 0.5 hr after the onset of the rapid autoxidation, whereas for γ-tocopherol at this time, there still is ca. 10% remaining with further decrease apparently occurring very slowly.

For assessing the extent of destruction of α-tocopherol by silica gel surface-generated initiators, α-tocopherol alone was coated on the silica gel, and the rate of disappearance was measured. The destruction of α-tocopherol by this means was found to be very small; for example, at the end of a 4 hr incubation, more than 95% remained unchanged.

In order to examine the effect of saturated fatty acids on the efficiency of α-tocopherol protection, monolayers with equimolar amounts of linoleic and palmitic acids containing 0.04 mole % of α-tocopherol (to linoleic acid) were autoxidized. The protective effect of α-tocopherol apparently is not influenced by the insertion of saturated fatty acid into the monolayers and an induction period similar in length to that for α-tocopherol-linoleic acid monolayers was observed. At the termination of the induction period, the autoxidation commenced with a rate the same as that for an equimolar linoleic acid-palmitic acid mixed monolayer(7).

An efficient bulk phase antioxidant (11) 2-(ω-carboxynonyl)-6-pentyl-*p*-hydroquinone was tested previously in the inhibition of monolayer autoxidation (1). In these preliminary monolayer autoxidation experiments, the hydroquinone derivative did not appear to be an effective antioxidant. We attribute this to the low solubility of the hydroquinone in hexane resulting in inadequate monolayer deposition or to binding of the hydroquinone to the surface of the silica gel in such a manner as to make it inaccessible as a free radical trapping agent. To circumvent these possibilities, the hydroquinone was modified by conversion to a monomethyl ether, IV. The mode of protection offered by this antioxidant is entirely different from that of the tocopherols. It does not introduce a definite induction period but rather reduces the overall rate of autoxidation considerably through a very long period (Fig. 4).

DISCUSSION

A concentration close to 0.05 mole % was chosen initially in studying the inhibition of peroxidation by α-tocopherol in monolayer autoxidation, with the understanding that ca. 2000 molecules of unsaturated fatty acid to 1 molecule of α-tocopherol is a well established ratio in mitochondrial membranes (14). The inclusion of 0.04 mole % of α-tocopherol in unsaturated fatty acid monolayers under our conditions introduces an induction period of nearly 5 hr at 60 C. The protection during the entire induction period is generally very efficient. In bulk phase inhibition, for phenolic antioxidants, it is generally believed that an optimum concentration for maximum length of induction period exists, and increasing the antioxidant beyond this concentration results in very little lengthening of the induction period (15). The length of the induction period produced by α-tocopherol in our case appears to be strictly proportional to the amounts incorporated at the concentration range tested (Fig. 1), although no attempt was made to determine an optimal concentration.

γ-Tocopherol in bulk phase produces an induction period almost twice as long as that of α-tocopherol (15). The protection rendered by γ-tocopherol in monolayers is similarly better with an induction period 1.4 times that of α-tocopherol. The length of the induction period introduced is also found to be roughly proportional to the amount of γ-tocopherol included. For phenolic antioxidants in bulk phase, the decreased antioxidant activity of α-tocopherol, as compared with that of γ-tocopherol, is generally considered as a direct effect of increased steric hindrance of the extra *ortho*-methyl group towards hydrogen atom abstraction by a peroxy radical (16).

The effectiveness of an antioxidant in the linoleic acid-silica gel model system may be determined in part by the oxidation-reduction potential of the antioxidant and in part by the availability of the antioxidant to peroxy radicals. The latter part is a function of the orientation of the adsorbed antioxidant and/or the mobility on the surface. In the case of the difference between the methoxyphenol derivative, IV, and α-tocopherol, it is not clear which feature is contributing the most to cause the observed difference.

The determination of residual α- and γ-tocopherol at various stages of induction period revealed that tocopherol was destroyed gradually throughout the entire induction period (Figs. 2,3). In both cases, antioxidants continued to disappear after the onset of the rapid autoxidation and decreased to a few percent when ca. 20% of the linoleic acid was oxidized. It has been shown that in the bulk phase autoxidation of methyl linoleate, the tocopherol retention (% of original amount added) is ca. 30% immediately prior to the start of the rapid autoxidation phase (17). Our results from monolayers suggest that the mechanism of inhibition operating is an extremely efficient one. With approximately the same initial concentration as in the bulk phase studies, the transition from the induction period to the rapid autoxidation phase is halted until the residual tocopherol drops down to a considerably lower level, ca. 10% of the original amount with both α- and γ-tocopherol. The transition from the induction period to the rapid autoxidation is further characterized by the attainment of a critical ratio of peroxide (produced in the induction period) to residual α-tocopherol, regardless of the initial antioxidant concentration. For methyl linoleate, this ratio is 160 to 180:1 (18,19). If the above mentioned critical ratio of peroxide to antioxidant in bulk phase is also important for the termination of the induction period in monolayer autoxidation, then the main reason for the longer induction period in the monolayers could be the slower accumulation of the hydroperoxides and other products capable of sustaining the free radical chains (5).

Two important questions demand answers at this point. 1) If the course of the reaction is different from that of bulk phase autoxidation, what are the initiators or the propagating radical species tocopherol is scavenging? 2) With a concentration of α-tocopherol as low as 1 molecule to 2000 molecules of unsaturated fatty acid in the adsorbed state, how could the protection be so efficient? The initiation by singlet oxygen was first considered in exploring the possibility of an active entity capable of diffusing freely through the surface to eventually encounter an immobile antioxidant. Another possible reactive oxygen species, superoxide anion radical, has been found to be unreactive towards cholesterol and unsaturated fatty acids (20), although it has been shown that two very reactive species, hydroxyl radicals and singlet oxygen can be generated from it (21). When α-tocopherol alone was coated on the silica gel surface, the extent of its destruction was less than 5% during a 4 hr incubation. This result seems to exclude all other silica surface-generated initiators except singlet oxygen. The possibility of participation of singlet oxygen and other initiators in this system is being investigated in this laboratory (22-25).

The oxidation products of α-tocopherol so far identified in previous linoleic acid monolayer autoxidation studies suggest that there are differences in the reactions from those in bulk phase. In bulk phase, the well established major products are α-tocopheryl quinone, dimer and trimer (26); whereas in monolayers, the products are mostly one to one addition products of oxidized α-tocopherol and linoleic acid and a small quantity of 2-phytyl-3,5,6-trimethyl-1,4-benzoquinone (9). The implication of this finding is not entirely clear, except to indicate that in monolayers the dimerization and trimerization are very much suppressed, and the phenoxy radical formed by the loss of a hydrogen atom from α-tocopherol, therefore reacts through loss of a methyl hydrogen atom to form either a quinone methide, which adds to linoleic acid, or by loss of a chroman ring hydrogen to produce the nonpolar quinone cited above (9).

For an antioxidant to be successful in adsorbed monolayers, mobility across the surface must be assumed for the fatty acid, the antioxidant, or a radical chain-carrying species. Without invoking mobility of at least one component, it seems very unlikely that 1

molecule of α-tocopherol could protect 2000-20,000 molecules of linoleic acid. This is the protection given by 0.04-0.004 mole % α-tocopherol (see Fig. 2).

Surface diffusion of adsorbed molcules in general has been dealt with sparingly in the past. It has been shown that small hydrocarbon molecules, such as benzene, are capable of behaving as a two dimensional gas when adsorbed on alumina (27). The diffusion coefficient of benzene was also reported to be only slightly lower than that for bulk benzene when adsorbed on silica gel (28). The mobility of adsorbed polar molecules, such as fatty acids or esters, apparently has never been studied. The heats of adsorption of these polar molecules are considerably higher than that of benzene (29); therefore, these require higher activation energy for the diffusion. Experiments to test the mobility of adsorbed fatty acid molecules under the conditions employed for the autoxidation are currently underway in this laboratory.

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Studies on Biosynthesis of Waxes by Developing Jojoba Seed. II. The Demonstration of Wax Biosynthesis by Cell-Free Homogenates

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ABSTRACT

The following enzyme activities were demonstrated in cell-free homogenates from developing jojoba cotyledons: 1) elongation of long chain acyl-CoAs in the presence of malonyl-CoA and NADPH (or NADH), 2) NADPH-dependent reduction of long chain acyl-CoAs to the corresponding alcohols, 3) esterification of long chain acyl-CoAs and the alcohols produced from them into wax, 4) elongation of stearoyl-ACP to eicosanoate and docosanoate as well as reduction to stearyl alcohol, 5) desaturation of stearoyl-ACP to oleate in the presence of reduced ferredoxin, and 6) incorporation of malonyl-CoA into long chain fatty acids and alcohols in the presence of added acyl carrier protein. These activities were associated entirely with the floating wax pad after centrifugation of the cell-free homogenate at 12,000 g for 20 min. The relevance of the above reactions (1-6) to wax biosynthesis in vivo is discussed. Production of oleate from acetate by enzymes utilizing ACP-thioesters as substrates followed by conversion of oleyl-ACP to oleoyl-CoA (via free oleic acid) for subsequent elongation, reduction, and esterification, is presented as the most probable in vivo pathway for wax biosynthesis. The substrate specificities of the elongation and reduction reactions utilizing acyl-CoAs as substrates are examined in terms of wax composition.

INTRODUCTION

Studies on the biosynthesis of C_{20} and C_{22} fatty acids, and in particular erucic acid, in plants are few (1-3). The only certain conclusions to be drawn from them are that erucic acid is produced from oleate by two C_2 additions and that malonyl-CoA is the C_2 donor. Our understanding of the biosynthesis of wax esters in plants comes mainly from studies on the unicellular organism *Euglena gracilis*, which uses wax esters as a storage lipid (4,5), and on cuticular wax ester biosynthesis (6). NADH-dependent acyl-CoA reductase activity has been observed in both cases, while several mechanisms for the esterification of acyl groups to fatty alcohols have been suggested.

The seed of the jojoba *Simmodsia chinensis (Link)* appears unique among higher plants in that it contains about half of the dry, mature seed weight as a liquid wax, which is composed principally of C_{20} and C_{22} monoenoic acids and alcohols (7,8). So far our studies on jojoba have shown that ^{14}C-labeled wax was produced from the incubation of slices of immature cotyledons with ^{14}C-acteate, ^{14}C-malonate or ^{14}C-glucose (9). Exogenous [1-^{14}C] acetate was utilized predominantly for the chain elongation of endogenous oleate to give [1-^{14}C]eicosenoate, [1-^{14}C]eicosenol, [1,3-^{14}C] docosenoate and [1,3-^{14}C]docosenol, whereas the label from [U-^{14}C]glucose was uniformly distributed along the chain of these fatty acids and alcohols. These data suggested the existence of metabolically separate pools of acetate and/or sites for de novo synthesis and elongation of acyl chains. It was further suggested that the de novo synthesis of oleic acid occurred on the ACP-track, while subsequent metabolism (i.e., chain elongation, reduction of acyl groups to alcohols, and esterification to produce wax) occurred on the CoA-track (*9-11*).

Investigations using fresh tissue from developing jojoba seeds are restricted to a period of approximately one month during which time the tissue is suitable for biochemical work. The emphasis of this study (June 1978) was to obtain active in vitro preparations in order to define the enzymic reactions responsible for wax biosynthesis.

MATERIALS AND METHODS

^{14}C-Substrates

[1-^{14}C]Decanoyl-, lauroyl-, myristoyl-, palmitoyl- and stearoyl-CoA thioesters (sp. act. 50-58 Ci/mol) and [1,3-^{14}C]malonyl-CoA (sp. act. 58 Ci/mol) were purchased from DHOM Products Ltd. (Hollywood, CA) and [1-^{14}C] oleic acid (sp. act. 56 Ci/mol) from Amersham Searle (Arlington Heights, IL).

Starting from oleic and *cis*-11-eicosenoic acids, respectively, [1-^{14}C] *cis*-11-eicosenoic acid and [1-^{14}C]*cis*-13-docosenoic acid were synthesized by chain extension of the corresponding mesylates with KCN and subsequent chain extension with K^{14}CN (sp. act. 48 Ci/mol, New England Nuclear, Boston, MA)

(12). After methanolysis, the ^{14}C-labeled methyl esters were purified by thin layer chromatography (TLC), and examined by argentation TLC, by gas liquid chromatography (GLC) with radioisotope monitoring by a gas flow proportional counter (referred to as radio-GLC) on a 10% SP-2330 packed column, and by reductive ozonolysis to check double bond position (13). By all criteria, the methyl esters were radiochemically pure (>95%). Chemical purity was ca. 70% as estimated by GLC on an OV-17 column. [1-^{14}C]*cis*-11-Eicosen-1-ol was prepared from methyl [1-^{14}C]*cis*-11-eicosenoate by reduction with $LiAlH_4$, and was purified by TLC.

[1-^{14}C] Acyl-CoA thioesters (sp. act. 3.5-5.5 Ci/mol) were prepared by the addition of the mixed anhydride, from the reaction of the [1-^{14}C] free acid (oleic, *cis*-11-eicosenoic or *cis*-13-docosenoic acid) with ethyl chloroformate, to the reduced form of coenzyme A, essentially according to the method of Young and Lynen (14). Product purity was checked by paper chromatography (Whatman's No. 1, developed in *n*-butanol/acetic acid/water 5:3:2 (v/v/v), free fatty acid R_f 0.95; acyl-CoA R_f 0.5-0.6), by $NaBH_4$ thioester reduction according to Barron and Mooney (15) and Nichols and Safford (16), by UV spectroscopy (absorptions at 233 nm and 260 nm corresponding to the thioester group and the adenine ring, respectively), and by the 5,5′-dithio-*bis*(2-nitrobenzoic acid) assay for free sulphydryl groups (17). The percent ^{14}C as free acid was 3-7%, the percentage of ^{14}C-labeled alcohol produced after $NaBH_4$ reduction, as measured by TLC, was 85-88%, while the amount of free sulphydryl groups present was less than 3%.

^{14}C-Stearoyl-ACP was prepared as follows. Acyl carrier protein (ACP) from *Escherichia coli* was purified to greater than 90% purity by the method of Majerus et al. (18). The ACP was acylated enzymically by the safflower system of Jaworski and Stumpf using [1,3-^{14}C] malonyl-CoA rather than [2-^{14}C] malonate (19). The reaction was stopped by the addition of 5% trichloroacetic acid (on ice), the precipitate was dissolved in 0.1 M piperazine-N,N′-*bis* (2-ethanesulphonic acid) buffer (PIPES), pH 5.7, and ammonium sulphate was added to 70% saturation. The precipitate was removed and the acyl-ACP was precipitated from the supernatant by the addition of trichloroacetic acid to 8% (w/v). The precipitate was redissolved in 0.1 M PIPES, pH 5.7 and was used without further purification. ^{14}C-Labeled acyl-ACP thus prepared generally was ca. 10-20% ^{14}C-palmitoyl-ACP and 80-90% ^{14}C-stearoyl-ACP. The final preparation contained ca. 50% acylated and 50% free ACP.

Subcellular Preparations from Jojoba Cotyledons

Developing jojoba seeds of undetermined age were the generous gift of Dr. D.M. Yermanos, University of California, Riverside. They were received between May 31st and June 26th. Most of the experiments reported were carried out on seeds harvested on June 21st and June 26th. Cotyledons removed from the seeds were finely sliced and gently homogenized in a chilled pestle and mortar with two volumes of chilled buffer consisting of 0.4 M sucrose, 7 mM β-mercaptoethanol, 5 mM sodium ascorbate and 0.1M 2-(N-morpholino)ethanesulphonic acid (MES) at pH 6.5. The resulting homogenate was filtered through two layers of cheesecloth to remove the coarser debris and then through one layer of Miracloth (Chicopee Mills, Inc., Milltown, NJ) to give a cell-free suspension as judged by light microscopy. For subcellular fractionation by differential centrifugation, the cell-free homogenate was spun at 12,00 g for 20 min at 5 C. The 12,000 g supernatant was centrifuged at 105,000 g for 1 hr at 5 C. The 12,000 g and 105,000 g pellets and the floating 12,000 g wax pad were resuspended in half the original volume of buffer. For sucrose density gradient fractionation, 2 ml of the cell-free homogenate was applied to the top of a linear 30%-55% sucrose gradient (12 ml) containing 5 mM $MgCl_2$ and 20 mM Tricine at pH 7.5, and was spun at 25,000 rpm in a SW-40 rotor for 5 hr.

Incubations

Incubations of ^{14}C-substrates with the cell-free homogenate (0.25 ml) in a total volume of 0.5 ml contained the following cofactor concentrations: ATP, 2 mM; NADH, 200 μM; $NADP^+$, 300 μM; glucose-6-phosphate, 2 mM; glucose-6-phosphate dehydrogenase, 0.4 units ml^{-1}; $MnSO_4$, 5 mM; $MgCl_2$, 5 mM; and defatted bovine serum albumin, 4 mg ml^{-1}. For measurement of fatty acid synthetase activity, [1,3-^{14}C] malonyl-CoA was diluted with cold malonyl-CoA to give a concentration of 60 μM, and ACP isolated from *E. coli* was added (8 μM). To assay for chain elongation, [1-^{14}C] oleoyl-CoA (30 μM) was incubated with unlabeled malonyl-CoA and acetyl-CoA (50 μM each), while [1-^{14}C] docosenoyl-CoA (30 μM) was incubated without any additional cofactors to measure reduction. In other incubations, the concentration of C_{10}-C_{18} saturated [1-^{14}C] acyl-CoA was 16-20 μM, while [1-^{14}C] eicosenoyl-CoA was 40 μM. Free [1-^{14}C] oleic acid (1 μCi, to give an incubation concentration of

35 μM) was added to 50 μl of BSA solution as the ammonium salt in 5 μl of aqueous ethanol prior to addition to the incubation, while [1-^{14}C]eicosenol was added to 50 μl of BSA solution in 5 μl of ethanol (final concentration 40 μM).

Incubations were for 1 hr at 27 C in a shaking water bath in open tubes. The reaction was terminated by the addition of *iso*-propanol (0.5-1 ml) followed by heating at 80 C for several minutes.

Stearoyl-ACP Desaturase Assays (20)

Each assay contained the following in a total volume of 0.5 ml: MES buffer, 50 mM, at pH 6.5; NADPH, 0.5 mM; defatted bovine serum albumin (BSA), 200 μg ml^{-1}; dithiothreitol, 1 mM; spinach ferredoxin, 100 μg ml^{-1} spinach ferredoxin-NADP^{+} reductase, 0.012 unit ml^{-1}; beef liver catalase, 1600 units ml^{-1}; enzyme preparation, 30-200 μl; and ^{14}C-stearoyl-ACP, 0.6 μM. The cofactors and added enzymes were preincubated for 10 min at 23 C in the absence of the substrate and the enzyme preparation prior to the full incubation at 23 C for 20 min. The reaction was terminated by the addition of 0.15 ml 8 M aqueous NaOH, carrier fatty acids added, and the mixture saponified by heating at 80 C for 20 min. After acidification and extraction into petroleum ether, the petroleum extract was methylated with diazomethane and the ^{14}C-labeled fatty acid methyl esters examined by radio-GLC on a 10% DEGS packed column as described previously (9).

Lipid Extraction and Analysis

Lipid material was extracted by the method of Hara and Radin (21). Petroleum ether (b.p. 30-60 C):*iso*-propanol, 3:2 (v/v) (6 ml) was added to the terminated incubation and the mixture allowed to stand at room temperature for several hours. Addition of 6.5% (w/v) aqueous Na_2SO_4 (3 ml) resulted in an upper petroleum-*iso*-propanol phase containing lipid materials and a lower aqueous phase containing acyl-CoA and acyl-ACP. The partition of acyl-CoA between the two phases was ascertained both in the presence and absence of 0.25 ml of the cell-free homogenate using [1-^{14}C] palmitoyl-CoA and [1-^{14}C]docosenoyl-CoA. The incorporation of radioactivity into the aqueous phase fell into the range 94-97%. A sample of ^{14}C-labeled phosphatidylcholine partitioned into the petroleum-*iso*-propanol phase to greater than 95%.

Part or all of the aqueous phase was saponified by heating with an equal volume of 10% (w/v) KOH in methanol for 1 hr at 80 C. After acidification and extraction into petroleum ether, the free ^{14}C-fatty acids were esterified with diazomethane and analyzed by radio-GLC on a 10% SP-2330 column. Part or all of the organic phase was subjected to ethanolysis as described by Duncan et al. (22) and the ethyl esters and alcohols recovered were analyzed by radio-GLC on a 14% SE-30 column. Short chain and hepatadecanoic acids were added as internal standards to the saponifications or ethanolyses when short chain (i.e., C_{10}-C_{14}) [1-^{14}C] acyl-CoA substrates were used. For analysis of the ^{14}C-labeled fatty acid and alcohol composition in each lipid class, the silica bands from TLC were scraped directly into the ethanolysis reagent. TLC and radio-GLC were essentially as described previously (9).

^{14}C-Acyl-CoA thioesters in the aqueous

TABLE I

Cofactor Requirements for Elongation of [1-^{14}C]-Oleoyl-CoA to C_{20} and C_{22} Products

Cofactors present	% Elongation
Total[a]	16
Total, less acetyl-CoA	17
Total, less malonyl-CoA	3
Total, less NADH	17
Total, less NADPH-generating cofactors	10
Total, less ATP	19
Total, plus ACP (8μM)	8
None	<1
Total[a]	14
Total, less NADPH-generating system, plus NADPH (0.3mM)	15
Total, less NADPH-generating system, plus grana reduction system[b]	0

[a]As described in Materials and Methods. Two separate experiments are shown in this table.

[b]NADP, 0.3 mM; 2,6-dichlorophenol-indophenol, 50 μM; sodium ascorbate, 10 mM; spinach ferredoxin, 0.1 mg ml^{-1}; spinach ferredoxin-NADP^{+} reductase, 0.012 unit ml^{-1}; and grana isolated from spinach leaves, 200 μg chlorophyll ml^{-1} (20). The incubation tube was irradiated under a 100 W light bulb.

TABLE II

Cofactor Requirements for Reduction of [1-^{14}C]Docosenoyl-CoA to [1-^{14}C]Docosenol

Cofactors present	% Reduction[a]
Total[b]	9
Total, less NADH	9
Total, less NADPH-generating system	0
Total, less ATP	5
Total, plus ACP (8μM)	7
None	0

[a][1-^{14}C]Docosenyl aldehyde was not detected.

[b]As described in Materials and Methods.

TABLE III

Substrate Specificities for Elongation and Reduction Reactions

[1-^{14}C]-Acyl Substrate	% Elongation[a]		% Reduction[a]	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
10:0-CoA	—	4	—	0
12:0-CoA	—	4	—	<0.5
14:0-CoA	5	1.5	2.5	1
16:0-CoA	1.5	0.5	9	3.5
18:0-CoA	23	9.5	6.5	3.0
18:1Δ9*cis*-CoA	16.5	9.0	2.5	2.0
20:1Δ11*cis*-CoA	4.5	—	6.5	—
22:1Δ13*cis*-CoA	0	0	9	4.5
18:0-ACP[b]	—	34	—	13

[a]Incubation conditions are as described in Materials and Methods. Experiment 1 was performed with seeds picked on June 22nd, experiment 2 with seeds picked on June 27th.

[b]Incubation concentration 2 μM.

phase were also examined by the Barron and Mooney and Nichols and Safford $NaBH_4$ reduction (15,16) with analysis of the resulting ^{14}C-alcohols by radio-GLC and/or TLC, and also by paper chromatography. Ozonolysis was carried out as described previously (9).

RESULTS

Cofactor Requirements for Elongation and Reduction

The cofactor requirements for the elongation of [1-^{14}C]oleoyl-CoA to C_{20} and C_{22} species, namely [3-^{14}C]eicosenoate, [3-^{14}C] eicosenol and [5-^{14}C]docosenoate, by the cell-free homogenates under the standard incubation conditions are given in Table I. Malonyl-CoA, and not acetyl-CoA, supplied the two carbon unit for elongation. NADPH, either added or generated in situ from $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, was the preferred reductant, although NADH was also acceptable. The $NADP^+$-ferredoxin-grana system, which generates reduced ferredoxin in the presence of light and which supplies reducing power to the stearoyl-ACP desaturase (20), inhibited elongation completely. ATP was not required for elongation while added ACP partially inhibited the reaction.

Table II shows the cofactor requirements for the reduction of [1-^{14}C]docosenoyl-CoA to [1-^{14}C]docosenol. NADPH was the obligatory reductant, as NADH could not replace the NADPH-generating system. Neither ACP nor ATP were necessary for reduction, although the results in Table II do suggest that ATP may be stimulating.

Further work is required to determine whether the cofactor concentrations used in this initial in vitro study are rate-limiting.

Substrate Specificity for Elongation and Reduction

Table III gives the substrate specificities for the elongation and reduction of [1-^{14}C]acyl CoAs, measured in two separate experiments. The enzyme activities were not restricted to physiological substrates, since saturated in addition to ω9-cis-monoenoic acyl-CoAs were reactive. The short chain (C_{10} and C_{12}) [1-^{14}C] acyl-CoAs were elongated only to the next higher two-carbon homologue, and were not reduced. Although elongation was not enhanced by the addition of ACP to the incubation medium, it was observed that when [1-^{14}C] decanoyl-CoA was incubated with exogenous ACP (8 μM), ^{14}C-stearate was produced in a 3% yield, while ^{14}C-palmitate or ^{14}C-myristate were not detectable.

Of considerable interest, stearoyl-ACP was readily elongated and reduced. In fact, ^{14}C-stearoyl-ACP (2 μM) gave the highest percent conversion (Table III). At the end of a 1 hr incubation the ^{14}C-label was distributed as follows[1]: in the aqueousphase (probably as acyl-ACP thioesters, as no free CoASH was added to the incubation); 31%, stearate; 20.5%, eicosanoate; 4%, docosanoate; and 1.5%, tetracosanoate: and in the organic phase; 21.5%, stearate; 13%, stearyl alcohol; and 8%, eicosanoate. Although the percent conversion data alone suggest that stearoyl-ACP is a more active substrate than stearoyl-CoA for both reactions, it is still too early to generalize on the specificity of the thioester moiety. The differences in substrate concentration resulted in 0.95 nmole of stearoyl-CoA being elongated

[1]Percent figures are given relative to the total ^{14}C added to the incubation (100%).

TABLE IV

Distribution of ^{14}C-Labeled Products between Different Lipid Classes

[1-^{14}C] Acyl-CoA Substrate[a]	^{14}C-Product	% of Total ^{14}C in						
		Wax	Free acid	Free alcohol	Polar lipids	Aqueous phase	Other[b]	Total
16:0	16:0 acid	9	19	2	13.5	43	3	89.5
	16:0 alcohol	1	0	6	0	0	1.5	8.5
	18:0 acid	tr	0.5	0	0.5	0.5	0.5	2
	Total	10	19.5	8	14	43.5	5	100.0
18:0	18:0 acid	4	13.5	1	9	41.5	1.5	70.5
	18:0 alcohol	1	0	3.5	0	0	2	6.5
	20:0 acid	2	1	0	1.5	11	0.5	16.0
	20:0 alcohol	0.5	0	1	0	0	0.5	2.0
	22:0 acid	1	tr	0	tr	2.5	0	3.5
	Total	8.5	14.5	5.5	10.5	55	4.5	100.0
22:1Δ13	22:1 acid	5	4	0	2.5	81.5	2	95.0
	22:1 alcohol	2.5	0	2.5	0	0	tr	5.0
	Total	7.5	4	2.5	2.5	81.5	2.0	100.0

[a]Incubation conditions as described in the Materials and Methods, with both elongation and reducing cofactors present.

[b]^{14}C-Material in the organic extract not identified.

and 0.3 nmole being reduced, compared with figures of 0.34 nmole and 0.13 nmole, respectively, for stearoyl-ACP, in a 1 hr incubation.

When free [1-^{14}C] oleic acid was incubated without cofactors for acyl activation (i.e., CoASH, ATP and Mg^{2+} ions absent), there was only a marginal formation of ^{14}C-eicosenoate (≤1%).

Incorporation of ^{14}C-Acyl and ^{14}C-Alkyl Groups into Wax and Other Lipids

The distribution of ^{14}C-labeled products among the lipid classes for [1-^{14}C]-palmitoyl-CoA, [1-^{14}C] stearoyl-CoA and [1-^{14}C] docosenoyl-CoA incubations in the presence of cofactors for both elongation and reduction is shown in Table IV. With palmitate and stearate, the major labeled polar lipid[2] was phosphatidylcholine (about half of the polar lipid radioactivity). For stearate the elongation product, [3-^{14}C] eicosanoate, was not detected in the phosphatidylcholine fraction. With ^{14}C-stearoyl-ACP as substrate, 8% of the total radioactivity was found in wax esters, 15.5% in free acids, 14% in free alcohols and only 2% in polar lipids, while 57% remained in the aqueous phase after extraction. Since the incubation of ^{14}C-stearoyl-ACP produced 13% ^{14}C-stearyl alcohol (Table III) and 14% free ^{14}C-alcohol, the labeled wax ester fraction must contain mainly ^{14}C-acyl groups, the implication being that acyl-ACP thioesters can serve as acyl donors in wax biosynthesis. This aspect will be examined more thoroughly when fresh tissue next becomes available for study in 1979.

A comprehensive substrate and cofactor specificity study on the wax esterification reaction remains to be done. However, Table V shows that [1-^{14}C] eicosenoyl-CoA was incorporated into wax in the absence of competing elongation and reduction reactions to the extent of 16%, and that there was only a marginal increase with the addition of oleyl alcohol as an acyl acceptor. The polar lipid fraction from [1-^{14}C] eicosenoyl-CoA incubations contained less than 1% ^{14}C-phosphatidylcholine and less than 0.5% ^{14}C-phosphatidylethanolamine. [1-^{14}C] Eicosenol, complexed to BSA, was incorporated into wax to the extent of 8%. This was not enhanced by the addition of stearoyl-CoA as an alkyl acceptor. [1-^{14}C] Oleic acid was not a substrate for esterification. ATP was not required for esterification.

Fatty Acid Activation

When [1-^{14}C] oleic acid was incubated in the absence of cofactors for activation, only 3% of the added radioactivity was found as acyl-CoA in the aqueous extract. The addition of CoASH (100 μM), ATP and Mg^{2+} ions resulted in 35% of the radioactivity as acyl-CoA after 1 hr, with 2.5% of elongated product. Thus, although free oleic acid is not a sub-

[2]"Polar lipid" is the term used to describe material from the organic extract remaining at the origin when Silica Gel G TLC plates were developed twice with petroleum ether/diethyl ether/acetic acid 90:10:0.5 (v/v/v).

TABLE V

Esterification of Substrates into Wax

[1-^{14}C] Substrate	Cofactors present[a]	Total organic extract	Percent of added ^{14}C in TLC Bands: Wax	Free acid	Free alcohol	Polar lipid
18:1 Acid[b]	NADH, NADPH, malonyl-CoA	ca. 100	<1	82	9	6
20:1-CoA	ATP[e]	43	16	17	2.5	3
20:1-CoA	ATP, oleyl alcohol (60μM)	53	19	21	4	5.5
20:1 Alcohol	ATP[f]	ca. 100	8	<1	87	5
20:1 Alcohol	ATP, stearoyl-CoA (40μM)	ca. 100	8	<1	86	4
18:1-CoA[c]	ATP, NADH, NADPH	68.5	6.5	30	4	20
18:1-CoA	No ATP	78.5	5	44	2.5	19
22:1-CoA[d]	ATP, NADH	27.5	5	14	2	4
22:1-CoA	No ATP	35.5	4.5	10.5	2	8.5

[a]Cofactor concentrations and other incubation parameters are given in Materials and Methods. NADP$^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were used to generate NADPH.

[b]As the cofactors for activation to [1-^{14}C]oleoyl-CoA were absent, negligible elongation and reduction occurred. The ^{14}C-distribution represents essentially that from [1-^{14}C]oleic acid.

[c]This ^{14}C-product distribution should take account of ca. 3% elongation, in the absence of malonyl-CoA, and 2% reduction to [1-^{14}C]oleyl alcohol.

[d]No [1-^{14}C]docosenol is generated in the presence of NADH alone.

[e]For wax formation the acyl acceptors are endogenous free alcohols.

[f]For wax formation the alkyl acceptors are endogenous acyl moieties.

strate for elongation or esterification into wax, the cell-free homogenate does possess fatty acid-CoA ligase activity.

Enzyme Activities Requiring Acyl-ACP Thioesters as Substrates

The incorporation of [1,3-^{14}C]malonyl-CoA into acyl and alkyl groups by the cell-free homogenate was 25.5% in the presence of added ACP and 10% without added ACP[3]. Incorporation into individual species was (the first percentage is for the incubation with ACP; the following, bracketed, percentage is for the incubation without ACP)[3]: C_{16} acid, 1.5% (0.5%); C_{16} alcohol, 1.0% (not detected); C_{18} acid, 10.5% (4.5%); C_{18} alcohol, 4.5% (1.0%); C_{20} acid, 4.5% (not detected); and C_{20} alcohol, 3.5% (4.0%). For both incubations, just under half the ^{14}C-lipid species partitioned into the aqueous phase on extraction, and were presumably acyl-ACP thioesters. Thus, in the organic phase, the alcohols accounted for over half of the ^{14}C-label. The C_{16}-C_{20}-labeled species were almost entirely saturated. The absence of monounsaturated products is probably due to the absence of ferredoxin and ferredoxin-NADP+ reductase in the incubation, curtailing stearoyl-ACP desaturase activity (19). The de novo fatty acid synthetase and palmitoyl-ACP elongase are ACP-track enzymes (10,11) in all other plant systems studied. These results show that this "ACP-track" biosynthesis is occurring and that it requires added ACP for maximal activity. The subsequent metabolism of ^{14}C-stearate (elongation and reduction) probably occurs on the ACP-track too. The reduction of ^{14}C-eicosanoate to ^{14}C-eicosanol extends the substrate specificity of the NADPH-dependent acyl reductase reported in a previous section. Degradation studies are now required to determine if the ^{14}C-label from [1,3-^{14}C]malonyl-CoA is at the carboxyl end or randomly distributed throughout the C_{18} and C_{20} species.

Stearoyl-ACP desaturation was demonstrated in the presence of ferredoxin, ferredoxin-NADP$^+$ reductase, and NADPH. Assays with 30, 100 and 200 μl of the cell-free extract gave 45, 40, and 34 pmole oleate min^{-1} ml extract^{-1}, respectively.

Acyl-ACP thioesterase activity in acetone powders from developing jojoba cotyledons has already been reported (24). The maximum rate of hydrolysis was observed for oleoyl-ACP among substrates having chain lengths from C_{12} to C_{18}.

Incubations with [1-^{14}C] Oleoyl-CoA and [1-^{14}C] Docosenoyl-CoA: Product Distribution and Time Courses

In a typical incubation of [1-^{14}C]oleoyl-CoA with the cofactors necessary for elongation and reduction 16.5%[1] elongation to C_{20}

[3]Percentages are corrected for 50% loss of ^{14}C-label from C(3) of [1,3-^{14}C]malonyl-CoA as $^{14}CO_2$.

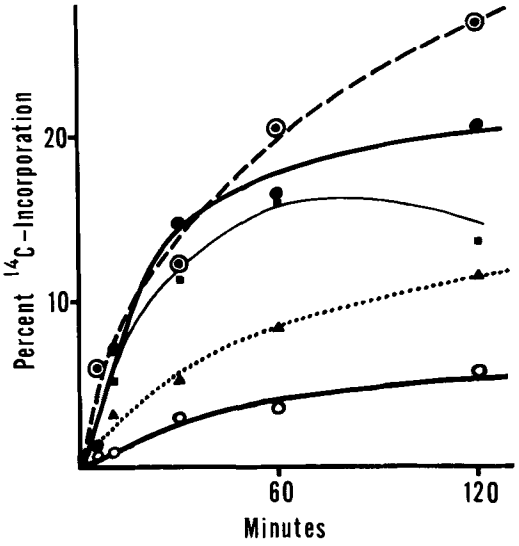

FIG. 1. Labeled reaction products from [1-^{14}C] oleoyl-CoA incubation with the cell-free homogenate as a function of time. Incubation conditions and cofactors are given in Materials and Methods. (●——●) C_{20} and C_{22} products. (○——○) Oleyl alcohol. (⊙- - -⊙) Polar lipid fraction. (■——■) Free fatty acids. (▲• • • ▲) Wax esters.

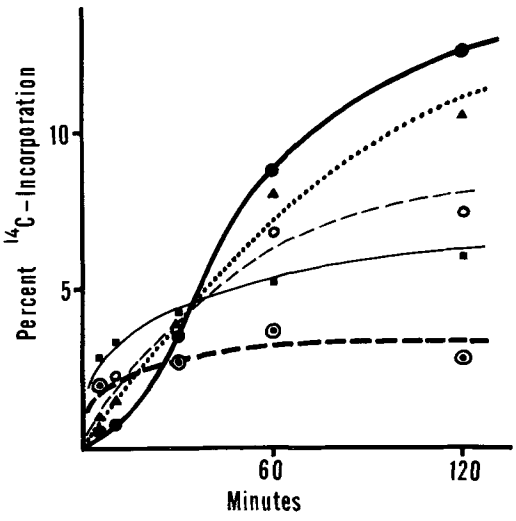

FIG. 2. Labeled reaction products from [1-^{14}C] docosenoyl-CoA incubation with the cell-free homogenate as a function of time. Incubation conditions and cofactors are given in Materials and Methods. (●——●) Docosenol. (⊙——⊙) Polar lipid fraction. (■——■) Free fatty acids. (○——○) Free alcohols. (▲ • • • ▲) Wax esters.

and C_{22} species and 3.5% reduction to oleyl alcohol occurred after 1 hr (Fig. 1). At this time, 40% of the radioactivity remained in the aqueous phase after extraction and partition, representing 7%[1] [3-^{14}C]eicosenoyl-CoA and ca. 0.5% [5-^{14}C]docosenoyl-CoA as determined by saponification and radio-GLC. The organic extract contained 3.5% [1-^{14}C]oleyl alcohol, 4% [3-^{14}C]eicosenol and 5% [3-^{14}C] eicosenoate as determined by radio-GLC after ethanolysis. The distribution of radioactivity between lipid classes, as measured by TLC, was 20.5% in polar lipids[2], 2.5% in free alcohols, 17.0% in free acids, and 8.5% in wax esters. A complete analysis of the distribution of the ^{14}C-labeled products within each lipid class after 1 hr is not available for this [1-^{14}C] oleoyl-CoA incubation. However, data from a separate experiment showed the wax ester fraction to contain labeled oleyl alcohol, oleate, eicosenol and eicosenoate in a 1:5:1:3 ratio. Furthermore, [3-^{14}C]eicosenoate was also present in the free acid and polar lipid fractions, but phosphatidylcholine, the major labeled species within the polar lipid fraction, contained only [1-^{14}C]oleate and not [3-^{14}C]-eicosenoate.

The ^{14}C-labeled acyl species, [3-^{14}C]eicosenoate and [5-^{14}C]docosenoate, produced from [1-^{14}C]oleoyl-CoA, were identified by radio-GLC of their esters on both nonpolar (14% SE30) and polar (10% SP2330) columns, and by reductive ozonolysis.

In a typical incubation of [1-^{14}C]docosenoyl-CoA with the cofactors necessary for reduction 9% [1-^{14}C]docosenol was produced after 1 hr (Fig. 2). There was a 27.5% incorporation of radioactivity into the organic extract, which contained all the reduced product. The distribution of ^{14}C between the lipid classes was 3.5% in polar lipids, 6.5% in free alcohols, 5% in free acids, and 8% in wax esters. Analysis of the composition of each lipid class (Table IV) showed that there was an approximately equal distribution of [1-^{14}C]docosenol between free alcohol and wax, though it should be stressed that this analysis was obtained in a separate experiment with seeds older than those used for the time course (Fig. 2) discussed above. Within the polar lipids, there was no major labeled species, such that a maximum limiting value of 0.5% could be placed on [1-^{14}C]docosenoyl-CoA acyl transfer into phosphatidylcholine or phosphatidylethanolamine.

^{14}C-Labeled docosenol produced from [1-^{14}C] docosenoyl-CoA (i.e., the free alcohol together with that released by ethanolysis) was identified by radio-GLC on SE-30 and SP-2330 columns, by TLC, and by ozonolysis.

Characterization of the ^{14}C-label remaining in the aqueous extract as acyl-CoA was by

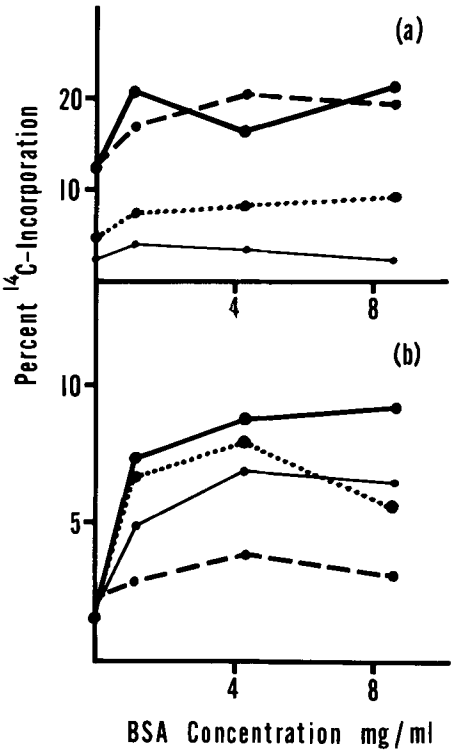

FIG. 3 The effect of BSA concentration on the incubation of (a) [1 ^{14}C] oleoyl-CoA and (b) [1-^{14}C] docosenoyl-CoA with the cell-free homogenate for 1 hr. Incubation conditions and cofactors are given in Materials and Methods. (• ◦ ◦ ◦ ◦•) (a) and (b), ^{14}C-Wax esters. (•— — — —•) (a) and (b), ^{14}C-Polar lipid fraction. (•———•) (a) C_{20} and C_{22} ^{14}C-labeled products, (b) [1-^{14}C] docosenol. (•———•) (a) [1-^{14}C] Oleyl alcohol, (b) ^{14}C-free alcohol.

several criteria. First, radioactivity was partitioned into the aqueous extract and free ^{14}C-fatty acids were released on base hydrolysis. Second, paper chromatography of aqueous extracts from [1-^{14}C] stearoyl-, oleoyl- or docosenoyl-CoA incubations showed in each case >90% of the label to be associated with a broad band at R_f 0.5-0.6, which co-eluted with authentic acyl-CoA. Neutral and phospholipids had an R_f ⩾0.9 in the *n*-butanol/acetic acid/water 5:3:2 (v/v/v) solvent system used. The band between R_f 0.4 and 0.7 was cut out and subjected to the Barron and Mooney $NaBH_4$ reduction to test for thioestrs. Recovery of ^{14}C-long chain alcohols was quantitative. The ^{14}C-distribution of the alcohols over chain length, as measured by radio-GLC, was similar to that for saponification of the total aqueous sample in each case. These results show that both the ^{14}C-labeled substrate and elongated products are present as acyl-CoA thioesters at the end of the incubation.

The product characterizations confirm the elongation and reduction reactions as:

$$[1\text{-}^{14}C]\text{oleoyl-CoA} \rightarrow [3\text{-}^{14}C]\textit{cis}\text{-11-eicosenoyl-CoA} \rightarrow [5\text{-}^{14}C]\textit{cis}\text{-13-docosenoyl-CoA}$$

$$[1\text{-}^{14}C]\textit{cis}\text{-13-docosenoyl-CoA} \rightarrow [1\text{-}^{14}C]\textit{cis}\text{-13-docosen-1-ol}$$

[1-^{14}C] Aldehydes were not detected by either GLC or TLC in incubations where [1-^{14}C] acyl-CoAs were converted to [1-^{14}C] alcohols. The detection limit was ca. 0.5% of the total added radioactivity.

A comparison of the product distribution and time course studies (Figs. 1 and 2) for [1-^{14}C] oleoyl-CoA and [1-^{14}C] docosenoyl-CoA incubations shows:

(1) Some enzymic hydrolysis of the acyl-CoA thioesters did occur , with the hydrolysis being greater for oleoyl-CoA than for docosenoyl-CoA, but in neither case was the substrate extensively removed.

(2) Oleate was more readily incorporated into polar lipids, and particularly phosphatidylcholine, than was docosenoate.

(3) The rate of docosenoyl-CoA metabolism (36% in 2 hr) was considerably slower than oleoyl-CoA metabolism (77% in 2 hr).

(4) ^{14}C-Labeled alcohols were not only esterified into wax, but also found in the free state. A pool of free alcohol available for esterification with acyl-CoA to produce wax had already been suggested from our earlier in vivo studies (9).

Further work is now required to establish enzyme assay conditions (i.e., linear rates, with the rates proportional to mg protein per assay) for these reactions of wax biosynthesis.

Reproducibility of Incubations and Effect of BSA

Experiments with early batches of seeds gave very variable results. In particular, homogenization of the cotyledonous tissue in a Braun blender equipped with razor blades (23) gave low or undetectable activities. Gentle hand homogenization of the tissue in a pestle and mortar was preferred. The addition of acyl-CoA substrates, complexed to defatted BSA, to the incubation was found to increase significantly percent elongation, reduction and incorporation of label into wax (Fig. 3) and also to remove variability, and was routinely used in further experiments. Homogenization of the cotyledons in the presence of defatted BSA (5 mg ml^{-1}) had the greatest stimulating effect, and this modification will be used in future work. The reason for the stimulation by BSA is not known.

TABLE VI

Jojoba Wax Ester Biosynthesis: Enzyme Activities in Subcellular Fractions Obtained by Differential Centrifugation

Fraction	% Incorporation of ^{14}C-malonyl-CoA into lipids	Stearoyl-ACP desaturase activity[a]	% Elongation of ^{14}C-18:1-CoA	% Reduction of ^{14}C-22:1-CoA
Cell-free homogenate	25	34	16	9
12,000 g Pellet	9	<3	<1	<1
12,000 g Wax pad	35	24	14	8
12,000 g Supernatant	---	<4	---	---
100,000 g Pellet	<5	—	0	0
100,000 g Supernatant	<5	—	0	0

[a]Expressed as pmole product min^{-1} ml of extract^{-1}.

Subcellular Fractionation

Differential centrifugation of the cell-free homogenate showed the overwhelming majority of lipogenic activity to be associated with the 12,000 g floating wax pad. This included both ACP-thioester and CoA-thioester substrate activities (Table VI). The small amount of activity observed in the 12,000 g pellet is probably associated with contaminating wax bodies, as GLC of this fraction after ethanolysis showed the presence of appreciable amounts of wax components. Sucrose density gradient fractionation did not result in distinct organelle bands being visible in the gradient. Again, essentially all the lipogenic activity resided in the floating wax pad, and not in the sucrose gradient or the pellet.

DISCUSSION

The in vitro studies reported here are the first demonstration of the enzyme activities responsible for wax biosynthesis in the developing jojoba cotyledon. They show that cell-free preparations from this tissue represent a viable system for further study and highlight manipulations necessary to obtain reasonable enzyme activities.

The demonstration of acyl-CoA elongase, acyl-CoA reductase and acyl-CoA-fatty alcohol ligase activities indicates that wax ester biosynthesis can proceed from oleoyl-CoA by the series of reactions shown in the lower right hand corner of Figure 4.

Our study is the first to show that erucic acid biosynthesis can occur via oleoyl-CoA and then eicosenoyl-CoA elongation. Previous studies have shown that malonyl-CoA is a cofactor for such elongations (2,3). Our experiments confirm this, rule out acetyl-CoA as a cofactor, and indicate NADPH to be the preferred reductant. However, the localization of elongation activity solely in the wax pad differs from the localization of erucic acid biosynthesis reported by Appelquist for *Brassica campestris* (2), where activity was found only in the "microsomal" fraction, and by Appleby et al. for *Crambe abyssinica*(3), where activity was found in the 800 g and 23,500 g pellets and in the fat pad.

The NADPH-specific acyl-CoA reductase, which produces free alcohol and is located in the jojoba wax pad, differs from other acyl-CoA reductase systems observed in plants. The activity studied by Khan and Kolattukudy in *Euglena gracilis* was microsomal and required NADH (4,5), while, in acetone powders from young broccoli leaves, Kolattukudy could separate two enzyme activities (6). An NADH-dependent reductase produced free aldehyde from acyl-CoA, while an NADPH-dependent fatty aldehyde dehydrogenase gave the free alcohol. Acyl-CoA reduction in the jojoba

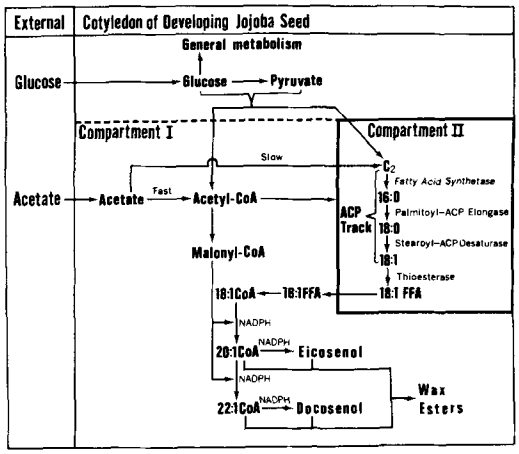

FIG. 4. Hypothesis for wax biosynthesis in the maturing jojoba cotyledon. The "ACP-track" enzymes shown in Compartment II require acyl-ACP thioesters as substrates, with malonyl-CoA supplying the C_2 unit for the fatty acid synthetase condensations and palmitoyl-ACP elongation.

TABLE VII

In vivo Enzyme Specificity Patterns for Chain Elongation, Reduction and Esterification, Derived from Jojoba Wax Composition[a]

% Alcohol in wax	Reduction to alcohol	Composite enzyme specificities: Elongation	Esterification into wax	% Acid in wax
0.5	18:1-OH ← 0.5%	18:1(9c)CoA → 5.5%	18:1WE	5.5
		↓ 94%		
22.0	20:1-OH ← 23.4%	20:1(11c)CoA → 38.2%	20:1WE	36.0
		↓ 38.2%		
23.0	22:1-OH ← 63.9%	22:1(13c)CoA → 22%	22:1WE	8.0
		↓ 13.9%		
4.5	24:1-OH ← 90%	24:1(15c)CoA → 10%	24:1WE	0.5

[a]% = Percentage conversion of acyl-CoA for one of three competing reactions.

cell-free homogenate did not produce significant quantities of free aldehyde. The absence of [1-^{14}C]docosenal upon incubation of [1-^{14}C]docosenoyl-CoA with NADH as the only reductant suggests the absence of an NADH-specific reductase to convert acyl-CoA to free aldehyde. The possible separation of the jojoba acyl-CoA reductase into two enzyme activities awaits further investigation.

Kolattukudy has suggested that wax ester biosynthesis can occur by three donor mechanisms: acyl transfer to the free alcohol from acyl-CoA thioesters, free fatty acids or phospholipids (6). Our results indicate that acyl-CoA is an acyl donor and free alcohol the acyl acceptor in the biosynthesis of jojoba wax. Another possible acyl donor may be the acyl-ACP thioester.

In vivo experiments on jojoba wax biosynthesis indicated two pools of acetate, one supplying the de novo synthesis of stearate and the other supplying the chain elongation of oleate (9). Acetone powders from maturing jojoba seeds showed acyl-ACP thioesterase activity, with oleoyl-ACP being the optimum substrate (24). From these considerations, a pathway for wax biosynthesis (Fig. 4) is suggested (9). It utilizes the concept of an "ACP-track, switching mechanism, CoA-track" proposed by Stumpf as a general pathway for plant lipid biosynthesis (10,11). The ACP-track reactions are de novo fatty acid synthesis, palmitoyl-ACP elongation, and stearoyl-ACP desaturation. The switching mechanism is the hydrolysis of oleoyl-ACP to oleic acid and then the conversion of the oleoyl moiety to its CoA-thioester by an acyl-CoA ligase. The subsequent metabolism to produce wax occurs on the CoA-track. The fact that all the requisite enzyme activities have now been demonstrated in vitro lends credence to this scheme. However, the observation of elongation and reduction of stearoyl-ACP was unexpected. The absence of stearoyl-ACP elongation or reduction would have confirmed our two-track hypothesis. Since ACP has the same functional group as CoA (i.e., 4-phosphopantetheine), an overlap of substrate specificity for these two types of acyl thioester for the elongase(s) and reductase(s) may explain our results, although the possibility of separate enzymes for acyl-CoA and acyl-ACP substrates cannot be ruled out. Obviously, further experimentation is required to resolve the question as to whether the subsequent metabolism of oleate to wax occurs on the ACP-track or the CoA-track or both. However, we believe our two-track scheme (Fig. 4) to be the most reasonable one to fit all the present data. Since jojoba wax has only traces of saturated fatty acid or alcohol components, it is clear that the stearoyl group, a potential precursor of saturated long chain acids and alcohols, never becomes associated with the elongation and reduction systems in the cell. Thus, while stearoyl-CoA and stearoyl-ACP are substrates for elongation and reduction in vitro, the intact cell does not utilize these substrates for this purpose, since stearoyl-CoA is never formed and stearoyl-ACP is presumably compartmented and serves only as the substrate for desaturation to oleoyl-ACP. Thus, the interpretation of the results from compositional studies coupled with in vivo and in vitro incubation data strongly suggest a two-compartment biosynthetic system (Fig. 4). Furthermore, our two-track hypothesis is also sup-

ported by the observation that the oleoyl switching mechanism coincides with the "boundary" between the acetate pools.

The substrate specificity for the monounsaturated acyl-CoAs fits very well the expected trends from an analysis of the wax composition. Examination of the mass composition of the wax suggests that as the chain length of the potential substrates increases (18:1 to 24:1) the elongation reaction becomes less favored while reduction becomes more favored (Table VII). Assuming that in vivo the subsequent metabolism of oleate to produce wax occurs on the "CoA-track," each acyl-CoA substrate can suffer one of three competing fates: chain elongation, reduction to the corresponding alcohol, or esterification into wax. Starting with 100 units of oleoyl-CoA supplied from the de novo synthesis reactions, this eventually ends up as 0.5 unit of oleyl alcohol, 5.5 units of oleate, 22.0 units of eicosenol etc. in the wax (i.e., the percent composition data from a typical wax analysis given in Table VII). The percentages given for each individual reaction in Table VII are a measure of the resulting or composite specificity at the steady state production of wax. For example, from the mass composition, it is apparent that 94 units of oleoyl-CoA must be elongated to eicosenoyl-CoA, and of this eicosenoyl-CoA 22 units are reduced to eicosenol (i.e., 23.4% conversion). The in vitro data show similar trends for elongation and reduction as are derived in Table VII from the wax composition: that is, as chain length increases (18:1 to 20:1 to 22:1), elongation becomes less favored while reduction becomes more favored (Table III). The in vitro trends reported in this study now require confirmation by a full evaluation of kinetic parameters (V_{max} and K_m values).

Localization of the various lipid-synthesizing activities within the cotyledon cell is a crucial aspect for a full understanding of wax ester biosynthesis. The floating wax pad from the 12,000 g spin of the cell-free homogenate contained the requisite activities to synthesize wax from malonyl-CoA, with negligible activity in other fractions. The floating fat layer has been reported as the major site for lipid biosynthesis in developing castor bean endosperm (25) and avocado mesocarp (26), while in the germinating jojoba cotyledon Huang and his coworkers have proposed that the first steps in wax degradation (lipolysis and fatty alcohol oxidation) take place at the wax body membrane (27,28). Electron microscopic studies clearly show that developing jojoba cotyledons contain both endoplasmic reticulum and plastids in addition to the wax bodies (29), and it is quite possible that membranous material is adhering to the wax pad during the preparation. A detailed morphological composition of the floating wax pad and definition of developing wax body membranes in the jojoba in terms of enzyme localization are clearly needed.

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Characterization and Composition of Sterols in the Free and Esterified Sterol Fractions of *Aspergillus oryzae*

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ABSTRACT

Free and esterified sterol fractions were isolated from *Aspergillus oryzae*, and their components analyzed mainly by gas chromatography-mass spectrometry. Cholesterol, brassicasterol, episterol (5α-ergosta-7,24[28]-dien-3β-ol), 4α-methyl-5α-ergosta-8[9],24[28]-dien-3β-ol, lanosterol and 24-methylene-24-dihydrolanosterol were well characterized, whereas 5-dihydroergosterol, sitosterol and 24[28]-dehydroergosterol were tentatively characterized. The principal sterol of the free sterol fraction was brassicasterol, and that of the esterified sterol fraction was episterol. Ergosterol, which is reported to be widely distributed in the fungi kingdom, was not detected.

INTRODUCTION

The lipid composition of fungi has not been investigated as well as that of other organisms. Especially no analyses of the lipids have been carried out concerning *Aspergillus oryzae*, yellow koji mold, which is popular in Japan to brew such foods as sake, shochu, miso, soy-sauce and so forth. We have examined the mycelial lipids of *Aspergillus oryzae* IFO 4290 in detail, and reported the structure and molecular species of the sphingolipids (1-3) and the chemical composition of the neutral acyl lipids (4) in previous papers. In this paper we describe the characterization and composition of 4-methylsterols and 4-demethylsterols in the free and esterified sterol fractions in *A. oryzae*.

Several studies have been done on sterols of fungi (5-8), but none on *A. oryzae*. Most of the fungi were examined only for 4-demethylsterols, and only a few for 4-monomethylsterols and 4,4-dimethylsterols (6).

EXPERIMENTAL METHODS

Thin Layer Chromatography (TLC)

TLC was performed using Silica Gel G plates, 250 μm. The solvents for TLC were: (A) hexane/ether (95:5, v/v), (b) hexane/ether (80:20, v/v), (C) benzene/ethyl acetate (10:1, v/v) and (D) chloroform/acetone (95:5, v/v).

Isolation of Sterol Lipids

A. oryzae was grown in Czapek liquid medium at 28 C; the mycelium (400 g wet weight) was harvested in the stationary phase (1), lyophilized, and extracted twice with 10 vol chloroform/methanol (2:1, v/v) and (1:2, v/v), respectively. After solvent removal and washing, the lipids (6.5 g) were applied to a silicic acid column (4 x 50 cm) and separated into nonpolar and polar fractions (1). The nonpolar lipids (5.1 g) were placed on a second silicic acid column (3 x 70 cm) (9). Steryl esters were eluted with hexane/benzene (85:15, v/v) and hexane/ether (95:5, v/v), and the three classes of free sterols (4,4-dimethyl, 4-monomethyl and 4-demethyl) were subsequently eluted with hexane/ether (70:30 and 50:50, v/v). Steryl esters were purified by preparative TLC with solvent A. The free sterol fractions were saponified to remove acyl lipids, and applied to a silicic acid column (2 x 30 cm) as described above. Free 4-methylsterol and 4-demethylsterol fractions were obtained by preparative TLC with solvent B. The 4-demethylsterol fraction was further purified by recrystallization from ethanol.

Hydrolysis of Steryl Ester

Steryl esters were saponified under reflux with methanolic 1 N KOH for 100 min. After cooling and addition of water, the reaction mixture was extracted with ether, the extract washed with water and concentrated to dryness to yield component sterols. This fraction was separated by TLC with solvent C into 4-methylsterols and 4-demethylsterols.

Trimethylsilylation and Acetylation of Sterol

Sterols were treated with either trimethylsilyl imidazole (10) or with the clear supernatant of dry pyridine/hexamethyl/disilazane /trimethylchlorosilane (1.0:1.3:0.8, v/v/v) (11) for 30 min at 60 C to convert them to the trimethylsilyl ether derivatives.

Sterols were acetylated in dry pyridine/acetic anhydride (2:1, v/v) for 1 hr at 37 C. The reaction mixture was then extracted with hexane to obtain the acetates (12).

Isolation of Major Component 4-Demethylsterols

The free 4-demethylsterol fraction and the

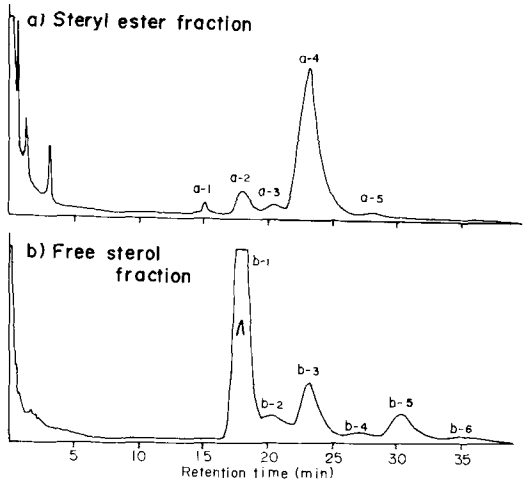

FIG. 1. Gas liquid chromatogram of 4-demethylsterols in the steryl ester and free sterol fractions from *A. oryzae*. Glass column, 0.3 x 200 cm, packed with 1.5% SE-30, 230 C.

component 4-demethylsterols of the steryl esters were purified by TLC with solvent C and separated on Silica Gel G-2% $AgNO_3$ with solvent D into several sterol classes (13). The isolated components were acetylated and further purified by preparative TLC with solvent C.

Infrared (IR) Spectrometry

IR spectra were recorded with an infrared spectrophotometer (IR-G type, Nippon Bunkō Kōgyo Co. Ltd., Tokyo) by use of 250 mg KBr pellets containing 2~3 mg of each lipid.

Gas Liquid Chromatography (GLC)

Sterols were analyzed with a Hitachi Gas Chromatograph (Model 063, Hitachi Seisakusho Co. Ltd., Tokyo). A glass tube 0.3 x 200 cm was used for the column, and N_2 as carrier gas. The column was packed with either 1.5% SE-30 on Chromosorb W (AW-DMCS) or 1.5% OV-17 on Chromosorb W(AW-DMCS). The former column was operated at 230 C and the latter at 250 C.

Gas Chromatography-Mass Spectrometry (GC-MS)

Analyses were performed with a Hitachi Gas Chromatograph-Mass Spectrometer (Model RMU-6MG, Hitachi Seisakusho Co. Ltd., Tokyo). The chromatograph was fitted with a glass column, 0.3 x 100 cm, packed with Diasolid ZS. The column was programmed from 200 C to 240 C at 2 C/min. The ion source temperature was 220 C, ionization voltage 20 eV, trap current 80 μA, and accelerating voltage 3.2 kV.

RESULTS

TLC of Sterols in the Nonpolar Lipids

TLC of individual sterol classes (not illustrated) showed that the Rf values of each agreed with those of the corresponding authentic standards prepared from rice bran (14). Moreover, all the sterols gave characteristic

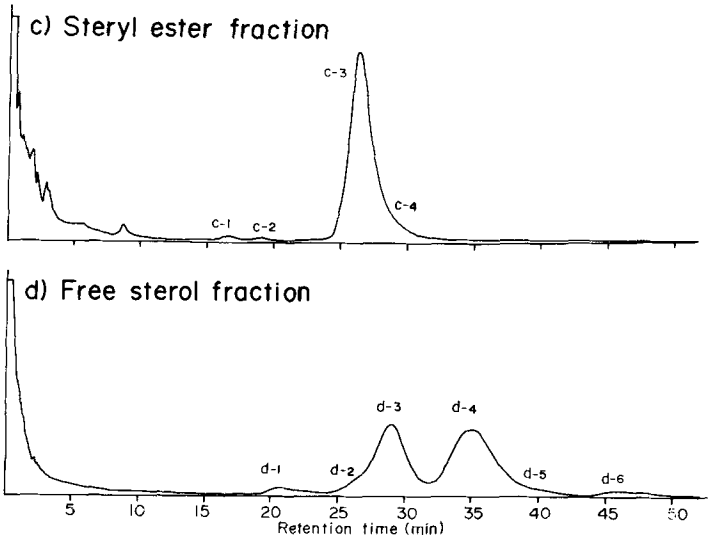

FIG. 2. Gas liquid chromatogram of 4-methylsterols in the steryl ester and free sterol fractions from *A. oryzae*. Analytical conditions were the same as described in Fig. 1.

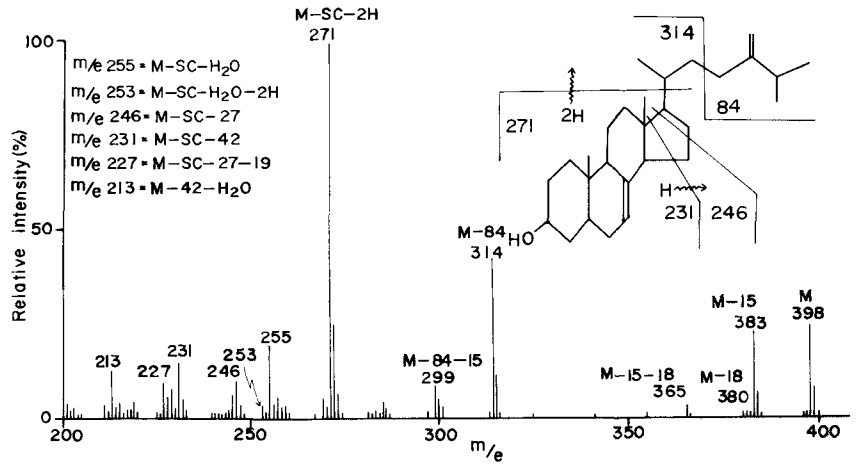

FIG. 3. Mass spectrum of episterol (peak a-4 in Fig. 1). Analysis by GC-MS. Glass column, 0.3 x 100 cm, packed with Diasolid ZS, temperature programmed from 200 C to 240 C at the rate of 2 C/min. The energy level of the ion source was 20 eV and the ionizing current 80 μA. SC: side chain, C-20~C-28.

color reactions with the sulfuric acid reagent. According to densitometric measurements, the proportions of free 4-demethylsterols, sterol esters and free 4-methylsterols in the nonpolar lipid fraction were found to be ca. 12%, 10% and 3%, respectively.

IR Spectra

IR specta of free 4-demethylsterols, free 4-methylsterols and steryl esters from *A. oryzae* (not illustrated) showed the usual absorption bands corresponding to these compounds. Peaks at 1640 and 890 cm^{-1}, characteristic of terminal methylene group, were also found in the latter two groups. The patterns of these spectra were similar to those of corresponding compounds isolated from rice bran (14).

TLC of Sterols from the Ester Fraction

TLC of the sterols from the ester fraction with solvent C gave four spots with Rf 0.51, 0.44, 0.34 and 0.30; the latter two were preponderant. The Rf values of those spots agreed with those of authentic 4,4-dimethylsterol (0.51), 4-monomethylsterol (0.44) and 4-demethylsterol (0.34 and 0.30). According to densitometric estimation of the chromatogram, the ratio of 4,4-dimethyl-, 4-monomethyl- and 4-demethylsterols was ca. 0.1:1:11.

GLC of Sterols

4-Demethylsterols. Gas liquid chromatograms of component 4-demethylsterols in steryl ester and free sterol fractions are shown in Figure 1. Five peaks were found in the steryl ester and six peaks in the free sterol fractions, respectively. The predominant peaks differed between the two fractions. No ergosterol, which is known to be widely distributed in fungi, could be recognized by either GLC or $AgNO_3$-TLC.

4-Methylsterols. Gas liquid chromatograms of component 4-methyl (4,4-dimethyl and 4-monomethyl) sterols in steryl ester and free sterol fractions are shown in Figure 2. Four peaks were found in the steryl ester and six peaks were detected in the free 4-methylsterol fractions. Again, predominant peaks differed between the two fractions.

Determination of the 4-Demethylsterols in Steryl Esters

In Figure 1, peaks a-1~4 were identified as follows, but peak a-5 could not be analyzed because of insufficient material.

Cholesterol (peak a-1). GLC and mass spectrometric data for the compound of this peak were consistent with those for cholesterol (5-cholesten-3β-ol) (15).

Brassicasterol (peak a-2). The mass spectrum for this compound (strong ions at M-43, M-43-18, M-side chain-H_2O and m/e 69) was consistent with that for a 5,22-diene sterol (15-17). Molecular weight and GLC data suggested this was brassicasterol (ergosta-5,22-dien-3β-ol) which was further confirmed by IR data (970, 840 and 800cm^{-1}, characteristic for C-22 and C-5 unsaturations) (18-20) of the pure material isolated by $AgNO_3$-TLC of the free sterol and acetate.

C_{28} Monoene and Diene Sterols (peak a-3). Although a distinct mass spectrum of peak a-3

was not obtained because of the small amount, molecular ions were observed at m/e 398 (C_{28} diene sterol) and m/e 400 (C_{28} monoene sterol). Other ions derived from the C_{28} diene, which were stronger than those from the C_{28} monoene, were m/e 271 (the base peak) favoring C-7 unsaturation (15,21), and M–43 and M–43–18 characteristic for C-22 unsaturation (15). Moreover, the relative retention time of authentic 5-dihydroergosterol (1.27, relative to cholesterol) (22) was similar to that of peak a-3 (1.28). Peak a-3, therefore, appeared to be principally composed of 5-dihydroergosterol (5α-ergosta-7,22-dien-3β-ol), in addition to a minor unidentified C_{28} sterol with one double bond.

Episterol (peak a-4). The mass spectrum of peak a-4 is shown in Figure 3. The spectrum exhibited the molecular weight ions (M^+, M-15, M-18 and M-18-15) indicating a C_{28} sterol with two double bonds. Diagnostic ions of M-84 and M-84-15 showed that one of these double bonds was at C-24[28](15). Ions at m/e 271, 255, 246, 231 and 213 indicated that another double bond was in the nucleus; moreover, the base peak ion at m/e 271 favored C-7 unsaturation (15,21). Further, in the mass spectrum of the trimethylsilyl ether derivative (not illustrated), strong ions at m/e 386 (M–C-23~C-27–H) and 343 (M–side chain–2H, the base peak), characteristic for C-7 and C-24[28] unsaturated sterols, were observed. From these fragment ions, peak a-4 was identified as episterol (5α-ergosta-7,24[28]-dien-3β-ol).

The component 4-demethylsterols from the steryl ester fraction were subjected to TLC on Silica Gel G as well as on $AgNO_3$-TLC, and acetylated to isolate the major component. When this was analyzed by GLC, a single peak corresponding to episteryl acetate was found. The ester was saponified, the free sterol purified and analyzed by IR. Bands at 1640 and 890 cm^{-1} (terminal methylene group at C-24 [28]), and absorptions at 847 and 820 cm^{-1} (C-7 unsaturation) (18) were found, which showed that the sterol was episterol.

Determination of the 4-Demethylsterols in the Free Sterol Fracion

In Figure 1, peaks b-1~5 were identified as follows, but peak b-6 was not because of insufficient material.

Brassicasterol (peak b-1). As GLC and mass spectrometric data of peak b-1 were consistent with those of peak a-2, peak b-1 was identified as brassicasterol.

C_{28} Monoene and Diene Sterol (peak b-2). As the retention time and mass spectrum of peak b-2 were in agreement with those of peak a-2 of steryl ester, this peak was presumed to be composed of 5-dihydroergosterol and an unidentified C_{28} sterol with one double bond.

Episterol (peak b-3). Peak b-3 was identified as episterol, being identical with peak a-4.

C_{29} Monoene Sterol (peak b-4). Although a distinct mass spectrum of peak b-4 could not be obtained because of insufficient material, a molecular ion was detected at m/e 414 suggesting a C_{29} sterol with one double bond. Strong ions at m/e 303(M–93–H_2O), 329(M–67–H_2O) and 396(M–18) suggested tha the position of the double bond was at C-5 (15). Peak b-4 is postulated to be sitosterol based on its mass spectrum and the retention time, which agreed with that of authentic sitosterol.

C_{28} Tetraene Sterol (peak b-5). The mass spectrum of peak b-5 had a molecular ion at m/e 394 suggesting a C_{28} sterol with four double bonds. Strong ions of M–(side chain + H_2O + 2H) at m/e 251 indicated that there were two double bonds in the side chain and nucleus, respectively (15). From the presence of diagnostic ions of M–43 and M–43–18, the position of one of the double bonds in the side chain was presumed to be at C-22 (15); moreover, ions of M–84 and M–84–15 suggested that there was another double bond at C-24[28] (15). The position of double bonds in the nucleus could not be determined from the mass spectrum. However, of the fungal C_{28} sterols with four double bonds, only 24[28]-dehydroergosterol (ergosta-5,7,22,24[28]-tetraen-3β-ol) and 14-dehydroergosterol (ergosta-5,7,14,22-tetraen-3β-ol) have been reported to be present in yeasts (23) and *Fungi imperfecti* species (24). Peak b-5 is, therefore, assumed to be 24[28]-dehydroergosterol.

Determination of the 4-Methylsterols in Steryl Esters

In Figure 2, peaks c-1, c-2 and c-4 could not be analyzed because of insufficient material, but peak c-3 was characterized as follows.

4α-Methyl-5α-ergosta-8[9], 24[28]-dien-3β-ol (peak c-3). The mass spectrum of peak c-3 had a molecular ion at m/e 412 (The base peak) suggesting a C_{29} sterol with two double bonds. The pattern of the spectrum was similar to that of 4α-methyl-5α-ergosta-8[9],24[28]-dien-3β-ol (16). Ions at m/e 328 (M–84, 4%) and 313-(M–84–CH_3, 6%), which indicated the presence of a 24-methylene group, were observed. Moreover, both the ions showed that there were one double bond and one extra methyl group in the nucleus. The position of the methyl group was deduced to be at C-4 from the detection of an ion at m/e 365(M–H_2O–29 1%) characteristic for a 4α-methylsterol (25). From these

TABLE I

Sterol Composition of Steryl Ester and Free Sterol Fraction from *A. oryzae* (%)

Sterols	Peak no.[a]	Steryl ester fraction		Free sterol fraction	
		A	B	A	B
4-Demethylsterols					
Cholesterol	a-1	1.0	1	—	—
Brassicasterol	a-2 and b-1	10.3	10	59.7	48
5-Dihydroergosterol + Monoenic C_{28} sterol	a-3 and b-2	3.0	3	7.1	6
Episterol	a-4 and b-3	85.4	78	18.7	15
Sitosterol	b-4	—	—	2.1	2
24(28)-Dehydroergosterol	b-5	—	—	11.3	9
Others	other peaks	0.3^{w}	$<1^{w}$	1.1^{x}	1^{x}
4-Methylsterol					
4α-Methyl-5α-ergosta-8(9),24(28)-dien-3β-ol	c-3 and d-2	96.0	8	6.8	1
Lanosterol	d-3	—	—	37.2	7
24-Methylene-24-dihydrolanosterol	d-4	—	—	45.4	9
Others	other peaks	4.0^{y}	$<1^{y}$	10.6^{z}	2^{z}

[a]See the gas liquid chromatograms in Figure 1 and 2. A: % for 4-demethylsterols or 4-methylsterols. B: % for the total sterols. w: peak 1-5. x: peak b-6. y: sum for peaks c-1, c-2 and c-4. z: sum for peaks d-1, d-5 and d-6.

fragment ions peak c-3 appeared to be 4α-methyl-5α-ergosta-8[9],24[28]-dien-3β-ol. Peak c-3 is a 7,24[28]-methylsterol appears to be excluded because its mass spectrum was different from that of 24-methylenelophenol (4α-methyl-5α-ergosta-7,24[28]-dien-3β-ol) (25), and the intensity of ions at m/e 285-(M−side chain−2H) and 328 (M−84), characteristic of a C-7 unsaturated sterol (25), were weak.

Determination of Component 4-Methylsterols in Free Sterol Fraction

In Figure 2, peaks d-2, d-3 and d-4 were characterized as follows, with peaks d-1, d-5 and d-6 not analyzed because of insufficient material.

4α-Methyl-5α-ergosta-8,[9],24[28]-dien-3β-ol (peak d-2). As the retention time and mass spectrum of peak d-2 agreed with those of peak c-3, peak d-2 was identified as 4α-methyl-5α-ergosta-8,[9]24[28]-dien-3β-ol.

Lanosterol(peak d-3). The mass spectrum of major peak d-3 exhibited ions at m/e 426(M^+, 42%) and 411(M−15, the base peak) indicating a C_{30} sterol with two double bonds. The pattern of other fragment ions (at m/e 313, 297, 295, 273, 270, 259, 255 and 241) was in good agreement with that of lanosterol(lanosta-8,24-dien-3β-ol) (15,16). Peak d-3 was, therefore, identified as lanosterol.

24-Methylene-24-dihydrolanosterol (peak d-4). In the mass spectrum of peak d-4, ions indicating a molecular weight were observed at m/e 440(M^+, 33%) and 425(M−15, the base peak). Moreover, ions at m/e 356(M−84, 34%) and 341(356−CH_3, 10%), respectively, showed the presence of a 24-methylene group (15). Ions at m/e 259 (32%) and 241 (25%) showed the presence of a 14α-methyl group (26) as well as lanosterol. From these fragment ions, the probable identity of peak d-4 was 24-methylene-24-dihydrolanosterol (24-methyl-5α-lanosta-8[9], 24[28]-dien-3β-ol), which was supported by IR spectroscopic data of free 4-methyl sterols (1640 and 890 cm^{-1} due to a terminal methylene group).

DISCUSSION

The sterol composition of the steryl ester and free sterol fractions calculated from the relative proportion of component 4-demethylsterol and 4-methylsterols, and the respective gas liquid chromatograms (Figs. 1 and 2) is shown in Table I. The principal sterols in the ester fraction were brassicasterol, episterol (78%) and 4α-methyl-5α-ergosta-8[9],24[28]-dien-3β-ol, whereas those in the free sterol fraction were brassicasterol (48%) and various amounts of five others. No ergosterol, which is known to be widely distributed and the most abundant sterol in fungi, was detected in *A. oryzae*. From the present experiments and analysis of fatty acids reported previously (4), the representative molecular species of steryl ester in *A. oryzae* could be characterized as episterol linoleate.

In the present study, identification of sterols was based on GC-MS and IR analyses, so that the stereochemistry at C-24 in the sterol has not been proved. To distinguish the C-24 epimers, an examination by high resolution nuclear magnetic resonance spectra should be undertaken in the future.

Sterols of the *Fungi imperfecti,* to which *A. oryzae* belongs, have been examined to some

extent (27). In some *Fungi imperfecti* previously examined, the principal sterols were ergosterol and 14-dehydroergosterol *(Aspergillus niger* (25)), ergosterol and 22-dihydroergosterol *(Aspergillus parasitiaus* and *Aspergillus flavus* (6)), and fungisterol (5α-ergosta-7-en-3β-ol) and ergosterol *(Pencillium claviforme* (6)). Moreover, the sterols in the rust fungi of *Basidiomycetes* and the aquatic *Phycomycetes* species, which produce no ergosterol (6,8), were identified as fungisterol (ergost-7-en-3β-ol), 5-dihydroergosterol or fucosterol (5,24[28]-stigmastadien-3β-ol). In the light of these facts, the significant point to be stressed here is that the sterol composition of *A. oryzae* is considerably different from that of the other fungi. In particular, brassicasterol, which was one of the major sterols in *A. oryzae*, has been detected only in a few dermatophytic fungi and the *Thichophyton* (6). Considering that C-5 and C-22 unsaturated sterols are not regarded as intermediates in the biosynthetic pathway from lanosterol to ergosterol in fungi and yeasts (6,8,28), and since no ergosterol was found in *A. oryzae*, a specific metabolic pathway appears to be present which causes the accumulation of brassicasterol in this organism.

The status and functional role of sterol lipids in fungi have not been made sufficiently clear. However, the association between the composition of the cell wall and sterols was discussed in *Phycomycetes* species (29). In previous papers we described the chemical characterization of sphingolipids which were present in comparatively large quantities and which also differed greatly from those of other fungi (1,3). These differences, as well as those of the sterol components discussed here, would make a study of the construction of the cell wall and cell membranes of *A. oryzae* particularly interesting.

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Studies on Cell Proliferation in Inguinal Adipose Tissue during Early Development in the Rat

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ABSTRACT

[2-^{14}C] Thymidine was injected into rats aged 3, 5 and 10 days, and incorporation of the precursor into deoxyribonucleic acid (DNA) of the inguinal fat tissue was measured for short time periods. Using chromatographic procedures to measure the distribution of thymidine and its metabolites in the soluble fraction of the tissue, degradation of the precursor was found to be similar at all ages. The data indicate that thymidine was more rapidly utilized for DNA synthesis in 3-day-old rats than in older animals. When ^{14}C-thymidine was injected in vivo and adipocytes and stromal cells were then separated from the inguinal tissue of 3-and 5-day-old rats, the incorporation into DNA was significant in both types of cells already 30 min after pulse labeling. Stromal cells took up twice as much of label as the adipocytes. Furthermore, real incorporation into DNA was found in the adipocytes when incubated in vitro in a culture medium supplemented with ^{14}C-thymidine. The possibility is discussed that early in postnatal life adipocytes might synthesize DNA for further cell division.

INTRODUCTION

Two questions are of particular interest to the investigator of adipose tissue development: is the number of adipose cells determined very early in the developing fat deposit and which cell types have the potential to multiply?

Some evidence suggests that cell number becomes fixed early in life both in obese humans and animals. The possibility of affecting cell multiplication in animals either by manupulating dietary intake or by hypothalamic lesions decreases as the animals grow older (1-3). The most interesting report on man is that early onset of obesity is associated with a greater increase in cell number than obesity beginning later in life (4,5). Most of these investigations on the cellularity of different adipose sites used counting and sizing techniques (6) which resulted in misinterpretation of the cell number in very young animals (7). Experiments using thymidine incorporation into (DNA) deoxyribonucleic acid as an index of cellular multiplication either were carried out with adult animals (8,9) or else labeling periods were too long to determine the real number and site of cells in the proliferative process (10). In addition, these experiments were performed with epididymal fat, which is not representative of the other adipose sites. Differences are observed in cellularity, in fat cell size, and in response to several stimuli between one adipose site and another (3,11, 12).

In the present study, we used the inguinal fat pad of newborn rats, which develops early and becomes macroscopically visible 12 hr after birth (13). With this tissue, it was possible to follow the different steps of development using the incorporation of ^{14}C-thymidine into DNA during short labeling periods. The kinetics of incorporation of thymidine vary with age and tissues and are influenced by the rate of degradation of the precursor after injection (14,15). Since no information was available on this point in the tissues of newborn rats, we also measured changes in labeled thymidine and its metabolites in the non-precipitable fraction of the whole tissue. Other experiments were performed on isolated adipocytes and stromal cells in vitro or after injection of thymidine in vivo to investigate whether cell multiplication is restricted to the stromal cells or whether adipocytes participate in the proliferative process during the early stages of development.

MATERIALS AND METHODS

Chemicals

Radioactive biochemicals: [2-^{14}C] thymidine (50 mCi/mmole) and [*methyl*-^{14}C] thymidine (50 mCi/mmole) were purchased from the Commissariat à l'Energie Atomique (Saclay – France); tissue culture medium 199 was obtained from Institut Pasteur (Paris – France). Collagenase and DNase I were obtained from Worthington Biochemical Corp. (Eurobio – France). Unlabeled thymidine, thymine, thymidine triphosphate (TTP), thymidine diphosphate (TDP), thymidine monophosphate (TMP) and β-Aminoisobutyric acid (AIBA) were obtained from Sigma (Eurobio – France). Other chemicals were obtained from Merck (Socolab – France).

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Animals

Three-, 5- and 10-day-old Sherman rats obtained from Janvier Breeding Laboratory (Le Genest – France) were used. In another set of experiments, adult animals (6 mo. old, weighing ca. 400 g) were used as controls. Animals were chosen which corresponded to average age and weight relationships determined from animals within the colony. These adult rats were fed ad libitum with a standard diet made up by "Extralabo," Pietrement Lab., Ste. Colombe, Provins, France. The composition of that diet was: Protein 25%; Fat 4%; Cellulose 6%; Minelras 9%; H_2O 12%; Vit. E 1.500 mg/100 kg; Vit. A 1.000 000 UI/100 kg; Vit. D_3 200.000 UI/100 kg. Radioactive compounds were administered by subcutaneous injection at a dose of 0.2 μCi per g body weight in 0.1 ml saline.

Experimental Procedures

The animals were decapitated 30, 60 and 120 min after the injection. The left and right inguinal fat pads were rapdily dissected, pooled and weighed. In all experiments, the whole tissue or the isolated cells were homogenized manually in a tight fitting glass grinder (Kontes, Polylabo – France). In experiments with whole inguinal tissue, total lipids were extracted three times with chloroform/methanol (2:1, v/v) according to Folch et al. (16). The methanol-water layer of the chloroform/methanol extracts contained 75% of the total free ^{14}C-thymidine and its phosphorylated derivatives and degradation products. This was shown by paper chromatography after evaporation of the extracts as described below. To assure complete removal of unincorporated thymidine and derivatives, the pellets containing the nucleic acids and proteins were dried with air and washed again three times with 10% cold TCA (no radioactivity was present in the last washing). The dry extracts obtained by the Folch extraction method and the combined TCA supernatants were measured separately for radioactivity. The total amount of label found in these fractions was called hereafter "total radioactivity in soluble fraction."

RNA and DNA were separated from the pellets by the method of Schmidt and Thannhauser (17). Aliquots of the RNA and DNA fractions were taken to measure the radioactivity. No label was found in the RNA fraction, showing that a good separation of the nucleic acids was achieved. The amount of DNA was determined by the colorimetric assay of Burton (18) adapted for low concentrations.

Parallel experiments were done using DNase digestion treatment to check whether the radioactivity of the precipitable fraction was only incorporated into DNA. After extraction by chloroform/methanol and subsequent TCA washing, the tissue residue containing nucleic acids and proteins was digested in 1N KOH for at least 15 hr at 37 C. In these conditions, DNA was resistant to hydrolysis as shown by Steudel and Peiser (19). The reaction was stopped by addition of 6N HCl and the precipitate was centrifuged. The pellets were incubated during 3 hr at 37 C in a medium containing Mg SO_4 (0.9 μmole), sodium acetate buffer (pH = 5, 150 μmoles), DNase (30 U/100 μg DNA) in a total volume of 1.8 ml. The reaction was stopped by 600 μl of 20% TCA. The DNA was determined in the supernatant by the Burton colorimetric method. A standard curve of DNA was treated in the same conditions as above. An aliquot of the DNA supernatant was measured for radioactivity as in former experiments.

In another set of experiments, the animals were killed 30 and 60 min after injection of ^{14}C-thymidine. The tissue was rapidly dissected and adipocytes and stromal cells were isolated from the whole tissue by the collagenase method of Rodbell with minor modifications (20). Pooled white fat was thoroughly minced with scissors, placed in closed plastic vials in 4 ml bicarbonate buffer (pH = 7.4) containing 3 mg/ml of crude collagenase. The bicarbonate buffer contained 2% bovine albumin instead of 4%, and glucose was omitted from the incubation medium. The incubations were carried out at 37 C in a shaking water bath during 40 min. At the end of incubation, the suspension of cells was diluted with fresh buffer, filtered through silk and centrifuged for 3 min at 200 x g. This procedure yielded two distinct cell pools, fat cells floating to the surface and stromal cells which settled. Both cell preparations were washed three times with the buffer without albumin and were homogenized as previously described. Lipids were extracted from the adipocytes with chloroform/methanol. The adipocytes and the stromal cells were washed with 10% TCA, and the nucleic acids were extracted from the pellets by the usual procedure. The [2-^{14}C] thymidine incorporation into DNA was measured in both cellular pools. In additional experiments, after incubation with collagenase, the separated cells were washed with phosphate-buffered saline (PBS), without $CaCl_2$ and $MgCl_2$ (pH = 7.4) and then once more with PBS-EDTA (0.02%) to remove the Ca^{++} ions of the Krebs-Ringer medium and prevent cellular aggregation.

In vitro studies: adipocytes and stromal cells

TABLE I

Various Aspects of Growth and Development of Inguinal Adipose Tissue at 3, 5 and 10 Days Postnatally

Age Days	Inguinal tissue total weight[a] mg	Total DNA[a] mg	Protein[a] mg/gm tissue	Lipids[a] mg/gm tissue
3	86 ± 2[b] (n = 31)[c]	0.128 ± 0.009[b] (n = 31)[c]	24.1 ± 1.0[b] (n = 8)[c]	421 ± 10[b] (n = 54)[c]
5	134 ± 7[b] (n = 24)[c]	0.218 ± 0.015[b] (n = 24)[c]	31.7 ± 1.5[b] (n = 12)[c]	561 ± 13[b] (n = 35)[c]
10	321 ± 11[b] (n = 14)[c]	0.281 ± 0.025 (n = 14)[c]	21.7 ± 0.8[b] (n = 10)[c]	669 ± 18[b] (n = 21)[c]

[a]Results are means ± SE of n rats.
[b]Differences between 3, 5 and 10 days in all data presented here are highly significant ($P<0.01$).
[c]Number of rats used in the experiment.

were isolated from the inguinal fat pads by the collagenase method, but a PBS (pH = 7.4) was used instead of the bicarbonate buffer. The two cell pools were washed four times with PBS and then were suspended in Medium 199 in Hanks BSS containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at pH = 7.4. Before incubation, the medium was gassed with 95% air 5% CO_2. The cell suspensions were incubated for 1 hr at 37 C in closed plastic vials containing 10 μCi of [2-^{14}C]thymidine in 8 ml culture medium. The vials were gently shaken during the incubation period. The reaction was stopped by cooling to 0 C and cell suspensions were rapidly washed twice with medium 199 and twice again with PBS buffer medium. Before extraction of the nucleic acids, special care was taken to completely remove the residual-free ^{14}C-thymidine by washing the pellets several times with cold 10% TCA. DNA was extracted from stromal cells and adipocytes as described previously. [2-^{14}C]Thymidine incorporation was measured in the DNA extracts.

The distribution of radioactivity was determined by paper chromatography (15,20) at different time intervals after injection and at different ages in the compounds which were present in the methanol-water layer after chloroform/methanol extraction. After evaporation of the chloroform/methanol extracts, the residue was treated by a mixture of ether/H_2O. In this procedure, the nucleotides and derivatives were recovered in the aqueous phase. This phase was concentrated by freeze-drying, and the samples were chromatographed on Whatman paper n°1 in the following solvent systems: (a) ETAA: 90% ethanol and 1M ammonium acetate containing 0.1M EDTA (70:30) and (b) EtAc form: upper phase from a mixture of ethyl acetate/water/formic acid (60:35:5). The radioactive spots were characterized by the use of unlabeled markers of thymine, thymidine, TMP, TDP, TTP and AIBA. The position of the spots was located with ultraviolet light (2537 Å), and the spots were eluted with 0.1M HCl. The amount of radioactivity present in individual spots was determined by liquid scintillation counting (15,21).

Radioactivity was measured in a liquid scintillation spectrometer (Nuclear Chicago Mark I). The degree of quenching among samples was corrected using quench curves.

The amount of proteins was determined by the method of Lowry et al. (22) in an aliquot of the KOH extract used for nucleic acid separation.

Total lipids were measured by the gravimetric method after evaporation of the chloroform/methanol extract.

Optical and electron microscopic studies were carried out with isolated adipocytes and stromal elements.

The results were analyzed by standard statistical procedures (Fischer t-test).

RESULTS

Adipose Tissue Growth and Chemical Composition (Table I)

Postnatal total weight increases varied significantly ($p<0.01$) by 56% between days 3 and 5 and by 140% between days 5 and 10.

A very rapid parallel increase in total DNA content was observed between the 3rd and 5th days (70%). After the 5th day, the total DNA content increased to a lesser extent (30%). Protein concentration was maximal at 5 days of age and then declined. The percentage of lipid rose regularly during the entire period studied. These results indicate that growth of inguinal

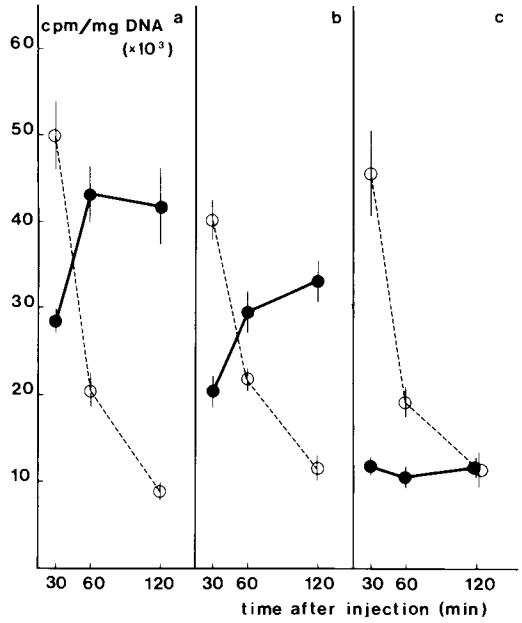

FIG. 1. Incorporation of [2-^{14}C] thymidine into the soluble fraction (○) and the DNA (●) of the inguinal tissue of (a) 3-day-old rats, (b) 5-day-old rats and (c) 10-day-old rats. Each point is the mean ± SE for 3 experiments carried out with pooled tissue from at least 3 animals.

fat tissue was for the most part correlated with cellular multiplication up to 5 days. Thereafter, the increment in adipose tissue was the result of both the increase in adipose cell number and the enlargement of cell size as shown by the fall of protein and the increase in lipid.

In Vivo Incorporation of Radioactive Thymidine in the Total Tissue

The data derived from these studies are shown in Figure 1 and Table II.

Thirty minutes after injection, the [2-^{14}C] thymidine incorporated into DNA of the whole tissue was high at 3 and 5 days of age (28,200 and 20,400 cpm/mg DNA, respectively, Fig. 1). At both stages of development, the time courses of incorporation were similar and attained a maximum of radioactivity at 60 min. On day 3, the difference of incorporation between 30 and 60 min was significant. Ten days after the animal's birth, the labeling of DNA was lower (ca. 11,000 cpm/mg DNA) and remained stable throughout the experiment. An inverse relationship between the disappearance of the radioactivity from the soluble compartment and the incorporation of the precursor into DNA as a function of time was observed in Figure 1 in 3- and 5-day-old but not 10-day-old rats.

TABLE II

Distribution of [methyl-^{14}C] Thymidine in the Soluble Components of Rat Inguinal Tissue during Postnatal Development[a]

Time after injection	Three days[b]			Five days[b]			Ten days[b]		
	30 Min	60 Min	120 Min	30 Min	60 Min	120 Min	30 Min	60 Min	120 Min
TMP[c] + TDP + TTP	1915 ± 323 (2.2)[e]	3460 ± 566 (7.5)[e]	1590 ± 133 (10.3)[e]	1585 ± 326 (2.7)[e]	1528 ± 93 (5.4)[e]	1012 ± 78 (8.8)[e]	1946 ± 388 (3.1)[e]	2882 ± 567 (6.6)[e]	1676 ± 187 (8.8)[e]
Thymidine	53297 ± 6463 (60.0)[e]	10765 ± 2246 (23.1)[e]	3335 ± 294 (21.7)[e]	26625 ± 484 (45.0)[e]	5482 ± 420 (13.6)[e]	2130 ± 257 (18.5)[e]	29964 ± 2770 (47.9)[e]	12173 ± 1248 (28.2)[e]	4461 ± 202 (21.0)[e]
Degradation Products[d]	26456 ± 2585 (30.6)[e]	25136 ± 2652 (55.0)[e]	7795 ± 1552 (49.8)[e]	24767 ± 386 (41.9)[e]	17221 ± 2320 (61.0)[e]	5341 ± 413 (50.5)[e]	23053 ± 1316 (37.0)[e]	22419 ± 1197 (52.1)[e]	11465 ± 1484 (53.4)[e]
Total radioactivity in soluble fraction	86271 ± 8081	46205 ± 7901	15553 ± 2277	59111 ± 845	28147 ± 3419	11486 ± 684	62430 ± 4147	43158 ± 3572	21403 ± 2151

[a]Results are expressed in cpm/mg DNA.
[b]Results are means ± SE of two experiments. Each experiment includes left and right pads from 3 animals.
[c]TMP, thymidine monophosphate; TDP, thymidine diphosphate; TTP, thymidine triphosphate.
[d]The compounds includes thymine, two unidentified products and amino-iso-butyric acid which was the main substance.
[e]Results in brackets represent percent of total soluble radioactivity in each component.

TABLE III

Incorporation of ^{14}C-Thymidine into DNA of Adipocytes and Stromal Cells Isolated from Inguinal Tissue, after Injection in vivo of the Precursor to 5-Day-Old Rats

Incorporation periods	Adipocytes[a] cpm/mg DNA	Stromal cells[a] cpm/mg DNA
30 Min	11500 ± 920 (n = 9)[b]	23100 ± 1390 (n = 9)[b]
60 Min	13000 ± 1500 (n = 7)[b]	24100 ± 960 (n = 7)[b]

[a]Results are means ± SE.
[b]n = Number of experiments carried out with pooled tissue from at least 7 animals. The experiments on adipocytes and stromal cells were always done in parallel.

TABLE IV

Incorporation of ^{14}C-Thymidine into DNA of Adipocytes and Stromal Cells Isolated from Inguinal Tissue of 3-Day-Old Rats: (a) Effect of Two Different Washing Procedures during Cell Fractionation and (b) Effect of Two Different DNA Extraction Methods

	30 Min		60 Min	
	Buffer + EDTA	Buffer - EDTA	Buffer - EDTA	
Incorporation periods	TCA extraction	TCA extraction	TCA extraction	DNase extraction
Adipocytes[a]	25310 ± 2700 (n = 4)[b]	23150 ± 2100 (n = 5)[b]	25700 ± 1250 (n = 7)[b]	20500 ± 2900 (n = 7)[b]
Stromal cells[a]	37630 ± 2100 (n = 4)[b]	33500 ± 1500 (n = 5)[b]	43100 ± 1700 (n = 7)[b]	40800 ± 1500 (n = 7)[b]

[a]Results are means ± SE.
[b]n = Number of experiments carried out with pooled rats from at least 12 animals.

The distribution of the radioactivity in the metabolites of the soluble compartment was measured using [methyl-^{14}C]thymidine. This precursor was used instead of [2-^{14}C]thymidine because with the latter all the degradation products were not labeled. With both precursors, no age changes occurred in the total radioactivity of the soluble fraction. A rapid decline in their radioactivity was observed between 30 and 60 min after injection. Two hours later, only 20% of the initial value was detected in the soluble fraction on days 3, 5 and 33% on day 10. The level of ^{14}C activity in the thymine nucleotides at various time periods after injection and at various ages appeared to be constant, whereas the radioactivity of free thymidine decreased. Between 30 and 120 min, this decline varied as a direct function of age. The radioactivity of the thymidine decreased 18-fold at 3 days, 12-fold at 5 days and only 5-fold at 10 days. Label present in the degradation products was constant between 30 and 60 min and then declined at all ages as a result of elimination. However, the relative proportion of the radioactivity present in these metabolites increased during the first 60 min and remained unchanged thereafter (Table II, results in brackets). Approximately 60% of the radioactivity in the degradation products was found in β-AIBA.

Thus, the decrease in the rate of labeled thymidine incorporation into DNA between days 3 and 10 was not the result of an increased rate of degradation of thymidine but a real reduction of DNA synthesis in the whole tissue.

Incorporation of ^{14}C-Thymidine into DNA of Isolated Adipocytes and Stromal Vascular Cells after Injection of the Precursor in vivo

We examined the tissue compartmentalization of radioactivity in order to provide further information about the sites of cell proliferation (Table III). The data indicate that a real incorporation of ^{14}C-thymidine into adipocytes DNA occured in 5-day-old rats even in the 30-min pulse period and remained at the same level 60 min after injection. The specific activity found in the stromal fraction was only double that of the adipocyte fraction. The similar values of incorporation found at 30 and 60 min were probably the consequence of the further 40 min incubation during cell dissociation.

In Table IV, we compared the values of

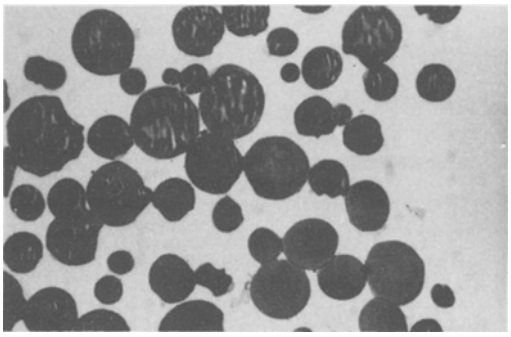

FIG. 2. Photomicrographs of free fat cells obtained from collagenase-treated rat inguinal adipose tissue. The cells were fixed in 1.7% glutaraldehyde in buffer-saline (0.1M sodium cacodylate buffer) and embedded in Epon-Araldite. Original magnification x 250.

DNA specific activity measured either after DNA digestion or after TCA extraction. In spite of slightly lower results obtained with DNase, no significant differences were found.

In order to check whether some contamination of the adipocyte fraction with stromal cells occurred, additional experiments were performed using PBS deprived of Ca^{++} and Mg^{++} and supplemented with EDTA in the washing procedures to avoid cellular aggregation (Table IV). Both cell pools incorporated ^{14}C-thymidine at the same level with or without EDTA.

Furthermore, stromal vascular cells were absent from fat cell preparations when examined by optical microscopy (Fig. 2).

In vitro ^{14}C-Thymidine Incorporation into DNA of Isolated Cell Fractions

In order to verify that the presence of the labeled DNA in the adipocytes was not due to a rapid differentiation occurring during the course of the experiment, we separated the cells in vitro before the incorporation of radioactive thymidine.

When stromal cells and adipocytes were incubated separately for 60 min with labeled thymidine in a culture medium, an appreciable amount of radioactivity was recovered in the DNA of both cell types (Table V). In the stromal cell types, the incorporation rate of thymidine was again double that of the adipocytes.

A parallel experiment using epididymal adipose tissue from 6-month-old adult rats was performed in order to determine the basal activity of a tissue which has ceased to multiply (1). Here, very low activity was observed in the DNA of the 2 cell pools, about 5 to 10 times lower than in the inguinal tissue of young rats (Table V).

DISCUSSION

The results presented here show that a high rate of cell multiplication and DNA synthesis occured between birth and 5 days after birth in the inguinal fat tissue of the infant rat. We observed that neither the penetration of the precursor into the cell nor its rate of degradation was modified during this period. In the inguinal tissue, 60% of the radioactivity was recovered in thymidine 30 min after the beginning of the experiment. In contrast, Chang and Looney (15) in an adult regenerating rat liver showed that, 2 min after precursor injection, 85% of the total radioactivity was found in the degradation products. The high level of radioactivity found in the DNA of the adipocytes after cellular separation supports the hypothesis of a real participation of these cells in the proliferative process of the young rat's inguinal tissue. The incorporation of thymidine into the DNA of the adipocytes has been a matter of discussion for a long time. It was assumed by many authors that it was a conse-

TABLE V

In vitro Incubation during 60 Min of Adipocytes and Stromal Cells with 1 μCl/1 ml ^{14}C Thymidine, after Separation of the Cells from Inguinal Tissue of Five-Day-Old Rats. Comparison with the Incorporation into DNA of Cells Isolated from the Epididymal Tissue of Adult Rats

Age	Adipocytes[a] cpm/mg DNA	Stromal cells[a] cpm/mg DNA
5 days	12800 ± 1200[b] (n = 9)[b]	29400 ± 2560[b] (n = 9)[b]
adults 6 months	2400 ± 300 (n = 3)[c]	3310 ± 820 (n = 3)[c]

[a]Results are means ± SE.
[b]Significantly different from the adult rats. $P<0.01$.
[c]n = Number of experiments carried out with pooled tissue from at least 10 rats aged three days and two adults.

quence of contamination of the adipose cells with stromal elements. Most of these experiments were conducted with adult animals, and the preparations probably contained connective and supportive tissue cells (8,9). However, 60 min after ^{3}H-thymidine injection, Greenwood and Hirsch (10) observed a real incorporation of the precursor into the DNA of adipocytes from epididymal tissue of 9-day-old rats. We could corroborate these results using inguinal tissue in the early stages of development and short labeling periods. The assumption of contamination by cellular aggregation could be discarded. Using a medium deprived of Ca^{++} and supplemented with EDTA during the washing procedures, we did not improve appreciably our results. Furthermore, taking as a basis the relative levels of radioactivity observed in the adipocytes and stromal cells, one would have to assume stromal contamination of at least 50%. Electron and optical microscopic studies with isolated adipocytes showed an homogeneous cell type presenting a characteristic spherical shape with a large central droplet and a flattened eccentric nucleus. These observations ruled out extensive contamination. In the adipocytes isolated from 5-day-old rats and incubated in vitro in a culture medium with ^{14}C-thymidine, we found label in the DNA. Hollenberg and Vost (8) did not find any radioactive thymidine in the adipocytes from epididymal tissue incubated in vitro. In these experiments, adult rats were used, and the authors reported that the cells were probably damaged during separation. The results presented here show that at an early stage of development, when the tissue is increasing especially by hyperplasia, adipocytes, like stromal cells, incorporated ^{14}C-thymidine into the DNA. This ability decreased as the animals grew older. The hypothesis, according to which the radioactivity accumulated in the adipocytes could originate in the very rapidly differentiating stromal cells, appeared unlikely. Indeed, the very short incorporation times used in our experiments do not warrant this interpretation if we take durations of various known cell cycles into consideration (23). Can one say, though, that these differentiated cells which actively incorporated thymidine into the DNA would be able to divide? One might suppose, with present knowledge, that the adipocytes having incorporated thymidine remained blocked in G 2 of the cell cycle. This phenomenon has been described for other cells (24). We can imagine that, under the influence of certain hormonal or nutritional stimuli, particularly in the young animal, these cells can become free of lipids and divide.

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Stimulation of Lipid Absorption in Young Rats by Cholesterol: Early Time Changes and Effects on Pentobarbital Sleeping Time

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ABSTRACT

Four groups of young male and female rats were fed a chow diet (0), chow plus 10% corn oil (F), chow plus 1% cholesterol (C), or chow plus 1% cholesterol plus 10% corn oil (CF) for 1, 2, 4 and 8 days. After 2 dats, male F, C and CF rats exhibited a shorter anesthesia period (-20 to -30%) when given pentobarbital. By 4 days, male F and C rats had pentobarbital sleeping times (PB-ST) 20% less than 0 rats. These effects were additive and CF rats had 40% shorter PB-ST. Reduction of PB-ST by cholesterol and corn oil was similar but slightly less in female rats. Liver lipid content doubled in 4 days in CF rats, and liver cholesterol was 4 times that of 0 rats. These changes and the increases in metabolism of barbiturate suggested changes in liver microsomal enzyme activities. Serum glutamic oxaloacetic and glutamic pyruvic transaminase, two enzymes reflective of liver damage, did not increase after 8 days on C, F or CF diets. Our results suggest that consumption of an animal sterol and a high lipid diet by laboratory rats, normally consuming a diet low in fat (3-4%), increases the ability of the animal to detoxify a barbiturate. Storage of absorbed dietary cholesterol in the liver may represent a major mechanism for maintaining extra hepatic cholesterol homeostasis.

INTRODUCTION

In almost all species, the liver is the major site for the metabolism of foreign chemicals (1). The barbiturates are a class of foreign chemicals which are strong depressants of the central nervous system and induce sleep in the treated individual. A standard dose of pentobarbital will produce a standard sleeping time and the duration of this period is primarily dependent upon the metabolism of the barbiturate by the liver. An increase in those liver microsomal enzymes which metabolize the barbiturate will be reflected in a shorter sleeping time. Conversely, any inhibition of the activity of these liver microsomal enzymes should be reflected in a longer duration of barbiturate sleeping time.

In recent studies on the effects of dietary cholesterol on lipid absorption in rabbits, we found that feeding cholesterol for 2-3 months induced very large increases in plasma and liver cholesterol and lipids (2). Similar experiments in rats involving feeding cholesterol for 3, 6 and 9 weeks also demonstrated increases in plasma and liver cholesterol and lipids, but these were of smaller magnitude (3). Since a 3-fold increase in liver lipids was observed in the rats at 3 weeks, it was obvious that significant changes in liver composition had occurred much earlier. These facts made it desirable to determine how early these cholesterol- and fat-stimulated alterations occurred in the liver. Accordingly, we fed 4 groups of weanling rats diets containing cholesterol and fat to determine a) the early sequence of changes in liver and plasma cholesterol and liver lipids and b) whether these changes in liver composition were associated with reduced barbiturate-induced sleep time as an indication of an altered level of activity of liver microsomal enzymes.

MATERIALS AND METHODS

Male and female weanling Sprague-Dawley rats, 21-23 days old weighing 45-55 g, were housed individually on a schedule of 12 hr of light and 12 hr of dark. Groups of rats were fed one of the following four diets: (O) ground laboratory chow, (C) chow containing 1% cholesterol, (F) chow containing 10% corn oil, (CF) chow containing 1% cholesterol and 10% corn oil.

The time sequence of the dietary effect upon pentobarbital sleeping time was studied at 2, 4 and 8 days. The sleeping time was defined as the time between injection of the barbiturate and restoration of the righting reflex. Sodium pentobarbital was injected intraperitoneally at a dosage rate of 24 mg/kg body weight.

After 1, 2, 4 and 8 days on the diet, rats in each group were killed by light etherization and jugular bleeding. Weighed samples of liver were frozen, later homogenized in chloroform/methanol, and the lipids were extracted by the technique of Bligh and Dyer (4). The resulting lipid extract in chloroform was stored at -20 C

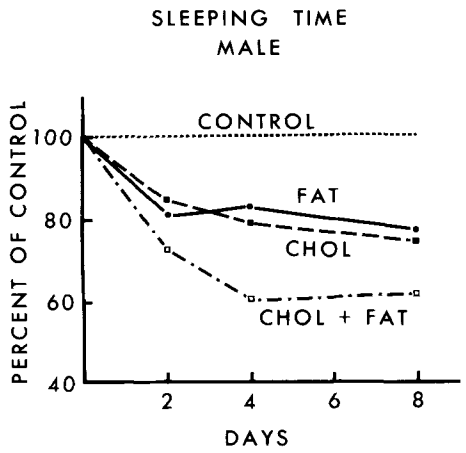

FIG. 1. Effect of 1% cholesterol and 10% corn oil diets on duration of pentobarbital anesthesia in weanling male rats fed for 2, 4 and 8 days. Significant AOV effects at $p<0.05$: 2 days, F; 4 days, C, F; 8 days, C, F. Each point represents the mean sleeping time of 6-8 rats. Controls slept 86 min; sleeping times are expressed as percentages of these control values.

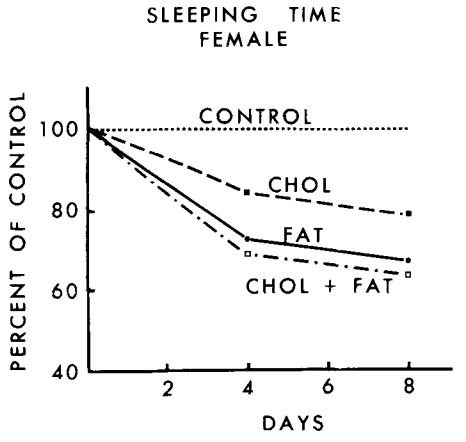

FIG. 2. Effects of 1% cholesterol and 10% corn oil diets on duration of pentobarbital anesthesia in weanling female rats fed for 4 and 8 days. Each point represents the mean sleeping time of 7-8 rats. Controls slept 80 min; sleeping times are expressed as percentages of these control values.

after addition of butylated hydroxytoluene (0.1% of estimated lipid weight) as recommended by Johnson (5). Liver lipids were determined gravimetrically on dried aliquots of the extracts.

Sodium heparin was used as anticougulant. Plasma was obtained by centrifugation of red blood cells at 1600 x g for 15 min at 4 C. Plasma cholesterol was determined on acetone/ethanol extracts (6) by the method of Pearson et al. (7). Free and total cholesterol in tissues were determined by the ferric chloride procedure of Zlatkis et al. (8) after digitonin isolation of the cholesterol (9).

Serum glutamic oxaloacetic transaminase (SGOT, EC 2.6.1.1) and serum glutamic pyruvic transaminase (SGPT, EC 2.6.1.2) are two serum enzymes which often increase as a result of liver damage, when a sufficient number of cells become damaged and membranes allow these enzymes to leak into the serum. SGOT and SGPT were determined by modifications of the procedures by Karmen (10) and Wroblewski and La Due (11) involving measurement of the change in NADH absorbance at 340 nm.

The experimental design was completely random with a factorial arrangement of treatments. Data was subjected to statistical analyses by the analysis of variance (ANOVA). When no significant interaction effect was present, significant main effects were determined. When a significant interaction effect was found, significant simple effects were determined.

RESULTS AND DISCUSSION

Pentobarbital Anesthesia

Two days after male weanling rats were placed on a diet containing cholesterol or fat, the duration of pentobarbital anesthesia was reduced ca. 20% (Fig. 1). No further reduction was observed at 4 or 8 days. The effects induced by cholesterol and fat were additive, and rats fed both cholesterol and corn oil exhibited sleeping times which were 60% of controls (Fig. 1). Pentobarbital anesthesia times were also determined in female weanling rats after being fed fat or cholesterol or a diet containing both constituents. Reduction of sleeping time after feeding fat or cholesterol was slightly less than in the males, and no greater decreases were observed in those rats fed both lipids (Fig. 2). At 4 days, none of the groups were significantly different from the controls; a fat effect approached statistical significance ($p\cong0.06$). At 8 days, all groups were different from the controls; the fat effect was apparent ($p=0.001$); a cholesterol effect approached statistical significance ($p\cong0.07$).

The demonstration that sleeping time was shortened indicated that the two lipids, cholesterol and corn oil, stimulated liver microsomal enzymes that metabolize pentobarbital. In classic studies, Selye (12) summarized the ability of several hundred drugs, insecticides, carcinogens, steroids and other foreign chemicals to stimulate drug metabolism in animals. Although cholesterol interaction with pentobarbital was not studied, cholesterol was found

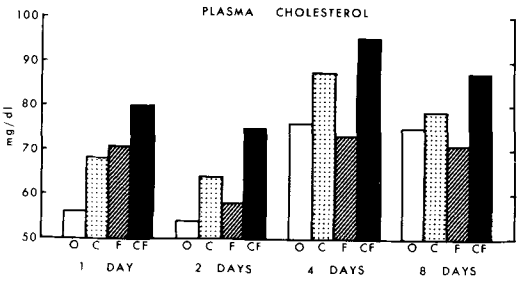

FIG. 3. Plasma cholesterol concentrations in young male rats fed diets containing no addition (O), 1% cholesterol (C), 10% corn oil (F), or 1% cholesterol plus 10% corn oil (CF) for 1, 2, 4 and 8 days. Significant AOV effects at $p<0.05$; 1 day, C, F; 2, 4 and 8 days, C. Bars represent mean values for six rats in each group.

to have no effect upon hexobarbital and cyclobarbital sleeping times, and a barely significant effect upon thiopental anesthesia in 100 g female rats (12). Ciaccio (13), in a review of drugs studied as stimulators of liver microsomal enzymes, has also cataloged cholesterol as having no effect. We are not aware of any reports of effects upon sleep time of corn oil or lipids per se, although both Brodie (14) and Mannering (15) have pointed out that compounds with low lipid solubility are poor inducers of drug-metabolizing microsomal enzymes. Potent inducers are, in general, highly soluble in organic solvents. While the molecular basis for liver microsomal enzyme induction is unknown, Conney (16) emphasized that the enzyme inducers are lipid-soluble molecules, capable of penetrating the microsomes. The shortened sleeping time of rats fed cholesterol, an animal fat, suggests the possibility that animals consuming this type of fat in their diet might be better able to metabolize a foreign chemical than animals subsisting on a plant diet. Whether herbivores or vegetarians are more susceptible to anesthetic action of drugs than carnivores is unknown, but Conney (16) has observed a similarity of effects in man and animals when liver microsomal enzymes are induced.

It is possible that the slightly shorter sleeping times observed in males is the result of a faster rate of metabolism of the barbiturate in male as compared to female rats. Sex differences have been reported in the activity of drug-metabolizing enzymes in liver microsomes (17). Quinn et al. (17) found that the metabolism of hexobarbital was similar in immature male and female rats, but between 5 and 6 weeks of age there was a decrease in hexobarbital action due to increased activity of the hexobarbital-metabolizing enzymes in the males. The rats of the present experiment were immature, but the greater stimulation of pentobarbital metabolism by cholesterol and corn oil in the males was suggestive of the initiation of sex differences in liver metabolism.

Blood and Liver Alterations

The effects of feeding the diets for 1, 2, 4 and 8 days upon plasma cholesterol of male weanling rats are shown in Figure 3. Increases in plasma cholesterol occurred when fat or cholesterol was fed, and these were apparent within the first 24 hr. Plasma cholesterol of the rats fed cholesterol was 23% greater than controls, 28% higher for rats fed the fat diet and 45% higher for rats fed both fat and cholesterol. The differences between controls and fat- and cholesterol-fed groups were less as the feeding continued, suggesting an adaptive mechanism for maintaining cholesterol hemeostasis in plasma as the rats aged.

Cholesterol- and corn oil-feeding caused only slight increases in liver weight during the short treatment periods of the present experiment (Table I), in contrast to a 20% increase observed when these diets were fed for 3 weeks (3). Changes in composition had occurred, however, and a progressive decline in water content was observed in the livers of rats fed cholesterol and fat: after 8 days, liver water concentration decreased by about 3%. An inverse relationship between water and lipid content was observed: as water content decreased, liver lipid increased. Rats fed both cholesterol and fat (Table I) showed a very marked synergistic effect: lipid and cholesterol increases were apparent after 24 hr and were significantly different after 2 days. Lipid content doubled at 4 days in rats fed both cholesterol and fat. Liver cholesterol content of rats fed cholesterol-fat was 2-fold greater at 2 days and 4-fold greater at 4 days than controls (Table I). In the male rats fed the control chow diet, almost all of the liver cholesterol was present as free cholesterol. After cholesterol-fat-feeding, all of the cholesterol deposited in the liver, above this basal amount of free cholesterol, was cholesterol ester.

Similar effects were observed when female weanling rats were fed the experimental diets for 4 or 8 days (Table II). Cholesterol-fat feeding caused a 35% increase in plasma cholesterol after 4 days, but as with the male rats, differences were less as feeding continued (27% increase at 8 days). After 4 and 8 days, liver water content declined 2 and 3%, respectively, while lipid content doubled. Total cholesterol

TABLE I

Effect of Dietary Fat and Cholesterol on Liver Composition of Male Rats

	Dietary group	Days on diet 1	2	4	8
Weight g	O	2.9 ± 0.2[a]	3.1 ± 0.1	4.2 ± 0.3	5.5 ± 0.2
	C	2.9 ± 0.2	3.1 ± 0.1	4.5 ± 0.3	5.0 ± 0.4
	F	2.8 ± 0.2	3.0 ± 0.2	3.8 ± 0.3	5.4 ± 0.1
	CF	2.7 ± 0.2	3.7 ± 0.1	4.6 ± 0.2	5.8 ± 0.4
	AOV	NS[b]	C x F	NS	NS
Water %	O	72.5 ± 0.2	71.8 ± 0.4	71.7 ± 0.5	71.2 ± 0.3
	C	72.2 ± 0.1	70.9 ± 0.3	71.1 ± 0.6	71.0 ± 0.4
	F	71.9 ± 0.2	71.9 ± 0.3	71.5 ± 0.2	70.6 ± 0.1
	CF	71.8 ± 0.2	70.8 ± 0.2	70.3 ± 0.5	67.8 ± 0.4
	AOV	F	C	NS	C x F
Lipid g/100 g	O	4.2 ± 0.2	4.2 ± 0.3	3.8 ± 0.2	4.2 ± 0.1
	C	4.5 ± 0.3	4.3 ± 0.1	4.7 ± 0.2	4.6 ± 0.1
	F	4.5 ± 0.2	4.5 ± 0.1	5.4 ± 0.1	5.1 ± 0.2
	CF	5.0 ± 0.2	5.4 ± 0.2	7.5 ± 0.5	8.9 ± 0.5
	AOV	NS	C x F	C,F, C x F	F, C x F
Total Cholesterol mg/g	O	2.8 ± 0.1	2.7 ± 0.1	2.0 ± 0.1	2.4 ± 0.1
	C	3.1 ± 0.1	3.2 ± 0.1	3.8 ± 0.3	4.2 ± 0.3
	F	3.0 ± 0.2	3.0 ± 0.2	2.9 ± 0.1	2.9 ± 0.1
	CF	3.7 ± 0.2	4.8 ± 0.3	8.8 ± 1.2	9.2 ± 0.8
	AOV	F, C	C, C x F	C x F	C, C x F

[a]Mean ± standard error for six rats.
[b]Not statistically significant at the 0.05 level.

doubled in 4 days and was 4-fold greater at 8 days. These compositional differences were not reflected by changes in liver weight.

Pathological changes were observed in the liver on gross examination after 4 days. Livers from rats fed either fat, cholesterol, or cholesterol and fat were lighter in color than controls and showed some mottling. Livers from rats fed cholesterol and fat for 8 days showed extensive mottling, probably due to fat infiltration.

The shortened pentobarbital sleeping time, the compositional changes observed (Tables I and II), and the altered appearance of the liver raised the possibility of liver cell damage. Serum glutamic-oxaloacetic and glutamic-pyruvic transaminase, two enzymes reflective of liver damage, were measured but showed no changes (Table III). It seems probable that the liver enzymes metabolizing pentobarbital were stimulated without cellular damage or with insufficient cell membrane damage to allow enzyme leakage into the serum.

Our results demonstrated that the rat responds very rapidly to the presence of cholesterol and fat in the diet. Within 24 hr, cholesterol- and fat-feeding produce significant increases in the lipid and cholesterol content of the liver. The rat usually does not respond to cholesterol-feeding with lipemia (18) and is considered to be a species resistant to dietary atherosclerosis. Drastic measures are required to produce atherosclerotic lesions. Commonly, poisoning of the thyroid with thiouracil and addition of bile salts are two features of rat atherosclerotic diets in addition to cholesterol and fat (18). Our data indicate that although cholesterol increases in the blood are relatively small, in contrast to those seen in the rabbit (2), cholesterol and lipid changes of great magnitude occur readily in the liver. Probably because of the resistance of the rat to atherosclerosis, we found few reports in the literature on short term cholesterol-feeding or on cholesterol-feeding without an atherogenic agent. Chanutin and Ludewig (19) reported an increase in liver cholesterol and lipids after feeding 2.5% cholesterol in a 20% fat diet for 2 days. Tsai and Dyer (20) fed adult rats a diet containing 2% cholesterol and 2% fat for 6 days and found an increase in liver cholesterol and long chain fatty acids, a measure of liver lipids.

Our studies suggest that the lipid-mobilizing effects mediated by cholesterol and the synergistic effects exerted by dietary cholesterol and fat, which we observed previously in rabbits, also occur readily in the rat. Cholesterol, or fat alone, produces relatively slight if any changes. Cholesterol and fat, however, exhibit a synergism and produce increases in liver cholesterol and lipid, a decrease in liver water concentra-

TABLE II

Effect of Dietary Fat and Cholesterol on Plasma Cholesterol and Liver Composition of Female Rats

	Dietary group	Days on diet 4	Days on diet 8
Plasma cholesterol mg/dl	O	80 ± 6[a]	59 ± 4
	C	92 ± 4	75 ± 4
	F	83 ± 5	65 ± 3
	CF	108 ± 9	75 ± 4
	AOV	C	C
Liver weight g	O	3.2 ± 0.1	4.5 ± 0.2
	C	3.6 ± 0.1	4.2 ± 0.2
	F	3.4 ± 0.2	4.7 ± 0.3
	CF	3.7 ± 0.2	5.2 ± 0.4
	AOV	C	NS[b]
Liver water %	O	69.8 ± 0.4	71.1 ± 0.2
	C	69.9 ± 0.4	71.0 ± 0.4
	F	70.2 ± 0.4	70.8 ± 0.5
	CF	67.8 ± 0.5	68.4 ± 0.7
	AOV	C x F	C x F
Liver lipid g/100 g	O	4.3 ± 0.1	4.5 ± 0.3
	C	4.6 ± 0.1	4.7 ± 0.1
	F	4.9 ± 0.1	5.2 ± 0.2
	CF	8.0 ± 0.4	8.7 ± 0.8
	AOV	C x F	C x F
Liver total cholesterol mg/g	O	2.6 ± 0.1	3.1 ± 0.3
	C	2.9 ± 0.1	3.8 ± 0.4
	F	3.1 ± 0.2	4.2 ± 0.3
	CF	5.9 ± 0.3	11.7 ± 1.7
	AOV	C x F	C x F

[a]Mean ± standard error for six rats.
[b]NS = Not statistically significant at the 0.05 level.

TABLE III

Glutamic-Oxaloacetic and Glutamic-Pyruvic Transaminases in Serum of Male Rats Fed Fat and Cholesterol for 8 Days

Dietary group	Glutamic-Oxaloacetic Transaminase[a]	Glutamic-Pyruvic Transaminase[a]
O	55 ± 3[b]	14 ± 1
C	55 ± 3	12 ± 1
F	56 ± 4	14 ± 1
CF	55 ± 3	12 ± 1
AOV	NS[c]	NS

[a]International Units per liter.
[b]Mean ± standard error for six rats.
[c]NS = Not statistically significant at the 0.05 level.

tion, and a shortening of pentobarbital sleeping time, thus indicating a stimulation of liver enzymes metabolizing pentobarbital. In contrast to the rabbit, blood cholesterol elevations are not maintained at a high level, but the liver rapidly exhibits large changes in composition. The rat is thus not a resistant species in the sense of no response to cholesterol. It is resistant to an increase in plasma cholesterol, and it does not readily develop atherosclerosis, but the rat's liver responds rapidly to dietary cholesterol and fat by accumulation of large lipid stores. Within 1 week, liver lipids double and liver cholesterol increases 4 times.

Since fat alone did not induce blood and tissue lipid increases, it appears that the rat is unable to absorb additional lipid from its diet. The addition of cholesterol resulted in large blood and tissue increases, indicating that the lipid-mobilizing effect is mediated by cholesterol. Cholesterol is probably converted to bile acids and salts in the liver, which then enter the usual enterohepatic circulation. These bile salts then aid in micellar and chylomicron formation and thereby stimulate an increase in the intestinal absorption of cholesterol and lipid from the diet.

Ostwald and her coworkers (21), in a series of studies on cholesterol metabolism in the guinea pig, have postulated that the accumulation of esterified cholesterol in the liver represents a major mechanism for maintaining

extrahepatic cholesterol homeostasis. In our short term studies in the rat, dietary cholesterol and fat caused a very large increase in cholesterol concentration in the liver but relatively small increases in plasma. Most of the increase in cholesterol can be accounted for as an increase in liver cholesterol ester. The data suggest that the maintenance of cholesterol homeostasis in the rat depends on liver accumulation of esterified cholesterol. Hepatic storage of excess cholesterol thus represents an important homeostatic control mechanism, allowing the blood and extrahepatic tissues to maintain normal cholesterol levels.

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Preparation of Cupric Palmitate Membrane, Its Characterization and Evaluation of Thermodynamically Effective Fixed Charge Density

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ABSTRACT

Membrane potentials have been measured across parchment-supported cupric palmitate membrane separating various 1:1 electrolytes at concentrations C_1 and C_2 such that $C_2 = 10\ C_1$. Membrane potential data have been used to calculate transference number of ions, permselectivity and also to derive the thermodynamically effective fixed charge density which is an important characteristic governing the membrane phenomena by utilizing the generally accepted and most widely used theory of Teorell-Meyer and Sievers as well as the recent theories for membrane potential of Kobatake et al. and Nagasawa et al. based on the principles of nonequilibrium thermodynamics. The values of charge densities derived from different theories were almost the same, confirming thereby the validity of the recently developed theories of membrane potential.

INTRODUCTION

Many biologically important substances resemble the membranous structures of the living cells. In recent years considerable attention has been focused on these structures in order to determine their properties, especially their permeability properties. Mueller et al. (1-3) generated the first bimolecular lipid layer membrane which has been used as a model by a number of investigators. Recently other complex membranes have been prepared by Liquori et al. (4-6), Hays (7), De Korosy (8), Lakshminarayanaiah and Siddiqi (9-11), Siddiqi et al. (12), Beg and coworkers (14-18), and utilized as a useful representation of living systems.

One of the most consistent properties of biological systems is the presence of a voltage across the cellular surfaces. The mechanism whereby this potential arises is still in dispute. Some consider it to be a diffusion potential while others suggest it to be an adsorption potential. Teorell (19-20), Meyer and Sievers (21-23) developed fixed charge theory, which is still regarded as the most pertinent starting point for the investigation of the acual mechanisms of the ionic or molecular processes which occur in the membrane phase. Based on the fixed charge concept, a number of mathematically rigorous equations for membrane potentials in recent years have been developed and their validity examined by taking simpler artificial membrane systems.

In this paper we describe the preparation of parchment-supported cupric palmitate membrane to be utilized as a biological model and the evaluation of its thermodynamically effective fixed charge density by the most recently, developed theories for membrane potential including those based on the principles of irreversible thermodynamics.

MATERIALS AND METHODS

Parchment-supported cupric palmitate membrane was prepared by the method of interaction suggested by Beg and coworkers (14-18). To precipitate cupric palmitate in the interstices of parchment paper (supplied by M/S Baird and Tatlock, London Ltd.) an aqueous 0.2 M cupric chloride solution was kept inside a glass tube, to one end of which was tied the parchment paper previously soaked in deionized water. This was suspended in a saturated sodium palmitate solution for 72 hr at room temperature (25 ± 0-1 C). The two solutions were interchanged later and kept for another 72 hr. The membrane thus obtained was thoroughly washed (4-5 times) with deionized water for the removal of free electrolyte. It was then cut into a circular disc form of radius 1.5 cm and clamped between two cylindrical half cells of capacity 50 ml each. The electrochemical cell of the type

$$\mathrm{Hg\text{-}Hg_2Cl_2} \left| \frac{\text{Satd. KCl}}{\text{agar}} \right| \frac{\text{Soln.}}{C_1} \left| \frac{\text{Mem-}}{\text{brane}} \right| \frac{\text{Soln.}}{C_2} \left| \frac{\text{Satd. KCl}}{\text{agar}} \right| \mathrm{Hg_2Cl_2\text{-}Hg}$$

was set up and was used for measuring electrical potentials arising across the membrane by maintaining a 10-fold difference in electrolyte concentration such that $C_2/C_1 = 10$ in the range 1×10^{-3} to 10^{-1} M using a Pye Precision

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TABLE I

Measured Membrane Potentials[a] E_m (Millivolts) Obtained across Cupric Palmitate Membrane for Various Electrolytes at Different Concentrations

Electrolyte concentrations eq./l	Membrane potentials (millivolts)		
	KCl	NaCl	LiCl
1/0.1	-4.05	-0.72	-15.80
0.5/0.05	-1.02	8.29	-14.00
0.1/0.01	+7.15	+1.27	-5.83
0.05/0.005	+17.07	+9.55	+1.50
0.01/0.001	+28.10	+17.90	+13.65

[a]Dilute solution side taken as positive.

TABLE II

Transference Number t_- of Coions Calculated from Membrane Potentials Measured across Cupric Palmitate Membrane for Various Electrolytes at Different Concentrations

Electrolyte concentrations eq.1/1	Transference number		
	KCl	NaCl	LiCl
1/0.1	0.53	0.58	0.63
0.5/0.05	0.51	0.57	0.62
0.1/0.01	0.44	0.49	0.55
0.05/0.005	0.36	0.42	0.49
0.01/0.001	0.26	0.35	0.38

Vernier Potentiometer No. 7568. The solutions in both the half cells were vigorously stirred by a pair of magnetic stirrers. The solutions were replaced by fresh solutions and, when there was no change in potential with the addition of fresh solution, it was taken as true membrane potential. The potentials could be reproduced within a few tenths of a millivolt. The salt solutions were prepared from the analytical grade reagent (BDH India) without further purification and using deionized water. Sodium palmitate used in the investigation was obtained by mixing 0.2 M aqueous caustic soda solution with 0.2 M palmitic acid solution previously recrystallized with ethyl alcohol.

RESULT

The measured values of membrane potentials across the cupric palmitate membrane in contact with various uni-univalent electrolytes at different concentrations C_1 and C_2 such that $C_2/C_1 = 10$ are given in Table I.

The values of the transference number calculated from the membrane potential measurements and using the Nernst equation are given in Table II. Thermodynamically effective fixed charge density of the membrane in contact with different electrolytes have been derived following the procedures of Teorell-Meyer and Sievers (19-21), Kobatake and coworkers (24-26) method, and the most recently developed method of Nagasawa et al. (27). The results are given in Table III.

DISCUSSION

When two electrolyte solutions are separated by a membrane, the mobile species penetrate the membrane and vaious transport phenomena are induced into the system. In particular, a potential is generated which depends upon the charge on the membrane and its porosity. When the membrane pores are too wide, any charge does little to generate good potentials, but if the pores are narrow a little charge on it gives a potential E according to the Nernst equation

$$E = \frac{RT}{F} \ln \frac{f_2 C_2}{f_1 C_1}$$

where C_1 and C_2 are the elctrolyte concentrations on either side of the membrane; other symbols have their usual significance.

The values of the membrane potential are smaller when the membrane is used to separate concentrated electrolyte solutions, and when it is separating dilute solutions the values are higher. Such behavior of the membrane is not peculiar to this system (9). The variation of

TABLE III

Derived Values of Mobility Ratio, and Effective Fixed Charge Density of Cupric Palmitate Membrane by Various Methods

Methods / Electrolytes eq./l.	TMS $\bar{u}/\bar{v}$	TMS $\bar{x}$ eq./l	Kobatake et al. Qx eq./l.	Nagasawa et al. $\bar{x}$eq./l.
KCl	1.0	1.1×10^{-2}	3.2×10^{-2}	1.5×10^{-2}
NaCl	0.3	9.3×10^{-2}	3.7×10^{-2}	1.7×10^{-2}
LiCl	0.6	8.0×10^{-2}	3.0×10^{-2}	1.8×10^{-2}

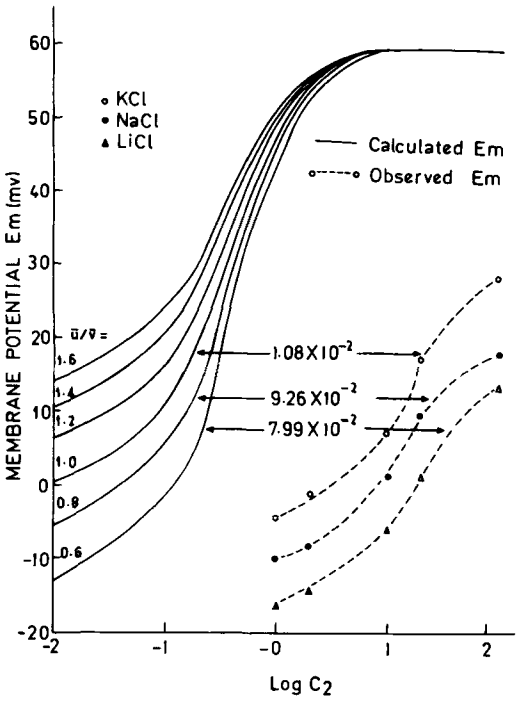

FIG. 1. Evaluation of the membrane charge density $\bar{X}$ and mobility ratio $\bar{u}/\bar{v}$. The smooth curves on the left are the theoretical concentration potentials for a cation selective membrane ($\bar{X}$ = 1) at different mobility ratio $\bar{u}/\bar{v}$. The experimental values of E_m for cupric palmitate membrane are shown by broken lines (see texts.)

membrane potential with electrolyte concentration may be attributed to the change in the selectivity character of the membrane for the ions of the elctrolyte at different concentrations. This is in agreement with the values of the transference number given in Table II.

Teorell (19-20), Meyer and Sievers (21-23) considered the membrane as a charged barrier. The total membrane potential was considered to be made up of two Donnan potentials at the two membrane solutions interfaces and a diffusion potential within the membrane. For a highly idealized system, these authors derived the following equation for membrane potential:

$$E_m = 59.16 \left[\log \frac{C_2 \left(\sqrt{4C_1^2 + \bar{X}^2} + \bar{X}\right)}{C_1 \left(\sqrt{4C_{22}^2 + \bar{X}^2} + \bar{X}\right)} + \bar{U} \log \frac{\sqrt{4C_2^2 + \bar{X}^2} + \bar{X}\bar{U}}{\sqrt{4C_1^2 + \bar{X}^2} + \bar{X}\bar{U}} \right] \quad (1)$$

Here $\bar{U} = (\bar{u}-\bar{v})/(\bar{u}+\bar{v})$; and $\bar{u}$ and $\bar{v}$ are the mobilities of the cation and anion, respectively, in the membrane phase, and $\bar{X}$ is the charge on the membrane expressed in equivalents/liter of imbibed solution. In order to evaluate this parameter for the simple case of a 1:1 electrolyte and a membrane carrying net negative charge of unity ($\bar{X}$=1), theoretical concentration potentials E_m existing across the membrane were calculated as a function of C_2, the ratio C_2/C_1 being kept at a constant value of 10 for different mobility ratios $\bar{u}/\bar{v}$ and plotted as shown in Figure 1. The observed membrane potential values with various 1:1 electrolyte solutions were also plotted on the same graph. The experimental curve was shifted horizontally and ran parallel to one of the theoretical curves. The extent of this shift gave log $\bar{X}$, and the parellel theoretical curve gave the value for $\bar{u}/\bar{v}$. The values for $\bar{X}$ and $\bar{u}/\bar{v}$ derived in this way for the membrane electrolyte systems are given in Table III. It may be mentioned here that this method (The TMS method) has been generally used and widely accepted for the evaluation of the effective fixed charge density of a membrane.

Kobatake and Kamo (24-25) derived another equation 2 based on fixed charge concept for membrane potential using a different set of assumptions; viz. (a) the contribution of mass movement is negligible (17), and (b) small ions do not behave ideally in a charged membrane (17).

$$E_m = \frac{RT}{F} \left[\ln \frac{C_2}{C_1} + (2\alpha - 1) \times \ln \frac{\sqrt{4C_2^2 + \phi^2 X^2} + (2\alpha - 1)\phi X}{\sqrt{4C_1^2 + \phi^2 X^2} + (2\alpha - 1)\phi X} \ln \frac{\sqrt{4C_2^2 + \phi^2 X^2} + \phi X}{\sqrt{4C_1^2 + \phi^2 X^2} + \phi X} \right] \quad (2)$$

where ϕ is a characteristic factor of the membrane-electrolyte pair, and represents a fraction of counterions not tightly bound to the membrane skeleton. The product ϕX is termed the thermodynamically effective fixed charge density of a membrane. Other terms have their usual significance. Equation 2 has the same functional form as that given by the TMS theory for the membrane potential E_m, i.e., Eq. 1 except that the thermodynamically effective fixed charge density ϕX of the membrane is used in place of stoichiometric fixed charge density $\bar{X}$. Equation 2 reduces to the TMS membrane potential for $\phi = 1$. Since it is somewhat troublesome to evaluate ϕX at an arbitrary external electrolyte concentration

from the observed membrane potential E_m using eq. 2, Kobatake and Kamo (24) have proposed a simple method using the following approximate equation for the diffusive contribution to the emf of a cell with transport

$$E_m = -\frac{RT}{F}(1 - 2\,T_{-app})\ln C_2/C_1 \qquad (3)$$

where T_{-app} is the transference number of coions in the membrane phase. Comparison of eqs. 2 and 3 yield

$$T_{-app} = \frac{1-2\alpha}{2} \frac{\ln\left(\frac{\sqrt{4\xi_2^2+1}+2\alpha-1}{\sqrt{4\xi_1^2+2}+2\alpha-1}\right)}{2\ln r} + \frac{\ln\left(\frac{\sqrt{(4\xi_2^2+1}+1}{\sqrt{4\xi_1^2+1}+1}\right)}{2\ln r} \qquad (4)$$

where

$$\xi = C/\phi X \text{ and } \gamma = C_2/C_1$$

On the other hand (17,18), the mass-fixed transference number of coions τ- in a negatively charged membrane immersed in an electrolyte solution of concentration C was defined by

$$T_- = vc_- / (uc_+ + vc_-) \qquad (5)$$

where C_+ and C_- are the concentrations of cation and anion, respectively, in the membrane phase. This equation was transformed to

$$T_- = 1-\alpha-\frac{\sqrt{4\xi^2+1}+1}{\sqrt{4\xi^2+1}+(2\alpha-1)} \qquad (6)$$

using the equations for the activity coefficients. mobilities of small ions in the membrane phase, and the equilibrium condition for electrical neutrality. The difference between T_{-app} calculated from eq. 4 and T_- from eq. 6 for various reduced concentrations was found to be less than 2%. Therefore, T_{app} and T_- were considered practically the smae. As a result, the apparent transference number T_{app} evaluated from the membrane potential data could be used for the determination of thermodynamically effective fixed charge density ϕX of the membrane at an average salt concentration C

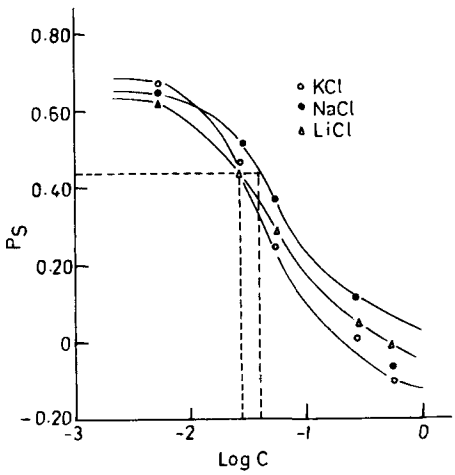

FIG. 2. Plots of P_S vs. log c for cupric palmitate membrane using various 1:1 electrolyte solutions. The dotted horizontal line shows $P_S = 1/\sqrt{5}$, and the vergical lines give the value of ϕX for the membrane-electrolyte system.

(i.e., $(C_1+C_2)/2$) using eq. 6. Rearrangement of equation 6 gave the definition of permselectivity P_s by the expression

$$\frac{1}{(4\xi^2+1)^{1/2}} = \frac{1 - T_- - \alpha}{\alpha-(2\alpha-1)(1-T_-)} \equiv P_s. \qquad (7)$$

This equation was used to find the permselectivity P_s from the membrane potential measurements using eq. 3. If the transport number of coions (T_- or T_{-app}) is zero, the membrane is perfectly selective and $P_s = 1$, while if the transport number of coions has the value in free solution, $P_s = O$. The values of P_s obtained using the right hand side of eq. 7 were plotted against log C. The concentration at which P_s becomes $1/\sqrt{5}$ (i.e., $\xi = C/\phi X = 1$) gives the value of the thermodynamically effective fixed charge density ϕX as demanded by the left hand side of eq. 7. Figure 2 represents a plot of P_s vs. log C for the parchment-supported cupric palmitate membrane in contact with various 1:1 electrolytes. The values of ϕX thus derived for the membrane-electrolyte systems are given in Table III.

Most recently, Tasaka et al. (27) derived another equation for the membrane potential existing across a charged membrane. The total membrane potential E_m was considered as the sum of a diffusion potential inside the membrane and the electrostatic potential difference between the membrane surfaces and electrolyte solutions on both sides of the membrane. The diffusion potential E_d was obtained by inte-

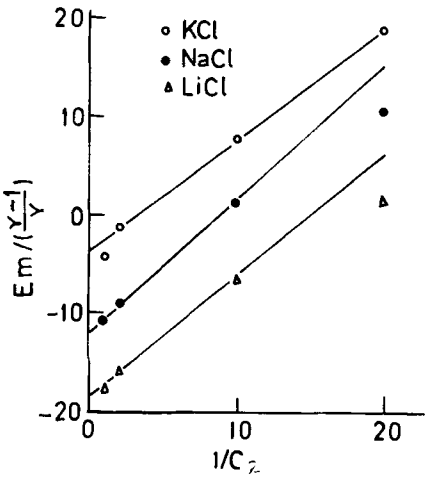

FIG. 3. Plots of membrane potential $E_m/(\gamma\text{-}1)/\gamma$ vs. $1/c_2$ for cupric palmitate membrane.

grating the basic flow equation for diffusion while the electrostatic potential was calculated from the Donnan's theory. At sufficiently high electrolyte concentrations, these authors derived the following approximate equation for membrane potential E_m

$$-E_m = \frac{RT}{F}\left(\frac{\gamma - 1}{\gamma}\right)\left(\frac{\phi X}{2}\right) 1/C_2 + \ldots \quad (8)$$

Equation 8 predicts a lienar relationship between E_m and $1/C_2$ from which ϕX can be calculated. A set of straight lines in Figure 3 are in full agreement with eq. 8. The values of ϕX derived from the slope of the lines are given in Table III.

Table III shows that the values of the charge densities evaluated from the various procedures are not much different from each other and that the values are comparable to those derived by the TMS theory. A slight difference in the values of ϕX may be ascribed to the different graphical procedures adopted for the evaluation. It may therefore be concluded that the recently developed theoretical equations for membrane potential are quite sound and that their use for the evaluation of effective fixed charge density of membrane is justified for at least the systems under investigation.

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The authors are grateful to Prof. W. Rahman, Head, Department of Chemistry, Aligarh Muslim University, Aligarh for providing the necessary research facilities and also to C.S.I.R. (India) for the award of fellowship to Akmal Husain and Badrul Islam.

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METHODS

High-Pressure Liquid Chromatography and Ultraviolet Spectrometry of Ketonic C_{27} Sterols

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ABSTRACT

A method for the separation of ketonic C_{27} sterols was devised, based on high-pressure liquid chromatography (HPLC) and ultraviolet absorption (UV). The adsorption column contained silica gel, particle size 10 μm, and the eluents were dichloromethane/*n*-hexane/ethyl acetate (94:5:1) and dichloromethane/ethyl acetate (99:1) followed by dichloromethane/ethyl acetate (3:1). The 5β-sterols were eluted before the 5α-analogs, sterols with isolated double bonds before conjugated carbonyl compounds, and ketones before hydroxy ketones. The effect of carbonyl groups on polarity depends on the position in the molecule and decreases in the order C-3 > C-6 > C-7. The ultraviolet absorption spectra of eleven sterols were determined, and their absorbance at 254 nm and at 280 nm was used for analyzing the column effluent with a dual detector system.

INTRODUCTION

Ketonic C_{27} sterols are important intermediates in animal (1) and plant (2) metabolism. Among the various analytical methods (3), the separation of individual metabolites by chromatography (4) prior to the application of nonspecific tests is presently the most useful procedure. Gas-liquid chromatography has greater resolving power than liquid-solid chromatography, but because it is unsuitable for the isolation of adequate amounts of radioactive metabolites, we have chosen liquid chromatography for our work on the biosynthesis and metabolism of plant sterols (5).

We have reported the separation of free sterols by high-pressure liquid chromatography (HPLC) earlier (6). Our present report deals with the HPLC and ultraviolet (UV) absorption of their ketonic derivatives. To our knowledge, the behavior of this group of sterols in HPLC and some of their UV spectra have not been studied previously. The structures of the eleven steroids examined in this connection are shown in Figures 1 and 2. In every case, R stands for the iso-octyl side chain of cholesterol.

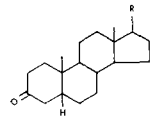
MATERIALS AND METHODS

The HPLC apparatus was assembled from commercially available components. The pump was of the dual-piston reciprocating type, Tracor Model 990 (Tracor, Austin, TX). The variable-wavelength detectors were: Tracor Model 970, set at 280 nm, and Altex/Hitachi Model 100-30 (Altex, Berkeley, CA), set at 254 nm. A dual-channel recorder, Linear Model 385 (Linear, Irvine, CA) was attached to the output of the detectors.

The column consisted of two stainless-steel chromatographic tubes (Alltech, Arlington Heights, IL), each 25 cm long and 4.6 mm I.D., packed with LiChrosorb Si-60-10 (E. Merck, Darmstadt, Germany), and connected in series. The column was prepared in our laboratory from a balanced-density slurry of the silica gel in a mixture of tetrabromoethane and tetrachloroethane. The slurry was packed into the tubes with a Haskel HPLC slurry packing unit, Model 29426. The efficiency of the two sections was determined by using dinitrobenzene as the test material and hexane as the eluent. Plate counts were 4000 for each section.

The inlet of the column was connected to a sample injection valve (Altex Model 905-23) and its outlet to the inlet of the Tracor detector. The outlet of that detector was connected to the inlet of the Altex detector. The effluent from the latter was then returned to the solvent reservoir. It was found that 100 ml of solvent could be recycled for at least 10

[1]Plant Biochemistry Research Unit.
[2]Instrumental Analysis Research Unit.
[3]Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.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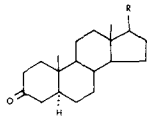
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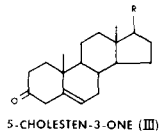
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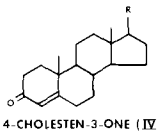
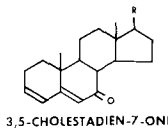

4-CHOLESTEN-3-ONE (IV)

FIG. 1. Structures of ketonic C_{27} sterols I-IV. R = $-CHMe(CH_2)_3CHMe_2$.

chromatograms before the background absorption showed signs of contamination.

All solvents were "Distilled in Glass" quality (Burdick and Jackson, Muskegon, MI). For Compounds I-IV, the eluent was dichloromethane/*n*-hexane/ethyl acetate (94:5:1), and for Compounds V-IX, the eluent was dichloromethane/ethyl acetate (99:1). After IX was eluted, both pump and chart were stopped and the eluent was changed to dichloromethane/ethyl acetate (3:1). Pump and chart were started again, and X and XI were eluted.

For the determination of the UV spectra, the vacuum-dried samples were weighed on a Cahn Model G Electrobalance (Cahn, Cerritos, CA) and dissolved in isopropyl alcohol. The concentrations were chosen to give suitable recordings when the solutions were examined, with pathlengths ranging from 0.5 to 100 mm, in a Cary Model 14 Recording Spectrophotometer (Varian, Palo Alto, CA). Since fixed-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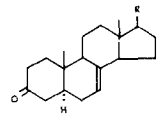
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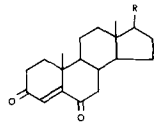
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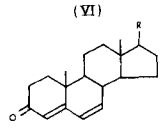
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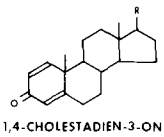
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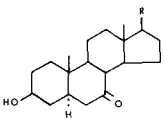
3β-HYDROXY-5α-CHOLESTAN-7-ONE (X)

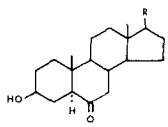
3β-HYDROXY-5α-CHOLESTAN-6-ONE (XI)

FIG. 2. Structures of ketonic C_{27} sterols V-XI. R = $-CHMe(CH_2)_3CHMe_2$.

wavelength detectors commonly used for HPLC operate at 254 and 280 nm, we determined the molar extinction at these two wavelengths.

RESULTS AND DISCUSSION

The molar extinctions, ϵ, at 254 and 280

TABLE I

Summary of UV Spectra of Ketonic C_{27} Sterols

Compd. No.	ϵ_{254}[a]	ϵ_{280}[a]	$\epsilon_{254}/\epsilon_{280}$	λ_{max}[a]	ϵ_{max}[a]	Literature λ_{max}	Literature log ϵ	Literature Ref.
I	16.7	21.8	0.766	281	21.9	285[b]	1.18[b]	7
				245	17.3	235[b]	1.13[b]	7
II	10.2	20.9	0.49	284.5	23.4	283[c]	1.36[c]	8
III	231	108	2.14	286	109	---	---	---
IV	9,500	56.8	167	241	17,500	240.5[c]	4.29[c]	9
				315	72	310[c]	1.80[c]	10
V	7,300	18,000	0.41	278	18,200	280[c]	4.33[c]	11
VI	240	35.3	6.8	245	286	---	---	---
VII	7,300	1,220	5.98	250	7,600	253[b]	4.05[b]	12
VIII	7,100	25,000	0.284	283.5	25,800	286[c]	4.43[c]	13
IX	12,700	1,920	6.61	244.5	15,100	245[c]	4.15[c]	14
X	6.24	30.4	0.21	291	36	287[cd]	1.6[cd]	15
XI	8.0	30.4	0.26	289	34.5	280[cd]	1.6[cd]	15

[a]Isopropyl alcohol.
[b]Ethyl ether.
[c]Ethyl alcohol.
[d]Acetate.

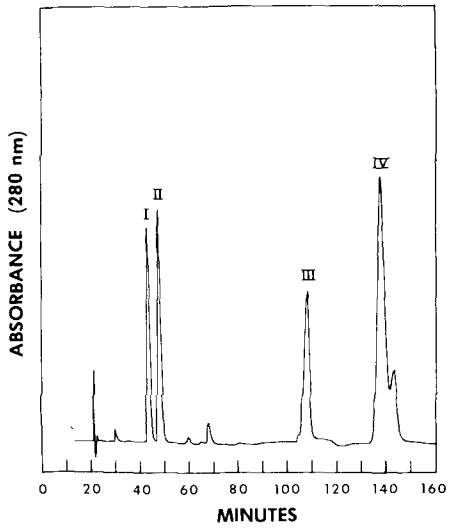

FIG. 3. Elution curve for ketonic C_{27} sterols I-IV (cf. Fig. 1). A mixture of 4000 μg I, 4000 μg II, 1100 μg III, and 160 μg IV in 50 μl dichloromethane/ethyl acetate (99:1) was applied to a 2-ft x ¼-in. O.D. column of LiChrosorb Si-60-10. Eluent, dichloromethane/*n*-hexane/ethyl acetate (94:5:1); flow rate, 9.5 ml/min; pressure, 550 psi. Detector at 280 nm; range 0.16; recorder speed, 6 cm/hr; span, 20 mV.

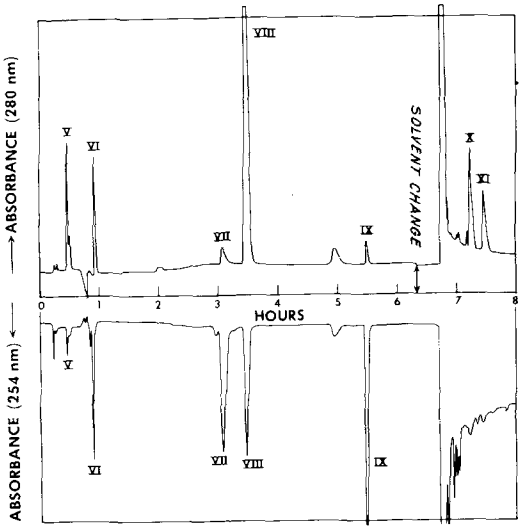

FIG. 4. Elution curve for ketonic C_{27} sterols V-IX (cf. Fig. 2). A mixture of 6.5 μg V, 1645 μg VI, 15 μg VII, 17.6 μg VIII, 12 μg IX, 4750 μg X, and 4500 μg XI was chromatographed under the conditions given for Fig. 3, with the following exceptions: the column effluent was passed through two detectors in series, the first one set at 280 nm and the second one at 254 nm. Dichloromethane/ethyl acetate (99:1) was the eluent until IX had emerged, when the eluent was changed to dichloromethane/ethyl acetate (3:1). Range, 0.32; recorder speed, 3 cm/hr.

nm, their ratio, the absorption maximum, λ_{max}, and molar extinction at the maximum of each of the eleven ketonic sterols in our study are compared with available literature values in Table I.

While most of the values determined by us essentially agree with the published values, the literature values for IV range from 14,000 to 19,500. Barton and Jones (9) improved the chromatographic purification of this compound, but they did not report the weaker R band at 310 nm (10). The literature value for ϵ_{max} of V seems too high, and we fail to find any evidence of an R band near 325 nm (11). We have found no literature values for the UV spectra of III, VI, X, and XI, but the published spectra of X acetate and XI acetate are comparable to ours.

The data will be useful for future quantitative analyses. Qualitatively, it is of interest that the conformational change from the 5β- to the 5α-epimers leads to the disappearance of the absorption band at 245 nm. With the greater purity now achievable by HPLC, even weak absorption bands, such as these, should be useful in solving conformational and structural problems.

The less polar ketones I-IV (Fig. 1) were separated by elution with dichloromethane/*n*-hexane/ethyl acetate (94:5:1) and detected at 280 nm (Fig. 3). The A/B *cis*-sterol I is less polar than the A/B *trans*-sterol II, as usual (16). The unsaturated sterols III and IV are more polar than the saturated ones and, among the former, the conjugated carbonyl compound IV is more polar than the steroid III with an isolated double bond.

For the more polar ketones V-XI (Fig. 4), detection at 254 nm as well as at 280 nm proved useful. Sterols with a high $\epsilon_{254}/\epsilon_{280}$ ratio (Table I), i.e., VI, VII, and IX, are more easily detected at 254 nm, whereas sterols with a low $\epsilon_{254}/\epsilon_{280}$ ratio, i.e., V, VIII, X, and XI, are best analyzed at 280 nm.

The ketones V-VIII are eluted at a lower concentration of ethyl acetate in dichloromethane than the hydroxy ketones X and XI. Among the 3-ketones, the least polar is VI, having an isolated double bond. Then comes the α, β-unsaturated ketone VII, which is followed by the heteroannular dienone VIII and, finally, the homoannular dienone IX. The position of V and VII in this chromatogram requires comment.

Compound V is the least polar sterol in this series, although it is a conjugated dienone. Compound VII is a diketone which turns out to be less polar than the monoketones VIII and

IX. Apparently, the effect of carbonyl groups on polarity depends on the position in the molecule and decreases in the order C-3 > C-6 > C-7. Thus, VI, a 3-ketone with an isolated double bond, is more polar than the 7-ketone V, although the 7-carbonyl group is conjugated. The effect on polarity of the carbonyl group at C-6 in VII is less than that of the double bond at C-6 in VIII. The slight difference between the effects of C-6 and C-7 is enough to permit the resolution of the position isomers X and XI.

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COMMUNICATIONS

Cholesteryl Ester and Triacylglycerol Fatty Acids in Type V Hyperlipidemia

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ABSTRACT

The fatty acid compositions of plasma cholesteryl esters (CE) and triacylglycerols (TG) from seven healthy individuals and five patients with type V hyperlipoproteinemia were determined. Very low density (VLDL), low density (LDL) and high density lipoproteins (HDL) were isolated. The lipids were extracted from the lipoproteins and the triacylglycerols and cholesteryl esters separated for analysis. The fatty acid compositions of triacylglycerols from healthy and type V individuals were very similar. The cholesteryl esters from type V patients had increased contents of palmitic and decreased amounts of linoleic and arachidonic acids as compared to the normal individuals. The fatty acid composition of the cholesteryl esters from the high density lipoproteins had the greatest deviation. The fatty acid compositions of the triacylglycerols from the two groups were similar. However, the triacylglycerols in all lipoprotein fractions contained more palmitic and oleic and less linoleic and arachidonic acids than the cholesteryl esters.

INTRODUCTION

The objective of this study was to determine if the fatty acid compositions of cholesteryl esters and triacylglycerols associated with very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoprotein (HDL) differs between healthy individuals and patients with type V hyperlipoproteinemia. The fatty acid composition of the triacylglycerols (TG) and cholesteryl esters (CE) will, in part, determine the ultrastructure of the lipoproteins, and any modification of lipoprotein structure could alter lipid transport.

The type V individual has quantitative differences in plasma lipids which are used to diagnose the disorder. The characteristics of fasting plasma from individuals suffering from type V hyperlipoproteinemia (HLP) are abnormally high TG concentrations, 10 to 20 times normal, in conjuction with normal to moderately elevated cholesterol levels (1). As a result, the concentrations of TG-rich particles, chylomicrons and VLDL are increased in the plasma, while there are decreases in LDL and HDL (1-3). Very little attention has been directed towards qualitative differences in fatty acids which might be associated with type V HLP. In this study, the fatty acid compositions of TG and CE were analyzed to obtain information, hopefully assisting in further elucidation of the disorders underlying lipid transport and metabolism in type V HLP.

MATERIALS AND METHODS

Seven clinically healthy individuals and five individuals suffering from type V HLP were used. The healthy individuals were drawn from the University of Connecticut faculty and staff and had no obvious medical abnormalities. The healthy individuals were all male. Initial selection of the healthy individuals included a normal lipoprotein pattern by agarose gel electrophoresis and a total fasting plasma cholesterol and triglyceride measurement. Type V plasma samples were obtained from the Lipid Clinic at the Methodist Hospital at Baylor College of Medicine in Houston, TX. Plasma from one female and four males with type V hyperlipoproteinemia were analyzed. Phenotyping was performed using the methodology of The Lipid Research Clinics (4), and hyperchylomicronemia was observed in all patients. In addition, measurements of post heparin lipolytic activity (PHLA) were done, but the results were questionable because of the high baseline TG concentrations; PHLA was shown to be qualitatively present by the electrophoretic strip technique.

A brief description of techniques used in this study follows. A more complete explanation has been presented in previous work published from this laboratory (5,6). Lipoprotein classes were isolated using a preparative ultracentrifuge based on the following densities: VLDL, 1.006 g/ml; LDL, 1.006-1.063 g/ml and HDL, 1.063-

TABLE I

Lipoprotein Fatty Acid Composition of Triacylglycerols
from Normal Individuals and Patients with Type V Hyperlipoproteinemia

Group	N	Fatty acids M %							
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Healthy VLDL									
Male	6	1.3 ± 0.9[a]	28.8 ± 2.8	4.4 ± 1.8	2.2 ± 1.0	42.7 ± 4.5	18.7 ± 3.5	1.1 ± 0.5	Tr[b]
Type V VLDL									
Male	3	2.8 ± 0.8	29.4 ± 1.5	7.7 ± 1.9	3.8 ± 1.1	38.5 ± 2.5	16.3 ± 1.2	1.0 ± 0.5	Tr
Female	1	Tr	31.6	5.4	3.4	41.8	14.4	Tr	1.3
Healthy LDL									
Male	6	1.3 ± 0.6	29.2 ± 1.9	4.2 ± 1.4	3.5 ± 1.3	43.9 ± 2.2	16.2 ± 2.6	Tr	1.0 ± 0.4
Type V LDL									
Male	3	3.1 ± 0.3	32.7 ± 2.4	6.4 ± 0.8	5.4 ± 1.1	38.5 ± 2.0	13.1 ± 2.3	Tr	Tr
Female	1	1.0	32.7	6.1	2.7	43.3	13.5	Tr	Tr
Healthy HDL									
Male	6	1.4 ± 0.5	27.8 ± 4.4	4.5 ± 1.5	3.5 ± 0.5	42.6 ± 2.5	15.9 ± 4.4	Tr	1.1 ± 0.4
Type V HDL									
Male	3	2.5 ± 0.2	29.3 ± 1.2	7.1 ± 0.8	4.9 ± 1.3	38.4 ± 2.1	14.7 ± 3.2	Tr	1.0 ± 0.1
Female	1	1.3	32.5	6.5	2.8	41.6	14.2	Tr	1.2

[a]± Standard deviation.
[b]Tr indicates fatty acid contributed less than 1 mole percent.

1.21 g/ml. Chylomicrons were isolated from type V plasma by layering with distilled water and centrifuging at 300 x g for 30 min. After removing the water layer, the plasma was layered again with water and centrifuged. Then plasma was layered with 1.006 g/ml density solution and centrifuged at 300 x g for 30 min. This process was repeated until the chylomicrons were removed. Purity of lipoprotein fractions was determined by agarose gel electrophoresis.

Lipids were extracted by the procedure of Folch *et al.* (7) and neutral and polar fractions separated on a Unisil column (Clarkson Chemical Co., Williamsport, PA). Cholesteryl esters and TGs were then separated by thin layer chromatography. Methyl esters of the fatty acids were made and analyzed using gas liquid chromatography. The column was packed with SP-2330 cyano-silicone coating on 100/120 Chromosorb (Supelco, Inc., Bellefonte, PA).

The results from the determinations of the cholesteryl esters fatty acids were statistically analyzed using multivariant analysis and significance taken at the .05 level as determined by Hotelling's T^2 (8). The dependent variables were the combined mole percents of 18:2 and 20:4 in VLDL, LDL and HDL. Multivariant analysis weighs the contribution of VLDL, LDL and HDL equally in determining difference between the type V plasma and normal plasma. The relative contribution of each lipoprotein class towards this difference then can be separated to determine if all the lipoprotein classes are effected equally.

RESULTS AND DISCUSSION

The mean age for the healthy individuals was 44 ± 8 yr, the male type V patients 53 ± 9 yr, and the female type V was 41 yr. The healthy persons had mean total plasma cholesterol (C) contents 254.4 ± 28.0 mg/100 ml and mean TGs were 86.8 ± 15.0 mg/100 ml. The values for the male type V patients were C 437. ± 53.6 mg/100 ml and TGs, 1053.5 ± 545 mg/100 ml. The female type V had C and TG concentration of 954 mg and 3890 mg/100 ml, respectively. The proportions of CEs were not determined but have been reported to be ca. 70% of the total for both normal and hypertriglyceridemic persons, although the types of the latter group were not given (9).

The composition of the TG fatty acids are given in Table I. There was little difference between the two test groups. These results may suggest that fatty acid compositions of total TGs are not a significant factor affecting lipid transport in the type V individual. This does

TABLE II

Fatty Acid Composition of Plasma Cholesteryl Esters in Healthy Individuals and Type V Hyperlipoproteinemia Patients

Group	N	Fatty acids M %						
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Healthy VLDL	7	Tr[a]	14.7[b] ± 3.1	2.2 ± 0.9	1.2 ± 1.0	23.2 ± 4.2	52.5 ± 6.9	Tr	4.9 ± 2.1
Type V VLDL									
Male	4	1.0 ± 0.6	16.9 ± 3.3	4.4 ± 2.1	1.4 ± 0.3	23.5 ± 1.1	47.4 ± 3.0	Tr	4.3 ± 1.2
Female	1	1.3	28.2	9.6	1.1	35.0	23.8	Tr	1.0
Healthy LDL	7	Tr	11.3 ± 2.1	2.1 ± 1.1	Tr	19.8 ±3.0	57.8 ±2.8	Tr	6.6 ± 1.8
Type V LDL									
Male	4	3.6 ± 3.7	14.8 ± 1.9	1.5 ± 0.6	1.3 ± 0.6	21.0 ± 2.4	49.5 ± 7.2	Tr	4.6 ± 1.6
Female	1	1.1	25.4	8.0	2.8	31.8	30.2	Tr	1.0
Healthy HDL	7	Tr	10.8 ± 2.8	1.9 ± 1.0	Tr	18.6 ± 4.2	60.1 ± 3.1	Tr	6.9 ± 2.8
Type V HDL									
Male	4	3.8 ± 2.2	21.1 ± 8.4	3.8 ± 2.1	2.2 ± 2.0	19.2 ± 1.7	41.9 ± 11.5	1.4 ± 1.2	4.6 ± 1.0
Female	1	Tr	22.0	10.2	1.0	29.0	37.6	Tr	Tr

[a]Tr indicates fatty acid content of less than 1 M %.
[b]± Standard deviation.

not eliminate the possibility of significant differences in structural isomers of TGs or influences therefrom.

The fatty acid compositions of plasma CE in healthy individuals and type V HLP patients are presented in Table II. In all three lipoprotein classes, the proportion of 16:0 increased while 18:2 decreased. These results are in agreement with the total plasma CE determinations made by Allard et al. (10). The female type V had the most extreme degree of deviation from healthy individuals. This may not be due to gender as the fatty acid composition of CEs in healthy males and females is similar (11). The female type V in this study had the highest plasma C and TG concentration which might account for the extreme variation in fatty acid composition of CEs.

In previous work it was found that the fatty acid compositions of CEs were altered to a greater extent than those TGs in the various HLPs (10). Our results support this observation at least with type V. Using multivariant analysis, the amounts of 18:2 and 20:4 in the CE from the individuals with type V hyperlipoproteinemia were significantly ($P < .05$) less than those from healthy individuals. The primary source of this variation was in the HDL fraction. The apparent unavailability of 18:2 and 20:4 in type V HLP, particularly the HDL, should be investigated further.

The amounts of fatty acids in the CEs and TGs were markedly different. The CEs in all lipoprotein fractions contained much less 16:0 and 18:1 and more 18:2 and 20:4 than the TGs, suggesting that there is no apparent precursor-product relationship between the CE and TGs.

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Lipoprotein Lipid and Protein Synthesis in Experimental Nephrosis and Plasmapheresis. I: Studies in Rat in vivo

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ABSTRACT

The incorporation of L-4,5-[^{3}H]leucine into the ultracentrifugally separated apolipoproteins of very low, low, and high density lipoproteins (VLDL, LDL, HDL) and into serum albumin was found three- to four-fold higher in nephrotic than in normal rats one hour after intravenous injection. Incorporation of leucine into the circulating lipids was negligible. Increases of similar magnitude were obtained in the incorporation of simultaneously injected 1,5[^{14}C]citrate into the lipids of VLDL, LDL, and HDL of nephrotic rats. Of the citrate carbons incorporated into serum and liver lipids, the proportion in cholesterol was higher in nephrotic rats when compared to normal rats. The incorporation of both precursors into total proteins and lipids of the liver vs. the incorporation into the lipoproteins was relatively lower in nephrotic than in control rats, indicating a preferential channeling into secretable products. The occurrence of enhanced new lipid synthesis in nephrosis was corroborated by the finding of markedly enhanced synthesis of lipoprotein-borne fatty acids and cholesterol from $^{3}H_2O$. These results point out that while leucine is not an efficient in vivo precursor of lipoprotein lipids in nephrosis, de novo lipogenesis proceeds from other precursors. Similar trend of changes, though of smaller magnitude, was elicited in rats after double plasmapheresis, 18 hr apart, when measured 3 hr after the second plasma withdrawal. This indicates that the loss of circulating proteins either by direct removal or through kidney lesion stimulates the compensatory hepatic response involving excessive lipoprotein synthesis. Time-course studies showed that peak incorporation of leucine and citrate into the protein and lipid components of lipoproteins, respectively, as well as into serum albumin, occurred coincidentally 3 hr after the second plasmapheresis, suggesting an interdependence of the enhanced protein and lipid synthesis.

INTRODUCTION

The experimental nephrotic syndrome of rats is characterized by hypoproteinemia, hyperlipoproteinemia, and increased hepatic synthesis of plasma proteins (1-6). The hyperlipoproteinemia is to a large extent due to an increased hepatic production of lipoproteins. This was demonstrated in antikidney serum nephrosis by increased incorporation of [^{14}C]-acetate and [^{14}C]leucine into the lipoproteins secreted by livers slices in vitro (7,7), and in aminonucleoside-induced nephrosis by the enhanced channeling of [^{14}C]glucose into the protein and lipid moieties of lipoproteins in vivo (9). Evidence is available that the hypoproteinemia resulting from plasmapheresis may also lead to an increased lipoprotein synthesis (10-11). Thus, the overproduction of lipoproteins both in nephrosis and plasmapheresis appears to be linked to the general compensatory stimulus for increased hepatic plasma protein synthesis.

We have approached the question whether the synthesis of fatty acids de novo may represent a source for the supply of lipoprotein lipid in nephrosis by studying the activities of rate-limiting, hepatic enzymes of the lipogenesis pathway (12). At the time of onset of amino nucleoside-induced nephrosis, an initial decrease in the activity of these enzymes was found coincidently with increased mobilization of free fatty acids (FFA) from peripheral tissues. Later, a rise in lipogenic enzyme activity was observed together with a fall in serum FFA, indicating a role for de novo lipogenesis.

Presently we intended to assess the importance of certain precursors in supporting the increased synthesis of the lipoprotein moieties in the intact rat with nephrotic syndrome. Citrate and $^{3}H_2O$ were used as precursors of lipids and leucine as a possible precursor of both proteins and lipids. Rats after plasmapheresis were also employed to test whether the observed changes are specially associated with the nephrotic kidney lesion or may also be evoked by direct removal of plasma proteins.

EXPERIMENTAL PROCEDURES

Animals

Male albino rats of Hebrew University strain, weighing 240 to 260 g, were used. They were fed ad libitum a local pelleted stock diet composed of 60% carbohydrate, 20% protein, 5% fat and 15% salts, cellulose and inert material by weight. Nephrosis was induced, as described previously (9), by six to seven subcutaneous injections of 2 mg/100 g aminonucleoside of puromycin (Sigma,). The rats were used 7 to 10 days after the last amino-

TABLE I

Time Course of Incorporation of Leucine and Citrate into Serum Total Proteins and Lipids in Normal Rats[a]

Time (min)	[^{3}H] in proteins	[^{14}C] in lipids
	dpm/ml	
5	725	24
30	6,270	288
60	14,375	709
90	15,900	558
120	16,880	651

[a]Data in the table are from a representative experiment, each value representing a mean of two rats injected with a mixture of labeled citrate and leucine. At the indicated times, the total serum lipids and proteins were isolated and counted as described in the Experimental Procedures.

nucleoside injection. At this time, the food consumption and several other metabolic parameters, such as liver glycogen content and lipogenic enzyme capacity, which became reduced during the nephrosis induction period, have returned to normal (12).

Plasmapheresis was performed by two withdrawals of about one-fourth of blood volume 18 hr apart. Heart puncture was performed under light ether anaesthesia, 5 ml of blood were taken out and 4 ml of a 50% suspension of homologous red blood cells in 0.9% NaCl injected through the same needle. Control animals were subjected to a procedure of withdrawal and reinjection of their own blood in the same syringe. If not otherwise stated, the rats were used 3 hr after the second blood withdrawal.

At the time of experiment, the nephrotic animals had serum triglyceride levels ranging from 460 to 1150 mg/dl and cholesterol levels between 240 to 540 mg/dl. Lipid levels in the plasmapheretic animals were within normal range (serum triglycerides from 65 to 144 mg/dl and cholesterol from 78 to 92 mg/dl). Serum albumin was 1.3 ± 0.2 g/dl in the nephrotic rats and 3.2 ± 0.2 g/dl in the plasmapheretic rats compared to 3.9 ± 0.1 g/dl in the control animals (mean ± SE for 20 animals in each group).

Injection of Labeled Leucine and Citrate and Separation of Lipoproteins

The rats received 10 μCi and 10 μmoles of 4,5-[^{3}H]leucine together with 10 μCi and 20 μmoles of 1,5-[^{14}C]sodium citrate by intravenous injection. The labeled leucine and citrate were freshly obtained products of the Radiochemical Centre, Amersham, England, of radiochemical purity>99%, as stated by the manufacturer. One hour later the rats were bled under light ether anesthesia and the livers

TABLE II

Incorporation of ^{3}H-leucine into Serum Apolipoproteins and Albumin and ^{14}C-citrate into Lipoprotein-Borne Lipids[a]

Fraction	Control (16)	Nephrosis (12)	Plasmapheresis (10)
		Leucine in proteins	
VLDL + LDL (d < 1.063)			
dpm/ml serum	383 ± 23	1,979 ± 138	786 ± 69
dpm/100 g body wt	1,916 ± 95	8,709 ± 636	3,773 ± 333
HDL (1.063 < d < 1.21)			
dpm/ml serum	1,213 ± 122	3,480 ± 321	2,112 ± 228
dpm/100 g body wt	5,942 ± 637	15,138 ± 1,429	10,032 ± 1,026
Albumin			
dpm/ml serum	9,950 ± 466	27,190 ± 1,730	15,624 ± 1,194
dpm/100 g body wt	46,570 ± 1,950	118,800 ± 8,280	75,000 ± 6,090
		Citrate in lipids	
VLDL + LDL (d < 1.063)			
dpm/ml serum	494 ± 41	1,946 ± 176	785 ± 69
dpm/100 g body wt	2,470 ± 210	8,660 ± 746	3,784 ± 328
HDL (1.063 < d < 1.21)			
dpm/ml serum	75 ± 8	212 ± 21	138 ± 12
dpm/100 g body wt	376 ± 42	940 ± 93	663 ± 55

[a]Values in the table are means ± SE for the number of rats given in parentheses. Blood was removed 1 hr after the intravenous injection of labeled leucine and citrate. In rats after plasmapheresis leucine + citrate were injected 2 hr after the second plasma removal and the incorporation measured 1 hr later. All differences from normal rats were significant at $P < 0.005$. For calculation of incorporation per 100 g body wt, the corresponding serum volume in normal, nephrotic and plasmapheretic rats was taken as 5.0, 4.4 and 4.8 ml, respectively (unpublished data).

TABLE III

Incorporation of ^{3}H-leucine and ^{14}C-citrate into Liver Lipids and Proteins[a]

	Control (16)	Nephrosis (12)	Plasmapheresis (10)
		Leucine in total proteins (x 10^3)	
dpm/g liver tissue	17.5 ± 1.6	25.4 ± 3.5	21.3 ± 1.8
dpm/100 g body wt	70.9 ± 6.3	143 ±19[b]	80.8 ± 8.6
		Citrate in total lipids (x 10^3)	
dpm/g liver tissue	2.6 ± 0.3	3.9 ± 0.6	3.5 ± 0.3
dpm/100 g body wt	10.5 ± 1.2	22.4 ± 3.3[b]	13.0 ± 1.3

aValues in the table are means ± SE for the number of rats given in parentheses. The livers are derived from the rats in which serum lipoproteins were investigated (Table II). The ratio liver/body wt was 4.0 ± 0.1, 5.6 ± 0.3, and 3.8 ± 0.2 in normal, nephrotic and plasmapheretic rats, respectively.

[b]Difference from control rats significant at $P < 0.01$.

removed. The extent of incorporation of leucine and citrate label into the lipid and protein components of the combined serum very low density + low density lipoprotein fraction (VLDL + LDL), high density lipoproteins (HDL), and albumin was measured after the flotation in a 50 Ti rotor of Spinco Model L3-50 ultracentrifuge. The separation procedure was essentially that of Havel et al. (13). The density of the medium was adjusted by the addition of solid KBr as described by Radding and Steinberg (8). Chylomicrons were removed first by centrifugation at 10,000 X g for 30 min. VLDL and LDL were then separated as one fraction floating at d = 1.063 g/ml after 24 hr of ultracentrifugation at 48,000 rev/min (140,000 X g) and 15 C. The VLDL and LDL were separated together due to the low relative concentration of the latter and the difficulty to sharply dissociate these two lipoprotein classes by flotation in the rat and because parallel studies were done with isolated perfused liver (Brenner and Shafrir, manuscript in preparation) which secretes negligible amounts of LDL. After slicing off the VLDL + LDL in the uppermost segment of the tube, the HDL were floated by ultracentrifugation of the infranatant portion at d = 1.21 g/ml and 48,000 rev/min for 48 hr at 15 C. The top fractions were washed once by diluting with 4 vol of KBr solution (at d = 1.063 and d = 1.21 g/ml, respectively) and recentrifugation for 24 hr. The HDL were subsequently dialyzed against 0.9% NcCl containing 1 mM Na_2 EDTA, pH 7.0 at 4 C.

The radioactivity in the protein and total lipid moieties of the isolated lipoproteins was measured after extracting with chloroform/methanol (2:1, v/v) according to Folch et al. (14). The phases were broken using 0.05% $MgCl_2$ solution acidified with 0.005 N H_2SO_4, and the chloroform extract washed with a synthetic upper phase containing the same concentration of H_2SO_4 and $MgCl_2$. This precaution was used to minimized the loss of lipids, including FFA, to the upper phase. After three washes, the volume of the extract was reconstituted by addition of methanol and portions were evaporated for the determination of lipid radioactivity in a liquid scintillation spectrometer. The sedimented protein was washed with methanol, dissolved in concentrated formic acid (15) and counted.

Separation of Albumin

The proteins in the d = 1.21 infranatant fluid were precipitated at 10% trichloroacetic acid (TCA) concentration, and washed 3 times with 5% TCA. The albumin was extracted from the precipitate by several treatments with a solution of 1% TCA in 95% ethanol (w/v) (16), reprecipitated by the addition of 3 vol. of ethyl ether, washed with ethyl ether and dissolved for counting in formic acid.

Lipid Synthesis from $^{3}H_2O$

Serum and liver lipid synthesis from $^{3}H_2O$ (Amersham Radiochemical Centre, England) was measured 4 hr after an intraperitoneal injection of 4 mCi isotonic tritiated water. The liver and serum were collected, portions extracted in chloroform/methanol (2:1, v/v) and washed 3 times as described. Portions of the extracts were evaporated, hydrolyzed by heating with 5 N KOH in ethanol (1:7, v/v). Fatty acids were extracted into heptane after acidification and counted. Cholesterol was precipitated and counted as digitonide (17). The synthesis of fatty acids and cholesterol was calculated using the incorporation factors of 13.3 and 18.9 μg atoms of ^{3}H per μmole fatty acid or cholesterol, respectively (18).

TABLE IV

Distribution of Citrate Label among Serum and Liver Total Fatty Acids and Cholesterol[a]

Rats	Serum		Liver	
	Fatty acids %	Cholesterol %	Fatty acids %	Cholesterol %
Control (16)	67.8 ± 2.9	17.4 ± 1.2	69.5 ± 3.1	10.4 ± 0.8
Nephrotic (10)	59.0 ± 3.6	22.8 ± 2.0[b]	58.0 ± 3.9[b]	14.9 ± 0.9[b]

[a]Values in the table are means ± SE for the number of rats in parentheses. Fatty acids and cholesterol were separated after saponification of the lipids (see Experimental) and the values represent percentages of the total lipid radioactivity.

[b]Difference from control rats significant at $P < 0.05$.

Portions of serum and liver from leucine + citrate-injected rats were treated similarly for determining the label incorporation into fatty acids and cholesterol.

Radioactivity in Proteins

Label incorporation into total serum or liver proteins was estimated by precipitating the proteins from serum or a 25% liver homogenate in 0.25 M sucrose with 10% TCA and washing the precipitate 3 times with 5% TCA and 2 times with ethanol/ether (3:1, v/v). The radioactivity in the proteins was determined after dissolving the precipitate in formic acid (15) and counting.

Determination of Leucine, Isoleucine and Citrate Contents

For this purpose, portions of liver were quickly frozen in liquid air and homogenized in 6% perchloric acid. Leucine and isoleucine were quantitated in a Beckman Amino Acid Analyzer. Citrate levels were determined by an enzymatic method using citrate lyase (19), which was purchased from Boehringer (Germany).

RESULTS

The time of one hour was chosen for the study of incorporation of [^{3}H]leucine and [^{14}C]citrate since at this time the incorporation of both precursors into serum total proteins and lipids seemed to plateau (Table I).

Table II shows the radioactivity of serum lipoproteins of nephrotic and plasmapheretic rats isolated by ultracentrifugal flotation. In nephrosis, more than four-fold increase was evident in [^{3}H]leucine incorporation into the protein moiety of the combined VLDL + LDL fraction and a three-fold increase in the HDL fraction. The increase in leucine-derived radioactivity in albumin was also about three-fold. The incorporation of [^{14}C]citrate carbons into the lipids of the VLDL + LDL fraction increased also about four-fold, and the radioactivity in the HDL lipids rose about three-fold.

The respective increases in leucine and citrate-derived label in the lipoproteins or albumin of rats after plasmapheresis were smaller than in nephrosis but all highly significant in comparison to control rats.

All incorporations have been calculated per ml of serum as well as per serum volume corresponding to 100 g body wt, since in the nephrotic rats the serum volume is lower than normal. This did not alter, however, the general trend of marked increase in the incorporation of leucine or citrate into the lipoprotein components or into albumin.

Nephrosis or plasmapheresis did induce only small increases in the amount of leucine-derived [^{3}H]label in liver proteins or citrate-derived [^{14}C] label in liver lipids (Table III). These increases were not significant in nephrosis when calculated per g liver wt, but become significant when expressed per liver wt corresponding to 100 g body wt as liver size increased in nephrotic rats. The increase in liver size appeared to be uniform with regard to its components since the defatted dry liver wt of normal and nephrotic rats was similar (29.6 ± 0.9 vs. 27.5 ± 1.0%, respectively). Thus, the incorporation of leucine or citrate into the liver total proteins or lipids, respectively, increased, in nephrosis, less than two-fold as compared with three- to four-fold rises in serum lipoproteins or albumin (Table II). The corresponding increases in the liver of plasmapheretic rats were not significant.

In several instances, cross incorporation of [^{14}C]label into the proteins and [^{3}H]label into the lipids of liver or serum was also checked both in control and nephrotic rats and was found negligible during the period of observation. Leucine label among the serum lipids was in the range of 2 to 5% of that of citrate, whereas citrate label in proteins was <1% of that of leucine.

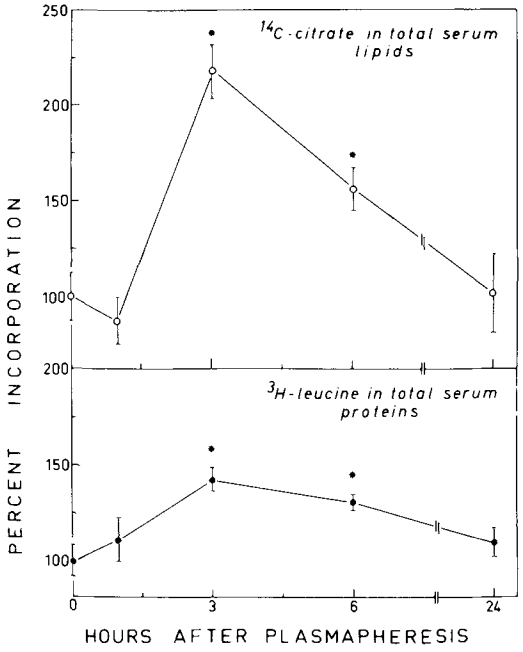

FIG. 1. Time course of the incorporation of citrate into total serum lipids and leucine into total serum proteins in rats after plasmapheresis, in relation to control rats. The two labeled precursors were injected together, one hour before the indicated times of sacrifice, starting from the second plasma withdrawal (see Methods). Each point represents a mean ± SE for 8 to 11 rats in each group. The asterisk indicates a statistically significant difference from the control rats.

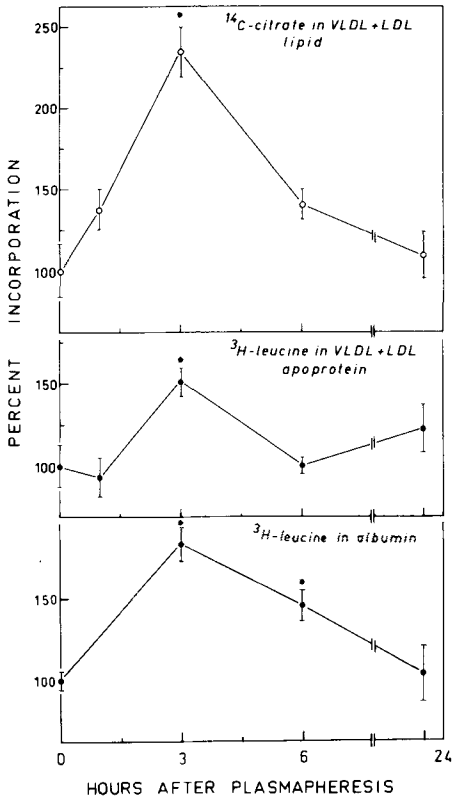

FIG. 2. Time course of rise in the incorporation of citrate into lipids and leucine into proteins of the ultracentrifugally separated d<1.063 g/ml serum lipoprotein fraction (VLDL + LDL) as well as into serum albumin, in rats after plasmapheresis. Each point represents a mean ± SE for 7 to 9 rats in each group. The asterisk indicates a statistically significant difference from the control rats.

When the lipids of serum and liver were separated (Table IV), the mean distribution ratio of radioactivity between fatty acids and cholesterol in serum and liver was 3.9 and 6.7, respectively, in normal rats and 2.6 and 3.9, respectively, in nephrotic rats. Thus, more citrate-derived radioactivity was channeled into cholesterol, in the nephrotic rats.

Phasmapheresis afforded the opportunity to follow the time course of the compensatory responses to the acutely produced hypoproteinemia. Figure 1 shows that the peak incorporation of leucine and citrate into the total lipids and proteins of serum occurred coincidentally 3 hr after the plasmapheresis. Percentagewise, the increase in incorporation, compared to control rats, was greater in the case of lipids than proteins. The increased rate of incorporation was still evident at 6 hr, but returned to normal 24 hr after the plasmapheresis. Figure 2 further illustrates the temporal coincidence in leucine and citrate label incorporation into the protein and lipid moieties of the ultracentrifugally isolated VLDL + LDL as well as into serum albumin.

To compare the precursor pools at normal and experimental conditions at which leucine and citrate incorporation was studied, the levels of endogenous leucine, isoleucine and citrate in serum and liver were determined. Table V shows that the levels of these precursors were lower in the nephrotic and plasmapheretic animals but usually not significantly and consistently so. Considering that the liver size in the nephrotic rats is greater, the expression of the precursor levels per 100 g body wt would tend to minimize these differences. Thus, the observed manifold rises in leucine and citrate incorporation into proteins or lipids were observed in absence of appreciable changes in liver precursor concentration, which might have significantly contributed towards incorporation increases.

To further document the occurrence of de

TABLE V

Serum and Liver Leucine, Isoleucine and Citrate Levels of Nephrotic and Plasmapheretic Rats[a]

Metabolite	Normal	Nephrosis	Plasmapheresis
		Serum, nmol/ml	
Leucine	118 ± 16	78 ± 9	86 ± 13
Isoleucine	83 ± 11	56 ± 8	63 ± 10
Citrate	79 ± 9	68 ± 8	63 ± 6
		Liver, nmol/g	
Leucine	219 ± 22	142 ± 10	309 ± 56
Isoleucine	159 ± 26	105 ± 12	225 ± 25
Citrate	197 ± 24	148 ± 19	175 ± 16

[a]Values are means ± SE for 7 to 10 rats determined at sacrifice.

TABLE VI

Serum and Liver Lipid Synthesis from ^3H_2O in Nephrotic and Plasmapheretic Rats[a]

	Control (6)	Nephrosis (6)	Plasmapheresis (6)
Serum fatty acids			
nmol/h ◦ ml	90 ± 9	750 ± 175[b]	198 ± 22[b]
nmol/h ◦ 100 g body wt	459 ± 51	3393 ± 718[b]	941 ± 109[b]
Liver fatty acids			
nmol/h ◦ g tissue	305 ± 46	646 ± 112[c]	424 ± 73
nmol/h • 100 g body wt	1248 ± 185	3389 ± 597[b]	1642 ± 364
Serum cholesterol			
nmol/h ◦ ml	9.3 ± 0.7	27.6 ± 1.6[b]	16.5 ± 1.1[b]
nmol/h ◦ 100 g body wt	46.2 ± 3.6	120 ± 7.3[b]	79.5 ± 5.4[b]
Liver cholesterol			
nmol/h ◦ g tissue	22.3 ± 1.9	29.6 ± 2.3[c]	26.8 ± 3.5
nmol/h ◦ 100 g body wt	88.7 ± 9.3	164 ± 13[a]	103 ± 13

[a]Values in the table are means ± SE for groups of 6 rats. 0.5 ml of isotonic tritiated water was injected i.p. and the rats killed 4 hr later. The plasmapheretic rats received the injection 1 hr after the second blood removal. Serum and liver lipid synthesis per 100 g body wt was calculated as explained under Tables II and III.

[b]Differences from control rats significant at $P < 0.01$ at least.

[c]Differences from control rats significant at $P < 0.05$.

novo lipogenesis in association with experimental hypoproteinemia, the incorporation of [^{3}H] from ^3H_2O into lipids was followed in nephrotic and plasmapheretic rats. Table VI shows that the rate of serum fatty acid synthesis in nephrosis exceeded that of control rats as much as 8 times (whether expressed per ml of serum or per 100 g body wt) and of cholesterol synthesis about 3 times. The content of newly synthesized fatty acids in the liver of nephrotic rats, expressed per 100 g body wt, was also significantly higher by a factor close to 3 and of cholesterol by a factor close to 2. In plasmapheretic rats, the rise in the synthetic rates of serum fatty acids and cholesterol was also significant but less conspicuous than in the nephrotic rats, whereas the increases in liver lipid synthesis did not reach the level of significance. These results corroborate those of increased incorporation of citrate carbon into serum and liver lipids of nephrotic and plasmapheretic rats (Tables II and III).

DISCUSSION

The enhanced lipoprotein production in intact nephrotic rats is indicated in our results of incorporation of simultaneously administered precursors into protein and lipid moieties of the lipoproteins. The increased flow of 1,5-[^{14}C]citrate carbons into the lipoprotein-borne lipids should be attributed to de novo fatty acid and cholesterol synthesis. The rise in 4,5-[^{3}H]leucine incorporation into apolipoproteins and albumin points to the coincidently increased hepatic synthesis of plasma proteins.

These conclusions are based on the premise that the specific activity and the pool size of these precursors in the control and hypoproteinemic animals were not appreciably dif-

ferent, which is indeed borne out by the determination of the levels of leucine and citrate in serum and liver (Table V). As a further precaution, leucine and citrate were injected in larger than tracer amounts to minimize possible fluctuations in the initial substrate concentrations between the normal and hypoproteinemic animals. Additional support for our conclusion comes from the fact that the pronounced increment in label incorporation was mainly evident in the circulating lipoproteins, whereas the incorporation into the hepatic lipids and proteins was only moderately elevated. The ratio of leucine label appearing in serum lipoproteins vs. that in total liver proteins (per 100 g body wt) was 0.11 in control rats and 0.17 in nephrotic rats. The same ratios for citrate label were 0.27 and 0.43, respectively (data of Tables II and III). Were higher precursor specific activities of citrate or leucine in nephrotic rats responsible for the higher incorporation value, the increases in protein or lipid label in serum vs liver would have been at a ratio similar to that in control rats. The preferential appearance of the label in the circulation in nephrosis and plasmapheresis strongly suggests a favored pathway into components to be exported from the liver in response to the hypoproteinemia. However, stimulation of incorporation into liver tissue proteins and lipids is also possible, particularly in view of the augmented liver size in nephrosis.

In accord with our parallel liver perfusion studies (Brenner and Shafrir, manuscript in preparation), it may be concluded that, both in vivo and in vitro, amino acids do not constitute an important substrate for the elaboration of the lipid moiety of the lipoproteins. Metabolic adjustments, yet to be fully elucidated, appear to divert amino acids from pathways of lipogenesis or gluconeogenesis in nephrosis (20). The lipid moiety of the lipoproteins seems to be supplied from other sources. As discussed elsewhere (12, Brenner and Shafrer, manuscript in preparation), accelerated trapping and recirculation of preformed, adipose tissue-derived FFA seems to represent one source. New lipid synthesis is also enhanced from glucose, shown in previous investigations to be an important precursor of the excessively synthesized lipoprotein lipids in nephrotic rats (9) and in a large proportion of nephrotic human patients (21). Our present results demonstrate the increased hepatic utilization in nephrosis and plasmapheresis of another lipogenic substrate, such as citrate which produces extramitochondrial acetyl-CoA, directly available for de novo fatty acid synthesis (22,23). Moreover, independent confirmation of the enhanced lipogenesis in nephrosis and plasmapheresis stems from the results of [^{3}H] incorporation from the in vivo administered tritiated water. Again, there was a predominant flow of the label into serum rather than liver lipids with close to three-fold increase in the distribution ratio in serum vs. liver lipids (data of Table VI). De novo fatty acid and cholesterol synthesis is demonstrated in these experiments, since [^{3}H] incorporation is known to result in random, NADH- and NADPH-mediated exchange of hydrogens along the fatty acid chain carbons during active fatty acid synthesis in rat liver (24).

It is of interest that the flow of citrate carbons and [^{3}H] to lipids is enhanced although the maximal capacity of the regulatory enzymes of fatty acid synthesis may not be adaptively increased, or even decreased, at the onset of the aminonucleoside-induced nephrosis (12). This transient effect on some enzymes is probably due to the retention, by aminonucleoside, of a fraction of the potency of its parent substance, puromycin, to inhibit the synthesis of selected proteins. However, the increasing availability of apolipoprotein acceptors seems to exert an overriding influence on the rate of flow of lipogenic precursors to fatty acids even if the maximal enzyme capacity is lower than normal. This is exemplified here by the increased in vivo incorporation of citrate and [^{3}H] to lipids and elsewhere by direct in vitro measurements of hepatic acetyl-CoA conversion to fatty acids (12). With longer time intervals after cessation of aminonucleoside injections (> 7 days), the activity of lipogenesis enzymes rebounds and at that time the share of preformed FFA in lipoprotein lipid elaboration decreases (12).

In rats, after plasmapheresis, the incorporation of leucine and citrate into serum lipids and proteins, though significantly increased, was less conspicuous than in nephrosis. The hypoproteinemia obtained by plasma withdrawal was less extensive and of shorter duration and most probably exerted a weaker stimulus for the replacement of plasma proteins. Nevertheless, the plasmapheresis-induced occurrence of enhanced lipoprotein and albumin synthesis points out that the hepatic response is not dependent upon protein loss specifically through the kidney.

In contrast to the nephrotic rats, the increased lipogenesis after plasmapheresis was not associated with hyperlipidemia. This again could be attributed to the short hypoproteinemic stimulus as well as to the fact that each of the two plasma withdrawals included the removal of lipoproteins. Mutiple bleedings over

a prolonged period do result in hyperlipidemia in dogs (10), rats (11) and rabbits (25).

The time course of [^{14}C]citrate and [^{3}H]-leucine label appearance in rat serum after plasmapheresis showed a temporal collusion of the incorporation of these two precursors into the lipid and protein moieties of lipoproteins as well as into serum albumin. The common peak, evident 3 hr after plasmapheresis, apparently resulted from a tight association of the stimuli for the enhanced synthesis of apolipoproteins and their complementary lipids. Likewise, in the nephrotic animals the magnitudes of increments in incorporation of leucine to apolipoproteins and citrate to lipoprotein-borne lipids were similar, one hour after the administration of the precursors.

Our conclusions from citrate and leucine incorporation data obtained here are generally in agreement with those of Takeuchi and Yamamura (11) who have measured leucine and acetate incorporation into liver slices of a highly selected group of rats subjected to a single plasmapheresis. In their experiments the incorporation of leucine and acetate into lipoprotein proteins and lipids was increased for up to 72 and 96 hr, respectively, with the onset of lipid synthesis lagging behind that of proteins. When apolipoprotein synthesis was inhibited by orotic acid, no increase in lipogenesis from acetate occurred. The results suggest that the enhancement of lipoprotein synthesis may persist longer after plasmapheresis when measured in an isolated liver, than in the whole animal. Although the lipogenesis from acetate cannot be strictly compared to that from citrate (23), the time course of the incorporation lends a strong support to the contention that the acceleration of lipogenesis in nephrosis, similarly to plasmapheresis, is secondarily linked to the excessive synthesis of apolipoproteins.

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Effect of Calcium on Absorption of Fatty Acid by Rat Jejunum in vitro

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ABSTRACT

The effect of Ca^{++} on jejunal osmiophilic particles was studied in a recirculating system which was not contaminated with plasma lipoproteins. An isolated, infused segment of rat jejunum was suspended in a bath of liquid paraffin. Transudate, containing osmiophilic particles, appeared like beads of sweat on the serosal surface, and fell to the bottom of the bath. In the range of 25-38 C, 30 C proved to be optimal for histological preservation of villous architecture. Production of transudate, 20 mg/min/g of jejunum, and transport of [^{14}C] oleate proceeded nearly linearly after the first 30 min. Necrosis of mid-villus and crypt cells became obvious by light microscopy after one hour. Therefore, transudate was collected between the period of 30-60 min. Shadow casting of transudates, produced when saline was infused, revealed that 86 ± 9 (SD) % of osmiophilic particles was < 800 Å in diameter; 13 ± 8% was 800 - 1000 Å; 0.4 ± 0.5% was 1000 - 2000 Å. Corresponding values were 58 ± 10, 25 ± 5, and 16 ± 5% when 5 mM [^{14}C] oleate + 2.5 mM monoolein was infused; 75% of the transported [^{14}C] appeared in triglyceride. Adding 2 mM Ca^{++} to the infusion doubled the transport of [^{14}C] triglyceride without increasing particle size further. We conclude that luminal Ca^{++} increases the absorption of luminal fatty acid by rat jejunum in vitro.

INTRODUCTION

We sought to confirm and to extend Strauss' observation that Ca^{++} enhances synthesis and transport of jejunal lipid particles (1,2). The in vitro system of Parsons and Volman-Mitchell (3) was adapted because it has the advantages of being relatively uncontaminated by small intestinal contents or by plasma lipoproteins.

MATERIALS AND METHODS

Materials

[1-^{14}C] oleic acid (New England Nuclear Corp., Boston, MA) was purified by autoradiography and thin layer chromatography. [1,2-^{3}H] polyethylene glycol, M.W. 4000 (New England Nuclear Corp.), oleic acid (NuChek Prep Inc., Elysian, MN), and 2-monoolein (Serdary Research, London, Ont.) were used without further purification. Na taurocholate was synthesized and purified (4).

Saline infusions consisted of (mM): NaCl, 85; KCl, 6; $NaHCO_3$ 40; Na taurocholate, 5; glucose, 28; [1,2,-^{3}H] polyethylene glycol, 0.5 mM, 0.2 μCi/ml; gas phase, 5% CO_2 - 95% O_2. [^{14}C] oleic acid, 5 mM, 0.2 μCi/ml, and 2-monoolein, 2.5 mM, were dispersed in saline infusions by brief sonication (Biosonik II, Bronwill Scientific, Rochester, NY, operated at about 1/3 of its maximum output for 2 min). The sonicated lipid mixture was slightly opalescent; 88% of [^{14}C] lipid mixture passed through a 1000 Å Millipore filter. Adding Ca^{++} to the lipid mixture increased turbidity: 51% and 10% of [^{14}C] lipid mixture passed through 1000 Å filters when the mixture contained 1 mM and 2 mM Ca^{++}, respectively.

Experimental Procedures

Male Sprague-Dawley rats, fasted overnight, were injected intramuscularly with sodium thiopental, 6 mg/100 g, and heparin, 250 units/100 g of body weight. Thirty min later, surgical anesthesia was induced with halothane.

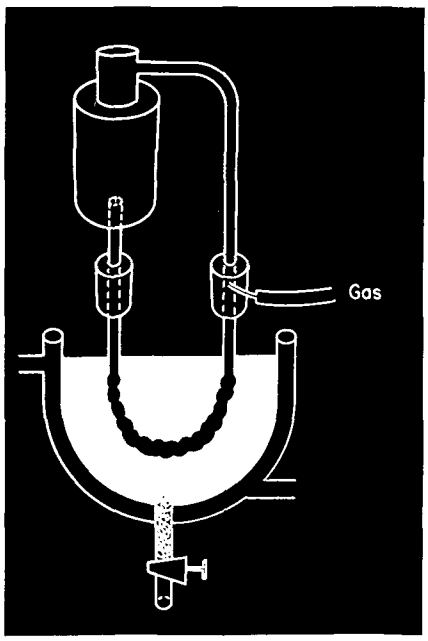

FIG. 1. Diagram of the infusion system.

TABLE I

Distribution of ^{14}C in Transudates[a]

Infusion	^{14}C Transported	Distribution of Transported ^{14}C[b]			
		Origin	DG	FFA	TG
Ca^{++} or Mg^{++}	nmoles/g tissue	% of recovered ^{14}C			
Neither (6)	250 ± 89	13 ± 7	3 ± 1	7 ± 2	76 ± 8
Ca^{++}, 1 mM (4)	470 ± 196	11 ± 6	6 ± 1	15 ± 7	61 ± 5
Ca^{++}, 2 mM (8)	565 ± 228[c]	9 ± 4	4 ± 3	12 ± 6	69 ± 7
Ca^{++}, 3 mM (8)	550 ± 107[c]	12 ± 11	5 ± 1	11 ± 6	70 ± 11
Ca^{++}, 4 mM (8)	530 ± 196[c]	7 ± 4	5 ± 1	10 ± 5	75 ± 9
Ca^{++}, 5 mM (6)	520 ± 159[c]	6 ± 2	4 ± 3	8 ± 3	79 ± 8
Mg^{++}, 1 mM (7)	390 ± 135	2 ± 2	6 ± 3	15 ± 11	70 ± 15
Mg^{++}, 2 mM (6)	330 ± 161	13 ± 7	7 ± 4	15 ± 7	69 ± 9

[a]Jejunal segments were infused for 1 hr with lipid mixture to which had been added Ca^{++} or Mg^{++} or neither. Transudates were collected between 30-60 min. The values are means ± SD with number of experiments in brackets.

[b]Radioactivity remaining in undesignated bands of chromaplates accounted for 3% of recovered ^{14}C. More than 85% of ^{14}C-transudate was recovered from chromatoplates. MG was not clearly separated from the origin.

[c]Significantly different from infusions without Ca^{++} (t test; $p < 0.01$).

A 15 cm segment of upper jejunum was cannulated at both ends and was flushed with 30 ml of saline infusion. While its mesentery was still intact, the cannulated segment was incorporated into a closed circuit system (Fig. 1) whose gaslift device circulated 25 ml of saline or of lipid infusion through the intestinal lumen continuously. The mesentery was cut, the serosal surface was washed with saline, dried gently with tissue paper, and the segment was placed in a bath of liquid paraffin. Transudate from the infused segment appears as sweat-like beads on the serosal surface, and sinks to the bottom of the bath (Fig. 1). Samples of transudate were separated from paraffin by centrifugation (1500 rpm for 5 min), and were weighed. ^{3}H and ^{14}C in 0.1 ml portions of transudate were measured to within ± 1% in a Beckman liquid scintillation counter. [^{14}C]-lipid was fractionated by cochromatographing portions of transudate with 12 μg of each of triolein, oleic acid, 1,2-diolein, and 2-monoolein in chloroform/methanol (1:1, v/v). Chromatoplates (Uniplate, Analtech, Inc., Newark, DE) were developed in hexane/diethyl ether/methanol/acetic acid (45:10:1:1, v/v), and stained with I_2 vapor. Bands of silicic acid were scraped into counting vials to which 10 ml of Aquasol (New England Nuclear Corp.) was added. Particles in samples of transudate were shadow-casted (5) without knowing previous experimental conditions; electron micrographs were taken, and the coded negatives were projected onto a grid so that diameters of particles could be measured.

At the end of an experiment, the jejunal segment was opened longitudinally, and was washed thrice in saline solutions. Its wet weight was obtained, and a piece of its mid-section was taken for light microscopy; the remaining segment was homogenized in chloroform/methanol (1:1) so that lipids could be extracted (6); the efficiency of extracting [^{14}C]oleic acid from a nonradioactive jejunal homogenate was 98.2%. The efficiency of the washing procedure was more than 98%; 3 segments of jejunum were exposed to a lipid mixture, and then they were immediately washed, weighed, and extracted with chloroform/methanol. 1.6% of ^{14}C-mixture remained with the washed tissue; 31% of this ^{14}C appeared in the free fatty acid region, 42% in the triglyceride region, and the remainder in the phospholipid and monoglyceride regions by thin layer chromatography.

Statistical differences between mean values of experiments in Table I were analyzed with Student's t test (7).

RESULTS

Effect of Temperature of Incubation

Histological slides of jejunal segments from pairs of rats which had been infused with lipid mixture at 25 C, or 30 C, or 33 C, or 38 C for 1 hr were coded and reviewed, Villous tips were better preserved, and absorptive cell injury was less at the two lower temperatures. We could not distinguish between 25 C and 30 C, so subsequent experiments were performed at 30 C.

Effect of Duration of Incubation

Jejunal segments from 4 animals were infused for 2 hr. After the first 30 min, produc-

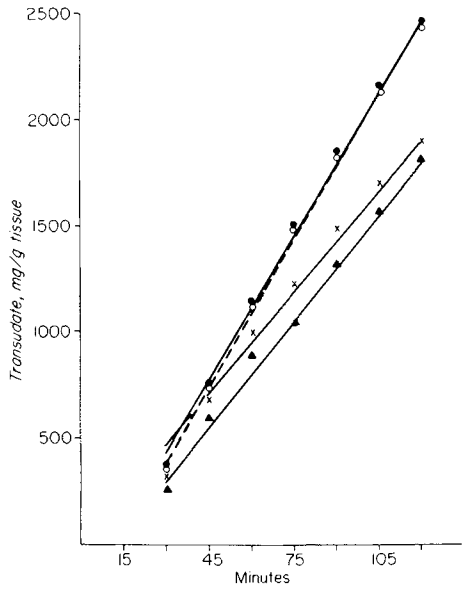

FIG. 2. Cumulative secretion of transudate per g tissue wet weight by 4 jejunal segments.

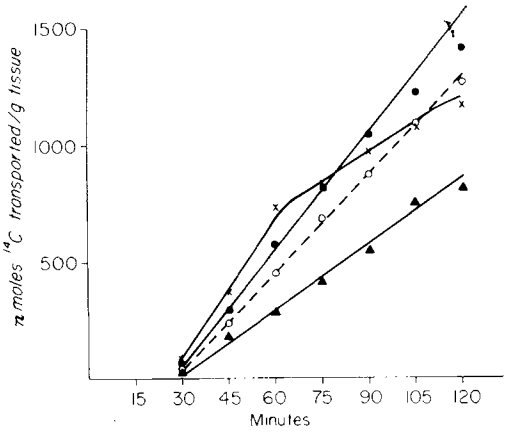

FIG. 3. Cumulative transport of ^{14}C per g tissue wet weight by 4 jejunal segments.

tion of transudate (Fig. 2), and transport of ^{14}C (Fig. 3) proceeded in a nearly linear fashion. However, histological exam of specimens from these segments which had been infused for 2 hr, as well as from segments infused for 90 min, revealed increased sloughing at villous tips and necrosis of absorptive cells in mid-villus and crypt regions. Therefore, transudate produced during the first 30 min of subsequent experiments was discarded, while that between 30 - 60 min was collected.

Production of Osmiophilic Particles

The majority of particles transported in response to infusions of saline had diameters less than 800 Å. Infusions of lipid prompted transport of chylomicron-sized particles (Table II). Additions of Ca^{++} or Mg^{++} did not further increase particle size.

Fractionation of Radioactivity in Transudate and in Tissue

^{14}C was not leached out of transudates by the paraffin bath. Less than 2% of ^{14}C was lost when 0.2 ml of [^{14}C] transudate was dripped through a 4 x 1 cm column of liquid paraffin.

[^{3}H] polyethylene glycol was added to the infusion mixture to detect leaks in intestinal segments. Experiments were discarded when the concentration of ^{3}H in the transudate was >10% of that in the infusion.

Adding Ca^{++}, 2-5 mM, but not Mg^{++}, 1-2 mM, to the saline-lipid infusion enhanced the secretion of ^{14}C by jejunal segments (Table I). About 70% of the transported ^{14}C was incorporated into triglyceride.

In the experiments in Table I, an average of 5.8 μmoles of [^{14}C] oleic acid were recovered from 1 g of tissue after one hour's infusion; 25% of the ^{14}C was in free fatty acid, and 62% was in triglyceride. Ca^{++} did not enhance the incorporation of ^{14}C into jejunal lipid.

Secretion of Protein by Jejunal Segments

Coded specimens of infusion mixtures and of transudates were kindly analyzed by radioimmunoassay for apoprotein A-1 by Dr. M. Fainaru of Jerusalem. Two segments infused with saline secreted 4.9, and 3.9 μg of apoprotein A-1/g tissue in 30 min, while 2 segments infused with lipid mixture secreted 4.4, and 5.5 μg/g tissue. No apoprotein was detected in the infusions. The possibility that these amounts of apoprotein A-1 were from contaminating plasma proteins was excluded by using albumin as a plasma marker. Albumin in transudates was determined by Dr. John Albers of Seattle who employed an immunodiffusion method able to detect 2 μg/ml. Two jejunal segments each transported 10 μg of albumin/g of tissue/30 min which may have denoted contamination with ca. 0.3 μliter of plasma. (The serum concentration of albumin in these rats was more than 3 g/100 ml). Such an amount of plasma would contain less than 0.3 μg of apoprotein A-1 (8).

DISCUSSION

In this simple system, jejunal segments infused with saline secreted osmiophilic particles whose diameters were primarily less than

TABLE II

Secretion of Osmiophilic Particles by Rat Jejunum[a]

Infusion	No. of rats	Percentage of particles		
		<800 Å	800-1000 Å	1000-2000 Å
Saline	6	86 ± 9	13 ± 8	0.4 ± 0.5
+ lipid	6	58 ± 10	25 ± 5	16 ± 5
+ lipid + Ca^{++}, 1 mM	7	57 ± 17	22 ± 6	18 ± 11
+ lipid + Ca^{++}, 2 mM	8	62 ± 15	21 ± 8	15 ± 7
+ lipid + Ca^{++}, 5 mM	5	48 ± 10	29 ± 6	21 ± 7
+ lipid + Mg^{++}, 1 mM	8	55 ± 11	24 ± 6	21 ± 7
+ lipid + Mg^{++}, 2 mM	6	51 ± 11	26 ± 4	21 ± 8

[a]An average of 340 particles was counted per animal. The values are mean ± SD. Incubation was for 1 hr at 30 C. Transudate was collected during the last half hour and was shadow casted.

800 Å. The addition of [^{14}C]oleate and monoolein to the infusion caused chylomicron-sized particles to appear and [^{14}C]triglyceride to be secreted in increased amounts. Appreciable contamination of the secreted particles by plasma protein was excluded.

We chose to examine the effect of Ca^{++} and Mg^{++} on uptake and transport of fatty acid because Strauss had reported that these ions affected lipid metabolism by sacs of rat intestine (1). In his experiments with everted jejunal sacs, 2.5 mM Ca^{++} plus 1.2 mM Mg^{++} caused fatty acids in mucosal fluid to appear as triglyceride in chylomicron-sized particles in serosal fluids (2). We confirmed that chylomicron formtion is enhanced by Ca^{++} which seemed to have an optimal concentration of 2 mM in our system; Mg^{++}, 1-2 mM, did not enhance fatty acid transport significantly. These observations on the effect of Ca^{++} may be relevant to understanding what causes steatorrhea in patients with hypoparathyroidism (9). Human postprandial small intestinal contents normally have 1-2 mM Ca^{++} (10). Investigation of Ca^{++} concentrations in small intestinal contents of hypoparathyroid patients might support the hypothesis that luminal Ca^{++} influences fatty acid absorption in man.

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Stereospecific Analysis of Glycerolipids of Egg Yolk of Japanese Quail (*Coturnix coturnix japonica*)

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ABSTRACT

Phosphatidylcholine, phosphatidylethanolamine and triacylglycerol were isolated from egg yolk of the Japanese quail. Fatty acid compositions at the two and three positions of glycerol in the glycerolipids were determined by stereospecific analysis employing phospholipase A_2. The distribution of the total number of carbon atoms in the fatty acid moieties of triacylglycerol was also quantitated by high temperature gas liquid chromatography. The distribution of acyl groups in each of the positions of the phosphatidylcholine, phosphatidylethanolamine and triacylglycerol was not random, and each position has a characteristic composition. The phosphatidylcholine and phosphatidylethanolamine had distinctive fatty acid distributions for position *sn*-2 of the triacylglycerol had a predominance of unsaturated fatty acids of which 18:1 (69.9%) was the major component. Position *sn*-3 contained 49.3% saturated fatty acids and was more saturated than position *sn*-1 by 8.1%. The experimentally determined distribution of the carbon numbers in triacyl glycerol deviated significantly from the distribution predicted by 1-random-2-random-3-random association of the fatty acids. The data suggest that in Japanese quail there is marked preferencial synthesis of some triacylglycerols.

INTRODUCTION

Considerable information is now available on the fatty acid composition of lipids from the egg yolk of Japanese quail (*Coturnix coturnix japonica*)(1-3), but most lipid analyst have concentrated on single specific lipid classes rather than systematically examining and comparing all the glycerolipids from the quail egg yolk.

Recently, Couch and Saloma (2) attempted to clarify the positional distribution of fatty acid in yolk triacylglycerols from Japanese quail. Because they used only lipase hydrolysis, they could determine only the fatty acids at the position *sn*-2. Although Ohtake et al. (3) have also proposed the positional distribution patterns for fatty acids in triacylglycerols from the quail egg yolk, the results were derived by calculation from the experimental data on the basis of 1,3-random-2-random theory. In the present study with lipids from the quail egg yolk, we have made stereospecific analyses of the triacylglycerol (TG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE).

EXPERIMENTAL PROCEDURES

Glycerolipids analyzed in the present study were from the same source described earlier (1). TGs were resolved from the other neutral lipids by preparative thin layer chromatography (TLC) on absorbent layers of Silica Gel G developed in a solvent system of n-hexane/diethyl ether/acetic acid (80:20:1, v/v). TG bands were corrected and eluted from the absorbent with chloroform/methanol (2:1, v/v). PC and PE were also isolated by preparative TLC of Silica Gel H developed in a solvent system of chloroform/methanol/water (65:25:4, v/v). The stereospecific analysis of TG was done by the method of Brockerhoff

TABLE I

Stereospecific Analysis of PC and PE[a]

	Position	16:0	16:0	18:0	18:1	18:2	20:4	22:6	UFA[b]
PC	*sn*-1	55.2	2.4	24.5	7.6	3.8	3.9	3.0	20.7
	sn-2	16.2	2.0	8.3	40.3	19.8	6.3	6.9	74.0
	Total/2	35.7	2.2	16.4	24.0	11.8	5.1	5.0	47.4
	Original	34.7	2.4	15.2	26.1	12.2	5.1	4.3	50.1
PE	*sn*-1	29.7	0.4	40.8	10.5	2.8	7.1	8.9	29.7
	sn-2	7.5	tr	13.2	24.4	13.8	15.9	22.0	76.2
	Total/2	18.6	tr	27.0	17.5	8.3	11.5	15.5	53.0
	Original	16.3	tr	14.0	18.9	6.3	12.0	17.8	55.0

[a]Fatty acids (%). PC = phosphatidylcholine and PE = phosphatidylethanolamine.
[b]Total unsaturated fatty acids.

TABLE II

Structural Analysis of Triacylglycerols[a]

Fatty acid	*sn*-1	*sn*-2	*sn*-3	Total[b] *sn*-1,2,3	Triacylglycerol original
14:0	0.7	0.3	tr	0.3	0.6
16:0	33.1	5.8	39.9	26.3	25.2
16:1	8.8	3.6	9.0	7.1	7.3
18:0	7.4	2.5	9.4	6.4	7.1
18:1	42.3	69.9	35.9	49.4	51.9
18:2	7.2	18.1	4.6	10.0	7.6
SFA[c]	41.2	8.6	49.3	33.0	32.9
UFA[d]	58.3	91.6	49.5	66.8	66.8

[a]Fatty acid (%).

[b]Total of fatty acid at *sn*-1,2,3 divided by 3.

[c]Total saturated fatty acids.

[d]Total unsaturated fatty acids.

(4,5), as modified by Christie and Moore (6). Digestion of diglyceride phenyl phosphates, PC and PE, were all done with phospholipase A_2 of *Crotalus adamanteus*(Sigma). Pancreatic lipase hydrolysis of the TG was carried out according to the Brockerhoff's procedure (4) for 100 mg samples to analyze fatty acids of position *sn*-2. All of the hydrolysis products of the digestion were separated by TLC on Silica Gel H with n-hexane/diethyl ether (1:1, v/v) as the solvent. Fatty acids liberated by hydrolysis were converted with methanol catalyzed by HCl to methyl esters, whereas 2-monoacyl glycerol, lyso and unreacted phenyl phosphates were transesterified. Methyl esters were analyzed by gas liquid chromatography (GLC) on diethylene glycol succinate polyester (8%) column (1). TGs were hydrogenated in methanol catalyzed by palladium black and analyzed by high temperature GLC on SE-30 (0.75% on Chromosolve W) stainless steel column (1 m x 3 mm inside diameter) programmed from 270 C to 330 C at 2 C/min (7). Inlet and detector were 350 C; nitrogen gas flow rate was 40 ml/min. All GLC analyses were performed on a JEOL JGC-20KFP instrument equipped with a dual flame ionization detector.

RESULTS AND DISCUSSION

Positional Distribution of Fatty Acids in PC or PE

The positional distributions of fatty acids in PC and PE, representing 73.4% and 19.4%, respectively, of the yolk phospholipids are given in Table I. In both classes of phospholipids, position *sn*-1 and *sn*-2 had quantitatively distinctive fatty acid composition, indicating a preferential association of certain fatty acids with one of the two acyl positions. Position *sn*-2 of PC was 50% more unsaturated than position *sn*-1 especially in predominance of C_{18} unsaturates. Fatty acids of position *sn*-1 of PC were much more saturated than those of position *sn*-1 of PE. Arachidonic (20:4) and 22:6 acids in position *sn*-1 of PE and PC were one-half the amount found in position *sn*-2. Fatty acids of position *sn*-2 of PE were 46% more unsaturated than *sn*-1, because of a higher proportion of C_{18} unsaturates at position *sn*-2. Linoleic (18:2) acid in position *sn*-2 of both phospholipids was 4 to 5 times higher than in position *sn*-1. Position *sn*-1 of both phospholipids contained only insignificant levels of polyunsaturated acids, whereas position *sn*-2 had a predominance of unsaturated fatty acids whose contents in PC and PE were 74 and 75%, respectively.

Positional Distribution of Fatty Acids in TG

Table II shows the distribution of fatty acids among the three positions of the TG. Each position has a quantitatively unique fatty acid composition, suggesting a nonrandom distribution of the fatty acids. However, the distribution pattern for fatty acids of TG had a different profile from that of the diacyl phospholipids. At position *sn*-1, the content of unsaturated fatty acids was greater than the content of saturated fatty acids by about 6 to 4, the predominant fatty acid at position *sn*-1 being 18:1. Position *sn*-2 had a greater content of unsaturated fatty acids than position *sn*-1 or *sn*-3; the proportion of unsaturated fatty acids to saturated fatty acids was almost 92 to 8 at position *sn*-2 with 18:1 being the predominant fatty acid. Position *sn*-2 possessed about 3 times more 18:2 than the other positions, whereas it contained the least amount of 16:0 and 18:0. The fatty acid composition in position *sn*-2 in

TABLE III

Comparison of Determined and Calculated Carbon Number of Triacylglycerol

	Percent carbon number[a]				
	48	50	52	54	56
TG (determined)[b]	5.0	27.9	56.4	10.6	tr
TG (calculated)[c]	1.9	23.5	47.8	25.6	—

[a]A carbon number represents the sum of carbon atoms in the 3 fatty acids esterified to glycerol.

[b]Analyzed intact by gas liquid chromatography.

[c]The 1-random-2-random-3-random distribution was calculated from the determined composition of the position *sn*-1, *sn*-2, and *sn*-3 of the triacylglycerol.

TG was in good agreement with the results of Couch and Saloma (2). Position *sn*-3 showed the greatest content of saturated fatty acids, the content of the latter being equal to the content of unsaturated fatty acids at this position. Several laboratories have demonstrated that position *sn*-2 from various tissue TGs of animals contained the highest percentage of polyunsaturated fatty acids, primarily 18:2 (8-10). It appears that TGs from the egg yolk of Japanese quail have approximately the same pattern of fatty acid distribution as those of animal tissues.

The carbon number distribution based on mol wt. of TG species is given in Table III. Generally, the determined values exhibited a higher percentage of the lower mol wt. species than those of the values calculated on the basis 1-random-2-random-3-random distribution of the fatty acids. The determined carbon number distribution of TGs did not show complete agreement with the calculated distribution. The great differences between expected and actual values occurred in the triacyl glycerols of carbon numbers 52 and 54. The random value for the C_{52} components was very much lower (47.8%) than the experimental value (56.4%), and the random value for the C_{54} component (25.6%) is very much higher than its experimental value (10.6%). This suggests that in Japanese quail there is marked preferential synthesis of the 16,18,18-triacylglycerols in relation to that for the 18,18,18-and the 16,16,18-triacylglycerols.

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Substrate Specificity of Flax Hydroperoxide Isomerase

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ABSTRACT

Incubations of the 13- and 9-hydroperoxides of linolenic acid with a flax acetone powder extract containing hydroperoxide isomerase resulted in the formation of 13-hydroxy-12-oxo-*cis*-9,15-octadecadienoic acid and 9-hydroxy-10-oxo-*cis*-12,15-octadecadienoic acid, respectively. The rate of formation of product from 13-hydroperoxy linolenic acid was 36 times that from 9-hydroperoxy linolenic acid. Analogous results were obtained with the 13- and 9-hydroperoxides of linoleic acid. The results demonstrated the substrate specificity of flax hydroperoxide isomerase.

Lipoxygenase (EC 1.13.11.12) catalyzes the enzymic oxidation of fatty acids containing *cis-cis*-1,4-pentadiene unsaturation. When linoleic acid is used as the substrate, the products of the reaction are 9-D-hydroperoxy-*trans*-10, *cis*-12-octadecadienoic acid (9-OOH 18:2) and 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-OOH 18:2). The ratio of the two isomers varies according to the source of the lipoxygenase (1).

In 1966 Zimmerman (2) showed that fatty acid hydroperoxides of linoleic acid could be further metabolized to α-ketols. Using a flaxseed preparation, Zimmerman and Vick (3) demonstrated that the 13-OOH 18:2 could be metabolized to 13-hydroxy-12-oxo-*cis*-9-octadecenoic acid (13,12-ketol 18:1). The enzyme that catalyzed this reaction was given the name hydroperoxide isomerase. They also isolated a minor compound which could be cleaved by periodate oxidation between carbons 9 and 10. This could have been 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid (9,10-ketol 18:1) formed from 9-OOH 18:2. Veldink et al. confirmed the formation of 13,12-ketol 18:1 from 13-OOH 18:2 using a flax extract. However, they were unable to detect the formation of 9,10-ketol 18:1 from 9-OOH 18:2. They concluded that flaxseed hydroperoxide isomerase was specific for the 13-OOH 18:2 (4).

Gardner et al. (5) reported that corn germ lipoxygenase oxidized linoleic acid to predominantly 9-OOH 18:2, which was subsequently isomerized enzymically to 9,10-ketol 18:1. When a mixture of 13- and 9-OOH 18:2 was used as a substrate, 13,12- as well as well as 9,10-ketol 18:1 were found among the products of catalysis by corn germ hydroperoxide isomerase. Similar findings were reported by Graveland et al. (6) with barley suspension. Barley suspension lipoxygenase oxidized linoleic acid to hydroperoxides in the molar ratio of 8:1 of 9-OOH to 13-OOH 18:2. The isomerization of these hydroperoxides resulted in a 9:1 ratio of 9,10- to 13,12-ketol. These results indicated that hydroperoxide isomerase enzymes from corn germ and barley suspensions were capable of utilizing both 13- and 9-OOH 18:2 as substrates, whereas flaxseed hydroperoxide isomerase utilized only 13-OOH 18:2 as substrate. Hydroperoxide isomerase activity has also been found in wheat germ (7) and in wheat flour (8); however, their substrate specificities are not known.

During our investigation of the metabolism of linolenic acid by a flax extract, we were able to demonstrate the isomerization of 13- and 9-hydroperoxides of linolenic acid to 13,12- and 9,10-ketol, respectively. Similar results were observed when 13- and 9-hydroperoxides of linoleic acid were used as substrates. The isomerization of the 9-hydroperoxide of linoleic acid to 9,10-ketol by a flax extract was in contrast to the findings of Veldink et al. (4).

MATERIALS AND METHODS

Chemicals

Linoleic, linolenic, and 9-hydroxy-hexadecanoic acid were obtained from Nu-Chek Prep Inc. (Elysian, MN); N-methyl-N-nitroso-p-toluenesulfonamide and soybean lipoxygenase (21,600 units/mg), from Sigma Chemical Co. (St. Louis, MO); 2% methoxyamine HCl in pyridine (MOX) and N,O-*bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA), from Pierce Chemical Co. (Rockford, IL); sodium borohydride, from Fisher Scientific Co. (Fairlawn, NJ); platinum oxide, from Matheson, Coleman and Bell (Norwood, OH); precoated Anasil HF silica gel thin layer chromatography plates from Analabs, Inc. (North Haven, CT); and 3% OV-210 on 100/120 mesh Gas-Chrom Q, from Applied Science Laboratories, Inc. (State College, PA). 9-Oxo-hexadecanoic acid, used as the internal standard in gas chromatographic analyses, was prepared by chromium trioxide oxidation (9) of 9-hydroxy-hexadecanoic acid.

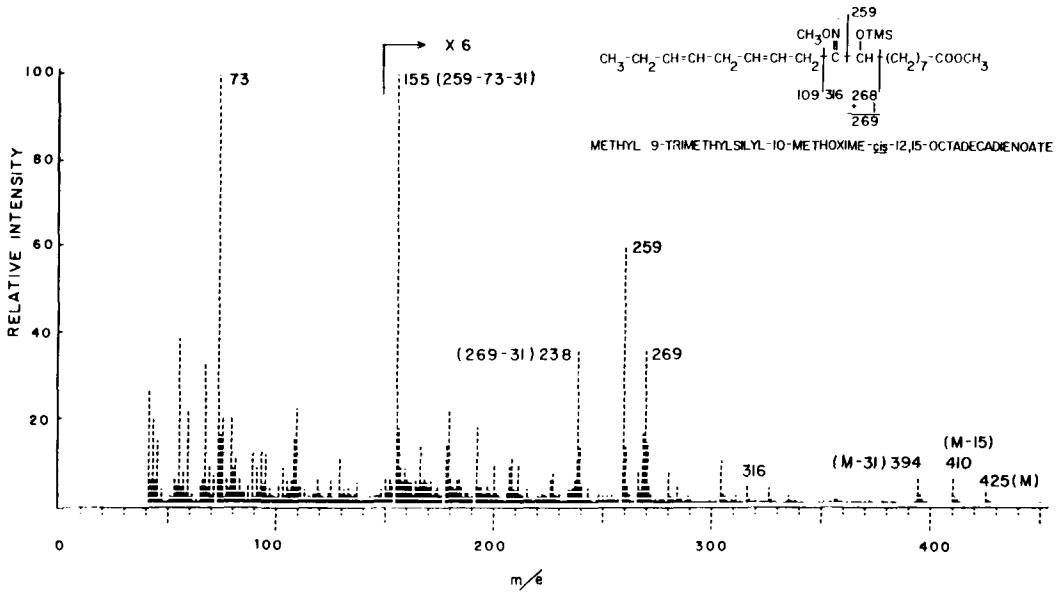

FIG. 1. Mass spectrum of the trimethylsilyl, methoxime derivative of methyl 9-hydroxy-10-oxo-*cis*-12,15-octadecadienoate formed from the 9-hydroperoxy isomer of linolenic acid by extracts of flax acetone powder.

Red firm tomatoes, *Lycopersicon esculentum* Mill., var. Sheyenne, were obtained locally.

Derivatives

Carbonyl and hydroperoxide functions were reduced by use of sodium borohydrode in methanol. Double bonds were reduced by hydrogenation with platinum oxide as the catalyst. Methoxime derivatives of carbonyl functions were made with methoxyamine HCl, and trimethylsilyl ether derivatives of the hydroxyl functions were made with BSTFA (10).

Preparation of 13- and 9-Hydroperoxide Substrate Solutions

Solutions of linoleic and linolenic acids (8 mM) were prepared according to the method of Surrey (11). Acetone powder extracts of flaxseed (*Linum usitatissimum* L., var. Summit) were prepared as described previously (3). The 13-OOH 18:2 and 13-OOH 18:3 substrate solutions were prepared by reaction of soybean lipoxygenase with the linoleic and linolenic acid solutions (12). Two mg of soybean lipoxygenase was dissolved in 2 ml of 10 mM borate buffer, pH 9.2; the enzyme solution was added to 20 ml of water, and reacted with 1 ml of 8 mM linoleic or linolenic acid solutions. The mixture was incubated for 30 min at 24 C under continuous flushing with oxygen.

Firm, red tomatoes were the source of lipoxygenase for reactions with linoleic and linolenic acid solutions to produce 9-OOH 18:2 and 9-OOH 18:3 (13). The fruit was peeled, the seeds removed, and the tissue washed. Then 10 g of tissue was diced and homogenized in 32 ml of 0.1 M acetate buffer, pH 5.5, with a mortar and pestle. The extract was centrifuged at 12,000 x g for 15 min; the 18:2 or 18:3 solution (4 ml) was added to the mixture and incubated for 30 min at 24 C under continuous flushing with oxygen. The major product of the mixture at this point was 9-hydroperoxide. This was determined by reducing the hydroperoxides to methyl hydroxystearates, preparing the trimethylsilyl derivatives with BSTFA, and analyzing by gas chromatography-mass spectrometry (GC-MS).

Preparative Enzymic Synthesis of 9,10-Ketol

The pH of the 9-hydroperoxide substrate solution of 18:2 or 18:3 was adjusted to 7.0 with 0.2 M K-phosphate buffer, pH 7.5. Flax acetone powder extract (8 ml) was added to the mixture and incubated for 1 hr at 24 C. The fatty acid products were extracted by the addition of 30 ml of chloroform/methanol (2:1, v/v), acidification of the mixture to pH 3.0 with 1 M citric acid solution, and flushing of the flask with nitrogen. After 30 min of stirring, 20 ml of chloroform was added, followed by 90 more min of stirring under nitrogen. The chloroform phase was removed and dried with anhydrous sodium sulfate. Then the solvent was evaporated at 40 C under

reduced pressure and the products dissolved in a minimum amount of diethyl ether. Esterification was accomplished with diazomethane. The methylated 9,10-ketol product was then separated by thin layer chromatography (chloroform/acetic acid, 100:1, three developments) or by gas chromatography with a 180 cm glass column packed with 3% OV-210 on Gas-Chrom Q, 100/120 mesh. The column was programmed from 170 to 220 C at 2 C/min.

Hydroperoxide Isomerase Assay

The initial concentrations of 9- and 13-OOH 18:3 substrate solutions were determined by use of UV absorbance at 234 nm and a molar absorptivity of 25,000 l $mole^{-1}$ cm^{-1}. Flax acetone powder extracts were then added to solutions of 9- and 13-OOH 18:3 of equal concentrations. A typical reaction contained 2 μmoles 9- or 13-OOH 18:3, 125 μg 9-oxohexadecanoic acid as internal standard, and 0.1 ml flax extract (0.5 ml for reaction with 9-OOH 18:3) in 25 ml total volume. After extracting with chloroform, the products were converted to saturated methyl esters, and quantified by gas chromatography. The reaction rate was determined from a graphical plot of concentration versus time. Initial rates were used in the determinations of the specific activities. Protein was assayed with Coomassie brilliant blue G250 dye by the method of Sedmark and Grassberg (14).

Instrumentation

Infrared spectra were obtained on a Perkin Elmer 337 spectrophotometer and ultraviolet spectra on a Beckman DK-2. Mass spectra were obtained on a Hewlett-Packard 5992A GC-MS system operated at 70 eV with the column prepared and programmed as described above.

RESULTS AND DISCUSSIONS

Tomato lipoxygenase is highly specific for synthesis of the 9-D-hydroperoxide. Incubationof linolenic acid with tomato homogenate gave a 9- to 13-hydroperoxide ratio of 96:4, similar to that reported by Matthew et al. (13). This ratio was determined by GC-MS analysis of the trimethylsilyl derivatives of the hydroperoxides, which showed large m/e ions at 259 $[M-(CH_2)_8CH_3]$ and 229 $[M-(CH_2)_7COOCH_3]$ resulting from chain cleavage on either side of the trimethylsilyl group of methyl-9-trimethylsilyl-octadecanoate; there were smaller mass fragments at m/e 315 $[M-(CH_2)_4CH_3]$ and 173 $[M-(CH_2)_{11}COOCH_3]$ resulting from chain cleavage on either side of the trimethylsilyl group of methyl-13-trimethylsilyl-octadecanoate. The 9- and 13-hydroperoxide ratio of 96:4 was estimated by comparison of the areas under the curves of the ions at m/e 259 and 229 with those at m/e 315 and 173 obtained by use of selected ion monitoring (15).

When the 9-OOH 18:3 solution was further reacted with flax acetone powder extract and the products analyzed by gas chromatography, the major product was a compound having the retention time of an α-ketol. The compound was derivatized by use of MOX and BSTFA and analyzed by GC-MS. Mass fragments at m/e 425 [M], 269 $[M-(CH_2)_7COOCH_3]$, 259, 316, and 109 indicated that the structure was methyl-9-trimethylsilyl-10-methoxime-*cis*-12,15-octadecadienoate (Fig. 1). This was supported by the infrared spectrum of the methyl ester of the underivatized compound. The significant structural characteristics were a secondary hydroxyl group (3494 cm^{-1}), unsaturation =CH- (3012 cm^{-1}), an ester carbonyl group (1741 cm^{-1}), and a ketone carbonyl (1714 cm^{-1}) (16). The absence of absorption between 950 to 1000 cm^{-1} indicated the absence of *trans* double bonds. Since the product of tomato lipoxygenase was predominantly 9-hydroperoxy-*trans*-10-*cis*-12,15-octadecatrienoic acid, the disappearance of the *trans* double bond established the point at which isomerization occurred in the hydroperoxide molecule, namely at carbons 9 and 10. The absence of UV absorption also supported the proposed structure as 9-hydroxy-10-oxo-*cis*-12,15- octadecadienoic acid.

Hydrogenation of 9,10-ketol 18:2, followed by derivatization with MOX and BSTFA, resulted in the formation of methyl-9-trimethylsilyl- 10-methoxime-octadecanoate. GC-MS analysis showed a molecular ion of 429; the increase of 4 mass units in the molecular ion was caused by the saturation of the double bonds at carbons 12 and 15. Other characteristic ion fragments were at m/e 414 [M-15], 398 [M-31], 273 $[M+H^+-(CH_2)_7COOCH_3]$, 259 $[M-CNOCH_3(CH_2)_7CH_3]$, and 170 [M-259].

The oxidation of linoleic acid with tomato lipoxygenase produced mainly 9-OOH 18:2. Further reaction of 9-OOH 18:2 with a flax acetone powder extract resulted in the formation of 9,10-ketol 18:1. Mass spectral analysis of this product as a methyl 9-trimethylsilyl-10-methoxime-*cis*-12-octadecenoate showed fragments at m/e 427[M], 412[M-15], and 396[M-31] (Fig. 2). Other characteristic fragments were at m/e 271 $[M-CHOTMS(CH_2)_7COOCH_3]$, 240 [271-31], 259 $[CHOTMS(CH_2)_7COOCH_3]$, 155 [259-31], and 168 [M-259]. The data clearly indicated the formation of a 9,10-ketol from the 9-hydroperoxide of linoleic acid.

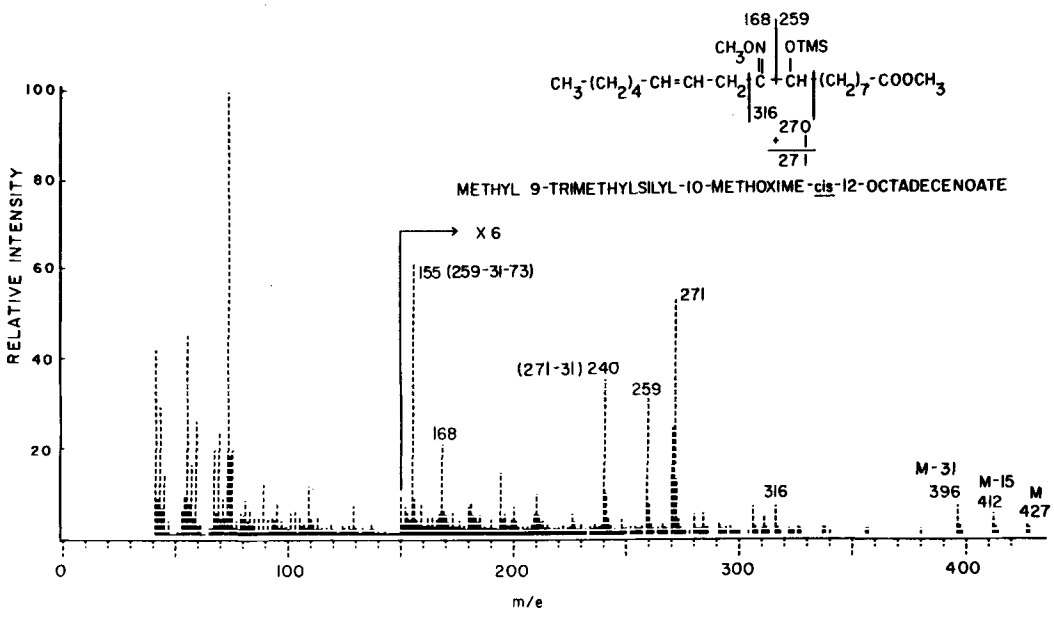

FIG. 2. Mass spectrum of the trimethylsilyl, methoxime derivative of methyl 9-hydroxy-10-oxo-*cis*-12-octadecenoate formed from the 9-hydroperoxy isomer of linoleic acid by extracts of flax acetone powder.

Although flax hydroperoxide isomerase was capable of utilizing 9-OOH 18:3 as a substrate, the specific activity was much lower than when 13-OOH 18:3 was used as the substrate. The specific activity with 13-OOH 18:3 was 690 nmoles/min/mg compared to 19 nmoles/min/mg with 9-OOH 18:3 (a ratio of 36:1).

Veldink et al. (4) prepared the hydroperoxide substrate of 18:2 using soybean lipoxygenase, which gave a 13- to 9-OOH ratio of 90:10. The low initial concentration of the 9-hydroperoxide and the low activity of flax hydroperoxide isomerase toward the 9-hydroperoxide may be the reason why they did not detect the 9,10-ketol as a product. It is evident from our findings that flax hydroperoxide isomerase, although not very active toward 9-hydroperoxides, is capable of utilizing both 13- and 9-hydroperoxides of linoleic and linolenic acids as substrates.

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Oxidation and Isomerization of Retinoic Acid by Iodine and Light: A Novel Preparation of All-*trans*- and 13-*cis*-4-Oxoretinoic Acid

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ABSTRACT

A mixture of all-*trans*-retinoic acid and iodine in heptane was irradiated. Two oxidation products were isolated by high performance liquid chromatography and identified as all-*trans*- and 13-*cis*-4-oxoretinoic acid by nuclear magnetic reasonance, ultra violet, Infrared spectroscopy, and mass spectral analysis. Under the same conditions, but without light, a mixture of all-*trans*- and 13-*cis*-retinoic acid resulted. The corresponding methyl esters were obtained when methyl all-*trans*-retinoate was used in place of all-*trans*-retinoic acid.

INTRODUCTION

The metabolism of vitamin A, in general, and retinoic acid in particular has been of interest in our laboratory for several years (1-3). Recent advances in high performance liquid chromatography (HPLC) and the development of high efficiency columns have provided the means for the rapid separation and purification of these labile compounds in the dark, at room temperature and without exposure to air. Procedures for the preparation of geometric isomers and oxidation products, the most commonly encountered derivatives of retinoic acid and related substances, were of particular interest to us. In this regard, Hubbard (4) has reported the isomerization of retinol, retinaldehyde, and retinaldehyde oximes under the influence of light and/or iodine. The major products resulting from the irradiation of all-*trans*-retinoic acid in the absence of iodine have been purified by HPLC and identified as geometric isomers of retinoic acid (5). These products account for at least seven of the eight possible *cis-trans* isomers that involve the 9, 11, and 13 double bond positions of retinoic acid. The present communication is concerned with the isolation and identification of the products generated by the treatment of all-*trans*-retinoic acid with iodine in the presence or absence of light.

EXPERIMENTAL

Distilled in glass solvents were obtained from Burdick and Jackson Laboratories Inc. (Muskegon, MI). Water was deionized, charcoal-filtered, and then glass-distilled. All-*trans*-retinoic acid and all-*trans*-4-oxoretinoic acid were obtained from Dr. W.E. Scott and Dr. B.A. Pawson, Hoffmann-La Roche, Inc. (Nutley, NJ). Methyl esters were prepared from retinoic acid and related products using diazomethane in methanol/diethyl ether solutions, essentially as described by Schlenk and Gellerman (6). Isomerates were prepared by irradiation with a fluorescent lamp (GE F-15-T8-CW). The irradiations were carried out in 22 ml borosilicate glass vials held at a distance of ca. 5 cm from the lamp. Samples were dissolved in heptane, and iodine, dissolved in heptane, was added as indicated. To terminate the iodine reactions, the samples were dried in the dark, with a gentle stream of nitrogen. The vials were partially submerged in a warm water bath to aid in rapid evaporation of the solvent and sublimation of the iodine. Such samples, once dry, were routinely redissolved in diethyl ether and redried to assure removal of iodine. Separations were performed by HPLC on a 0.46 x 25 cm bonded, reverse phase, octadecylsilane (ODS) column (Partisil 10-ODS, Whatman Laboratory Products, Clifton, NJ) with methanol/water, 70:30 (v/v) as solvent as previously described (5). Cochromatography with all-*trans*-4-oxoretinoic acid was on a Partisil PXS ODS-2 column with methanol/0.01 M acetic acid (75:25). Purified samples were subjected to mass spectral analyses by way of the direct probe inlet on a low resolution mass spectrometer (LKB-9000, LKB Instruments, Inc., Rockville, MD) as previously described by Waller (7), Lin et al. (8) and Reid et al. (9). Nuclear magnetic resonance (NMR) spectra were obtained using a Varian XL-100 (15) spectrometer (Varian, Palo Alto, CA). Samples were dissolved in carbon tetrachloride. Infrared spectroscopy (IR) was performed with a Perkin-Elmer (Norwalk, CT) Model 457 grating

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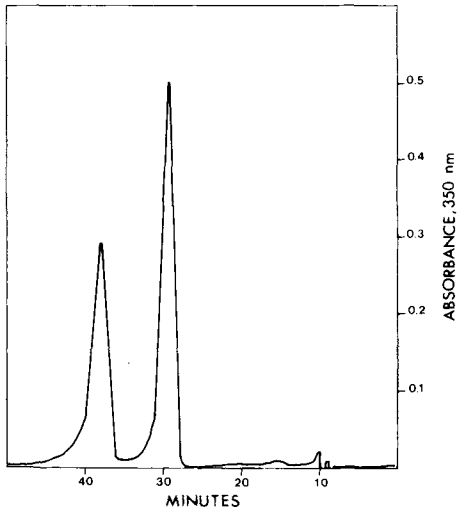

FIG. 1. HPLC of the products resulting from a mixture of all-*trans*-retinoic acid (130 μg/ml) and iodine (25 μg/ml) in heptane incubated for 3 hr in the dark. Compounds were separated on a reverse phase column with methanol/water (70:30) at a flow rate of 0.4 ml/min and 24 C. Compounds eluting at 29 and 38 min were identified as 13-*cis*- and all-*trans*-retinoic acid, respectively.

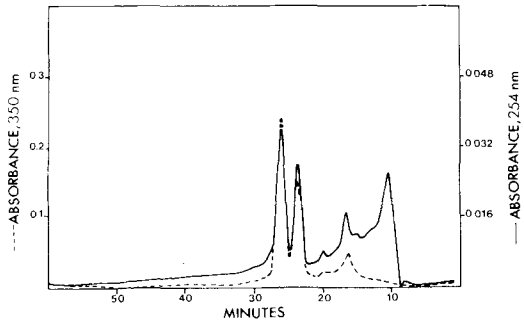

FIG. 2. HPLC of the methylated products resulting from a 2½ hr. irradiation of a mixture of all-*trans*-retinoic acid (130 μg/ml) and iodine (25 μg/ml) in heptane. Chromatography conditions were as described in Figure 1. Compounds eluting at 24 and 27 min were identified as the methyl esters of 13-*cis*- and all-*trans*-4-oxoretinoic acid, respectively.

infrared spectrophotometer using micro KBr pellets.

RESULTS AND DISCUSSION

Iodine-Dark Reaction

In the absence of light and iodine, solutions of either all-*trans*-retinoic acid or methyl all-*trans*-retinoate in methanol, diethyl ether or heptane can be stored at room temperature for extended periods of time without significant change. The addition of iodine to all-*trans*-retinoic acid in heptane in the dark resulted in a rapid change leading to a mixture of two compounds. The two compounds were separated by HPLC (Fig. 1) and identified as the starting material, all-*trans*-retinoic acid, which eluted at 38 min, and 13-*cis*-retinoic acid, which eluted at 29 min. The 13-*cis*-retinoic acid was identified by its UV (λmax 354 nm) and NMR spectra (10,11). The mass spectra of the two compounds (M^+ = *m/e* 300) were indistinguishable and similar to that which has been previously reported (8). Correspondingly, methyl all-*trans*-retinoate and methyl 13-*cis*-retinoate were obtained when methyl all-*trans*-retinoate was the starting material.

The UV spectra of the two acids were of interest. Robeson et al. (12) reported a marked blue shift in the absorbance maximum of retinoic acid in ethanol if great care was not exercised in the preparation of the solvent. This shift was observed in the present study using glass-distilled methanol in which all-*trans*-retinoic acid had an absorbance maximum at 337-338 nm. However, when scanned on-line in aqueous methanol as they passed through the detector, these same samples showed no such shift. On-line UV scans indicated absorbance maxima of 350-351 nm for the all-*trans*-isomer and 353-354 nm for the 13-*cis*-isomer.

Iodine-Light Reaction

When a mixture of retinoic acid and iodine in heptane was irradiated, several products more polar than retinoic acid were separated by HPLC. The same qualitative results were obtained for the iodine-light reaction with either methyl all-*trans*-retinoate or all-*trans*-retinoic acid, followed by subsequent methylation of the reaction products. Since these compounds were more easily handled as methyl esters, the major products were isolated and characterized as methyl esters. A profile obtained with the methylated derivatives of the iodine-light reaction is shown in Figure 2. The compounds eluting at 24 and 27 min were collected and purified by HPLC using methanol/water (60:40) which resulted in retention times of 34 and 40 min, respectively.

The UV spectra of these two compounds were very similar. The on-line scans indicated absorption maxima in the vicinity of 353-355 nm and 278-280 nm for both compounds. The ratio of the peak absorbances ($A_{350}/_{280}$) was about 2.2-2.5. The mass spectral fragmentation patterns of these two compounds were also qualitatively indistinguishable, indicating that they were, in all probability, geometric isomers

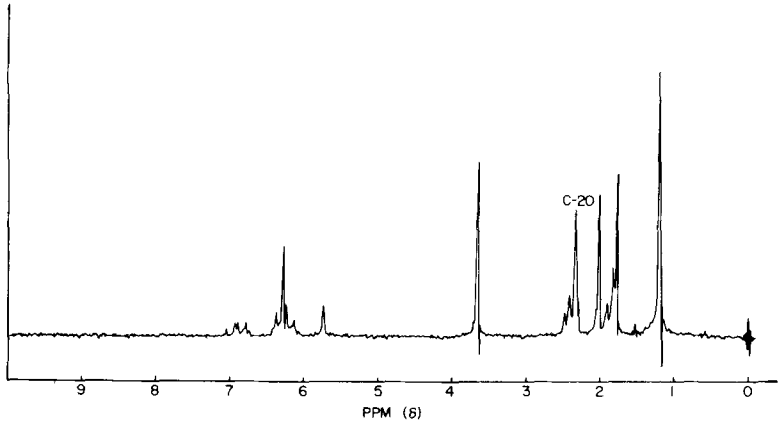

FIG. 3. Nuclear magnetic resonance spectrum of the compound eluting at 27 min in Figure 2 and identified as methyl all-*trans*-4-oxoretinoate.

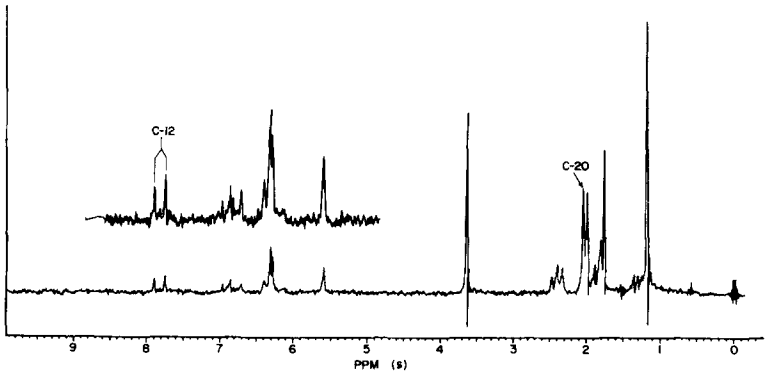

FIG. 4. Nuclear magnetic resonance spectrum of the compound eluting at 24 min in Figure 2 and identified as methyl 13-*cis*-4-oxoretinoate.

of the same compound (8). The molecular ion (*m/e* 328) was consistent with these compounds being oxidation products of methyl retinoate (M^+ = *m/e* 314), which had added a single oxygen atom and lost two protons. The molecular weight and the absorbance at 280 nm are consistent with a ketone structure. The presence of a ketonic function was further supported by the presence of two distinct carbonyl absorbances at 1650 and 1700 cm^{-1} in the infrared spectra. Again, the spectra of the two compounds were essentially identical.

The NMR spectra of the peaks are presented in Figures 3 and 4. Based on these spectra, the site of oxidation is restricted to either carbon 2 or 4 in the cyclohexenyl ring. The location of the oxygen on either carbon 2 or 4 was supported by the conversion of the methylene multiplet of methyl retinoate to a pair of triplets rather than a pair of singlets as would be required by a 3-oxo structure. This triplet character is readily seen in Figure 4, where the upfield shift of the C-20 signal has left a triplet centered at ca. δ 2.4 clearly visible. The second triplet is partially hidden by the C-18 signal and centered at ca. δ 1.8. The assignment of the oxygen to C-4 was based on the fact that the downfield shift (18 Hz) of the *gem*-dimethyl signal (C-16 and C-17) was comparable in extent to that observed in a model 4-oxo compound (13).

Additional support for the 4-oxo structure is provided by Rao et al. (14) who prepared methyl 4-oxoretinoate by exposing methyl retinoate to active manganese dioxide in light petrol. THe NMR spectra shown in Figure 3 is essentially identical to that reported by them for methyl 4-oxoretinoate (14). In addition to the UV, NMR, IR and mass spectral analysis, when all-*trans*-retinoic acid was irradiated in heptane in the presence of iodine as described in Figure 2, the compound identified as all-

trans-4-oxoretinoic acid cochromatographed with the sample obtained from Hoffmann-La Roche, Inc. This compound was one of several 4-oxo derivatives or retinoic acid that were reported to have been isolated from rat urine (15).

In the present study, a comparison of the spectra in Figures 3 and 4 reveals an upfield shift of the C-20 methyl signal from δ 2.33 (Fig. 3) to δ 2.06 (Fig. 4) and a downfield shift of the C-12 doublet from δ 6.4 (Fig. 3) to δ 7.82 (Fig. 4). These changes are characteristic of the 13-*cis*-configuration (10,11). Thus, based on the data presented here and by previous workers, the two compounds eluting at 24 and 27 min (Fig. 2) were assigned the structures of the methyl esters of 13-*cis*- and all-*trans*-4-oxoretinoic acid, respectively.

We have not isolated products resulting from the irradiation of retinol or retinaldehyde in the presence of iodine. These and similar studies can now be simply and fruitfully pursued along many different avenues of interest using HPLC analysis. This potent analytical tool has opened the way to an effective study of this biologically significant family of compounds and of polyene compounds in general.

ACKNOWLEDGMENTS

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Phospholipid Metabolism in *Neisseria gonorrhoeae:* Phospholipid Hydrolysis in Nongrowing Cells

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ABSTRACT

Hydrolysis of cell envelope phospholipids was demonstrated in cells of both autolytic and nonautolytic strains of *Neisseria gonorrhoeae* that were labeled during growth in the presence of [^{3}H] acetate. The label incorporated into the cellular phospholipids was located exclusively in the fatty acid acyl side chains. Labeled cells were incubated for 2 hr in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, pH 8.5, containing various additions, and then examined for distribution of ^{3}H in lipids. Ca^{++} selectively stimulated the deacylation of phosphatidylethanolamine (PE), whereas Mn^{++} stimulated the deacylation of phosphatidylglycerol (PG). Hydrolysis of phosphatidylethanolamine by phospholipase A was accompanied by the accumulation of lysophosphatidylethanolamine (LPE) and free fatty acids in the cells. Free fatty acids accumulated to a greater extent than lysophosphatidylethanolamine , suggesting that the latter was further hydrolyzed to glycerophosphorylethanolamine (GPE) and free fatty acids by a lysophospholipase. Methanol, ethanol, propanol, and isopropanol, added at concentrations which inhibited growth by 50%, stimulated phospholipase A, but not lysophospholipase activity. Differences in heat inactivation, metal ion requirements, and pH optima suggested that phospholipase A activities with phosphatidylethanolamine or phosphatidylglycerol as substrate and lysophospholipase may be separate enzymes.

INTRODUCTION

Gonococci release free fatty acids (FFA) and lysophosphatidylethanolamine (LPE) into the medium during growth (1). The release of these products suggests that phosphatidylethanolamine (PE), a major cell envelope component of *Neisseria gonorrhoeae,* is hydrolysed by phospholipase(s) (1). Further evidence for hydrolysis of PE in gonococci has been shown by the cellular accumulation of LPE in stationary phase cultures (2), and in cells which were suspended in buffer and lyophilized (3). In *N. gonorrhoeae,* a cell envelope phospholipase A has been shown to be highly active with PE (3). Similar enzymes have been found in association with the outer membrane of other gram-negative bacteria (4,5). A recent investigation (6) has shown that hydrolysis of PE does not occur during autolysis of *N. gonorrhoeae.* The present study examines factors which influence the hydrolysis of endogenous phospholipids in whole cells of *N. gonorrhoeae,* and presents evidence that phospholipid hydrolysis occurs in both autolytic and nonautolytic strains of gonococci.

MATERIALS AND METHODS

Organism and Growth Conditions

Neisseria gonorrhoeae strains JW-31 (autolytic) and CS-7 (nonautolytic) were employed. The properties of these strains have been previously described (7,8). Cells were grown at 37 C in liquid medium containing IsoVitaleX (1% v/v), $NaHCO_3$ (420 mg/l), and 0.5% (w/v) glucose (final pH = 7.2) as described (9). Growth was monitored turbidometrically in nephelometer flasks using a Klett Summerson colorimeter, filter No. 54 (540 nm).

Radioactive Labeling of Phospholipids

To radioactively label phospholipids, cells were grown to the mid-log phase (120-130 Klett units), then centrifuged and resuspended in fresh medium containing [^{3}H]acetate. Resuspension of cells in fresh medium is necessary since the metabolism of glucose results in significant accumulation of acetate in the medium (8). [^{3}H] Acetic acid, sodium salt (686 mCi/mmole; New England Nuclear Corp., Boston, MA) was added at 5 μCi/ml. Cultures were incubated for 40 min, then centrifuged, resuspended in fresh medium and incubated for an additional 10 min to facilitate incorporation of any residual extracellular [^{3}H]acetate or ^{3}H-labeled intracellular pools.

Distribution of Radioactivity in Phospholipids

The relative distribution of ^{3}H in the glycerol backbone and in the fatty acid acyl groups of the labeled phospholipids was determined as follows. A 50 ml culture of labeled cells was centrifuged and the cell pellet extracted with a 1:1 mixture of chloroform/

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TABLE I

Hydrolysis of Endogenous Phospholipids In *N. gonorrhoeae* Suspended in Buffer[a]

Conditions[b]	Strain	^3H recovery (% of Time 0)	Percent of ^3H recovered as[c] PE	PG	LPE	FFA
Time 0	JW-31	100	72.6	22.4	2.5	1.1
pH 8.5	JW-31	87	71.3	21.7	1.3	4.5
pH 8.5, Mg^{++}	JW-31	94	62.1	21.7	2.8	12.0
pH 8.5, Ca^{++}	JW-31	93	36.2	17.4	7.3	36.9
pH 8.5, Mn^{++}	JW-31	92	45.1	10.7	3.4	38.1
pH 8.5, Fe^{++}	JW-31	74	54.3	15.8	5.4	15.3
pH 8.5, Zn^{++}	JW-31	94	66.6	19.9	3.9	5.6
pH 8.5, Ca^{++}, NEM	JW-31	90	35.8	17.1	7.6	37.4
pH 8.5, Ca^{++}, Hg^{++}	JW-31	80	62.9	17.8	6.3	10.1
pH 6.0	JW-31	91	72.6	23.5	1.3	2.0
pH 6.0, Mg^{++}	JW-31	90	71.2	24.0	1.0	1.5
pH 6.0, Ca^{++}	JW-31	94	65.3	21.6	3.7	7.3
Time 0	CS-7	100	70.3	26.1	2.0	0.3
pH 8.5	CS-7	88	67.2	22.9	3.5	4.7
pH 8.5, Mg^{++}	CS-7	94	63.0	25.0	2.8	7.7
pH 8.5, Ca^{++}	CS-7	98	49.8	21.9	6.5	19.9

[a] [^3H]acetate-labeled cells were incubated at 37 C for 2 hr in HEPES buffer, pH 8.5 or 6.0 with various additions and ^3H distribution in lipids was determined as described in Materials and Methods.

[b] Divalent cations were added at a final concentration of 20 mM. $HgCl_2$ and NEM were added at a final concentration of 10 mM. $HgCl_2$ was added to growing cells 10 min prior to suspension in buffer.

[c] The total amount of ^3H applied to the chromatograms at 0 time varied between experiments, but was typically 2.5-3.5 x 10^5 CPM. The ^3H recovery from the chromatogram at 0 Time was taken as 100%, and the relative percent of ^3H present in the various lipid spots was calculated. The values reported are averages of 2 or more experiments. The deviation from the averages was ± 1-5%.

methanol as described below. The lipid extract was concentrated, and phospholipids were isolated by thin layer chromatography (TLC) on a 20 x 20 cm silica gel plate (see next section for procedure). The areas corresponding to PE and PG were identified, and the [^3H] lipids were recovered from the scraped gel by elution with chloroform/methanol. The extract was evaporated to dryness, resuspended in water and [^3H]PE or [^3H]PG extracted into chloroform by the Bligh-Dyer procedure (10). The chloroform extract was evaporated, subjected to alkaline methanolysis as described by White (11), then extracted with equal volumes of chloroform and water. After phase separation, the chloroform and aqueous layers were assayed for radioactivity, and analyzed by TLC to determine the distribution of ^3H in the reaction products.

Assay of Phospholipid Hydrolysis

The assay was a modification of the procedure of Grossman et al. (12) and measured the hydrolysis of endogenous phospholipids in intact cells as a function of incubation conditions. Cells were labeled with [^3H]acetate, and adjusted to a standard cell density (100 Klett units). A series of 10 ml samples were withdrawn and immediately centrifuged (3,400 x g for 3 min). The supernates were decanted and the pellets (containing ca. 2.0 mg cells dry wt., and 3-4 x 10^5 CPM of ^3H) were suspended in 4 ml of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer and incubated at 37 C under the conditions specified. Immediately (0 time) or after incubation for 2 hr, the suspensions were centrifuged (10,000 x g for 10 min) and the cell pellets and supernates recovered. To assay hydrolytic activity, the cell pellets were suspended in 10 ml of a 1:1 (v/v) mixture of chloroform/methanol and lipids were extracted at 25 C with shaking for 60 min in 125 ml capped flasks. The cell residue was removed by centrifugation, and the entire lipid extract was withdrawn and evaporated to dryness at 25 C with air flow. The resulting lipid residue (ca. 2.5-3.5 x 10^5 CPM of ^3H) was dissolved in a minimal amount of chloroform/methanol/water (2:1:0.1) and quantitatively applied to precoated, glass-backed, 5 x 20 cm silica gel plates.

PE, PG, LPE, glycerophosphorylethanolamine (GPE), cardiolipin, oleic acid, palmitic acid, tripalmitin, dipalmitin, monopalmitin, and phosphatidic acid (Sigma Chemical Co., St. Louis, MO) were employed as standards.

Chromatograms were developed in a single dimension for 2 hr in a mixture of chloroform/methanol/water (65:25:4, v/v), then air-dried. Fatty acids were visualized by short wave ultraviolet light and phospholipids detected by exposure to iodine vapor. PE and LPE were identified by staining with ninhydrin. Lipid spots were scraped from the plates and placed in scintillation vials. One ml of methanol/water (2:1) and 10 ml of scintilation fluid (13) were added to the vials, and radioactivity was determined in a liquid scintillation spectrophotometer. Controls with [^{3}H] toluene indicated that the methanol/water mixture quenched 8.5%; no significant quenching was observed in the presence of unlabeled phospholipids or free fatty acids.

The percent of total ^{3}H associated with each of the lipid classes was determined before (time 0) and after incubation of cells. Most of the ^{3}H (>96%) was localized in five spots having R_f values which corresponded to PE (R_f = 0.67), PG (R_f = 0.47), LPE (R_f = 0.32), oleic and palmitic acids (R_f = 0.95, and neutral lipid (R_f = 0.98-1.00) standards. Criteria used to further identify PE, PG, and LPE isolated from gonococci by a similar extraction and chromatographic procedure have been previously described (3).

Identification of Free Fatty Acids

Incubation of labeled cells in buffer, under conditions which promoted the hydrolysis of PE and PG, resulted in a dramatic increase in radioactivity associated with a spot at R_f = 0.95. To identify this labeled material, the gel at R_f = 0.95 was scraped from thin layer plates and eluted with chloroform/methanol. The solvent was evaporated, and the lipid residue was rechromatogramed in a single direction in a solvent system (14) consisting of petroleum ether/ethyl ether/glacial acetic acid (90:15:2, v/v). Spots were visualized, scraped, and counted as above. Under these conditions, 96% of the radioactivity recovered was localized in a spot at R_f = 0.48. This spot migrated with the oleic and palmitic acid standards, and was clearly separated from standards of monopalmitin (R_f = 0.02), dipalmitin (R_f = 0.24), and tripalmitin (R_f = 0.66). Fatty acids in this spot were identified by gas liquid chromatography according to the method of Wene et al. (15). The major fatty acids were 16:0 (38.0%); 16:1 (33.9%) and 18:1 (21.9%). Therefore, the spot which migrates with R_f = 0.95 in the chloroform/methanol/water solvent system appears to represent [^{3}H]acetate-labeled fatty acid acyl groups released by deacylation of phospholipids, and hereafter is referred to as the free fatty acid (FFA) fraction.

RESULTS

Distribution of Label in Phospholipids

During exponential growth of *N. gonorrhoeae* in media containing glucose, exogenous [^{14}C]acetate is not oxidized to $^{14}CO_2$ but is incorporated primarily (ca. 78%) into the lipid-containing fraction of the cell (7,8). Relatively small amounts of ^{14}C were associated with wall material (6%), and with protein (8%) and nucleic acids (3%). From the restricted pattern of acetate incorporation, one would expect [^{3}H]acetate to be incorporated into the fatty acid acyl groups but not into the glycerol backbones of phospholipids. [^{3}H]-Labeled PE and PG were isolated from cells labeled during growth with [^{3}H]acetate, deacylated by mild alkaline methanolysis, and the products identified as described in Materials and Methods. After deacylation, 97 and 98% of the ^{3}H activity of PE and PG, respectively, was recovered as free fatty acids.

Table I (0 time sample) shows the relative amounts of ^{3}H associated with various phospholipid classes in cells labeled with [^{3}H]acetate. The phospholipid patterns of strains JW-31 and CS-7 were similar. The major labeled phospholipids were PE and PG. LPE was a minor constituent of the total phospholipids and the amount of ^{3}H in cell-associated FFA was very low. A small amount of ^{3}H (less than 1%) migrated with an R_f value corresponding to neutral lipids. This fraction was not further identified and is not reported since the ^{3}H activity of this fraction was similar before and after incubation of cells in buffer.

The relative amounts of PE, PG, LPE and FFA in strains JW-31 and CS-7 were similar to those reported for other strains of *N. gonorrhoeae* (2,3,16). Other investigators (2,3,16) have reported that cardiolipin is present in gonococci in low concentrations (<2%). Using two dimensional TLC with n-butanol/acetic acid/water (60:20:20, v/v) as the second solvent (17), we could not detect [^{3}H]labeled cardiolipin in exponential phase cells of strains JW-31 and CS-7. Other studies (2) have indicated that the relative percent of cardiolipin in *N. gonorrhoeae* is increased during the stationary phase of growth.

Phospholipid Hydrolysis in Cells Suspended in Buffer

Phospholipase A activity was previously demonstrated in *N. gonorrhoeae* strain 2686 by incubating cell envelope preparations with exogenous radioactively labeled phospholipids (3). The present study investigates the hydroly-

sis of endogenous phospholipids in whole cells of *N. gonorrhoeae* JW-31 and CS-7.

Table I shows the effect of pH and of divalent cations on the hydrolysis of endogenous phospholipids. Phospholipid hydrolysis occurred in both strains, the highly autolytic JW-31 and the nonautolytic CS-7, and was both pH and divalent cation-dependent. At pH 8.5, in the absence of divalent cations, there was minimal hydrolysis of PE. At pH 8.5, in the presence of Ca^{++}, the relative percentage of PE decreased markedly, and the relative percentages of cell-associated LPE and FFA concomitantly increased. When cells were incubated at pH 6.0, phospholipid hydrolysis was very low as compared to pH 8.5 and was only minimally stimulated by Ca^{++}. Other divalent cations stimulated phospholipid hydrolysis to different degrees and appeared to selectively stimulate the hydrolysis of PE or of PG.

While incubation of cells in buffer, pH 8.5 containing Ca^{++}, resulted in extensive hydrolysis (ca. 50%) of PE, a high percentage (94%) of the total ^3H activity initially present (time 0) was recovered in the cell pellet following incubation. This suggested that the products of PE hydrolysis, LPE and FFA, remained cell-associated and were not released into the buffer. It appears that LPE and FFA accumulate in cells incubated in buffer. Although it is not known if these products are reutilized for phospholipid synthesis, this appears unlikely in cells suspended in buffer without an energy source. However, under growth conditions, the products of PE hydrolysis do not accumulate, but are reutilized for phospholipid synthesis (data to be published).

The premise that LPE and FFA remain cell-associated and are not selectively released into the buffer was further investigated by analysis of the buffer supernates. Supernates were extracted by the Bligh-Dyer procedure (10) and analyzed for ^3H distribution in lipids as described above. These studies indicated that the ratio of FFA, LPE, PE, and PG in the supernates was similar to that in the cell pellets. The total amount of ^3H found in buffer supernates was ca. 3-12% of the activity at time zero.

In several cases, the amount of ^3H that was recovered by extraction of the cell pellet was significantly lower than that recovered from zero time cells. The lower recovery of ^3H (87%) following incubation of cells at pH 8.5 in the absence of divalent cations correlates with the observation that these conditions promote cell autolysis (6,9). Cell lysis probably results in the release of membrane fragments which are not recovered by centrifugation. The low recovery (74% and 80%) of ^3H following incubation of cells with Fe^{++} or Hg^{++} might be due to the interaction of these metals with lipids to form salts which have decreased solubility in chloroform/methanol.

Table I also shows the effect of sulfhydryl poisons on phospholipase activity in strain JW-31. When cells were incubated for 10 min with Hg^{++}, then suspended in buffer, the hydrolysis of PE was markedly inhibited. In contrast, the addition of N-ethylmaleimide (NEM) to the buffer did not inhibit phospholipid hydrolysis. Hg^{++}, but not NEM, also inhibited autolysis and the hydrolysis of peptidoglycan in cells suspended in buffer (9). It is possible that in situ, hydrolytic enzymes associated with the cell envelope have essential sulfhydryl groups which are accessible to Hg^{++} but not to NEM. However, we cannot exclude the possibility that inhibition of hydrolysis of cell envelope components by Hg^{++} is a secondary effect of general Hg^{++} toxicity.

Estimation of the Relative Activities of the Enzymes Catalyzing Phospholipid Hydrolysis

Table II expresses the data of Table I in a form which estimates the relative activity of phospholipase A and lysophospholipase under various conditions. Estimation of enzyme activity is based on the fact that ^3H was exclusively localized in the fatty acid acyl groups of phospholipids. Preliminary studies indicated that the decrease in PE and PG (phospholipase A activity), and the increase in FFA from hydrolysis of LPE and LPG (lysophospholipase activity) were linear over a 2 hr period in cells incubated at pH 8.5 in the presence of Ca^{++} (data not shown). In view of the complexity of the system, and since kinetic studies were not performed for other conditions, the relative enzyme activities are expressed as a net hydrolysis over a 2 hr period. Phospholipase A activity was calculated (equation i) as the net hydrolysis of either PE or PG (expressed as a percent) in 2 hr.

$$(i) \quad \frac{\%PE_0 - \%PE_2}{\%PE_0} \quad \text{or} \quad \frac{\%PG_0 - \%PG_2}{\%PG_0}$$

The effect of divalent cations on the relative activity of phospholipase A is summarized in Table II. In the absence of divalent cations, activity was minimal. In the presence of Ca^{++}, phospholipase activity was greater with PE than with PG as substrate and was higher in strain JW-31 than in strain CS-7. In the presence of Mn^{++}, phospholipase A activity was higher with PG than with PE. PE and PG were hydrolyzed to a similar extent in the presence of Fe^{++}. Mg^{++} and Zn^{++} were much less effective than Ca^{++}, Mn^{++} or Fe^{++} in stimulating phospho-

TABLE II

Estimation of Phospholipase Activities as Net Hydrolysis of Phospholipids in Whole Gonococci[a]

Strain	Additions	Phospholipase A Net Hydrolysis of		Lysophospholipase Net Hydrolysis of	
		PE	PG	LPE	LPE + LPG
JW-31	None	1.8	3.1	---	---
JW-31	Ca^{++}	50.1	22.3	73.6	72.9
JW-31	Ca^{++}, Hg^{++}	13.4	20.5	21.8	25.8
JW-31	Mg^{++}	14.5	3.1	94.3	76.9
JW-31	Mn^{++}	37.8	52.2	93.4	88.8
JW-31	Fe^{++}	25.2	29.5	68.3	14.1
JW-31	Zn^{++}	8.3	11.2	53.3	5.9
CS-7	None	4.4	12.3	---	---
CS-7	Ca^{++}	29.1	16.1	56.0	42.5
CS-7	Mg^{++}	10.3	4.2	78.1	78.5

[a]Activities (x 100) were estimated from the data in Table I and are expressed as net hydrolysis of phospholipids over a 2 hr period. Phospholipase A activity was calculated using equation (i); lysophospholipase activity was calculated using equations (ii) and (iii).

lipase activity. Incubation of strain JW-31 with Hg^{++} markedly inhibited the Ca^{++}-activated phospholipase activity with PE as substrate, but only slightly inhibited activity with PG as substrate.

The calculation of lysophospholipase activity is based on the premise that LPE and FFA remain cell-associated and are not reutilized under nongrowth conditions. In the absence of further metabolism of LPE, one-half the loss of ^3H activity in PE will equal the gain in LPE. Deacylation of [^3H]LPE will yield unlabeled GPE and [^3H]FFA. Similar considerations hold for the hydrolysis of PG. The data in Table I indicate that, in the presence of Ca^{++}, the increase in [^3H]LPE is much less than one-half the decrease in [^3H]PE; further, the increase in [^3HFFA is greater than one-half the combined decrease in [^3H]PE and [^3H]PG. Thus, further hydrolysis of [^3H]LPE and [^3H]LPG to [^3H]FFA, and unlabeled GPE and GPG, is suggested. Further hydrolysis of lysophospholipids over a 2 hr period was estimated by two methods: (a) from LPE accumulation (observed vs. theoretical) (equation ii) and (b) from FFA accumulation (equation iii).

$$\text{(ii)}\quad \text{Hydrolysis of LPE} = \frac{\tfrac{1}{2}(\%PE_0 - \%PE_2) - (\%LPE_2 - \%LPE_0)}{\tfrac{1}{2}(\%PE_0 - \%PE_2)}$$

$$\text{(iii)}\quad \text{Hydrolysis of LPE plus LPG} = \frac{(\%FFA_2 - \%FFA_0) - \tfrac{1}{2}[(\%PE_0 - \%PE_2) + (\%PG_0 - \%PG_2)]}{\tfrac{1}{2}[(\%PE_0 - \%PE_2) + (\%PG_0 - \%PG_2)]}$$

Hydrolysis of LPE as estimated from LPE accumulation (equation ii) was roughly similar in strains JW-31 and CS-7. Hydrolysis of LPE appeared to be somewhat greater in the presence of Mg^{++} and Mn^{++} than in the presence of Ca^{++}, Fe^{++} or Zn^{++}. LPE hydrolysis could not be estimated in the absence of divalent cations because of the low activity of phospholipase under these conditions.

The combined hydrolysis of LPE plus LPG was estimated by comparing the combined decreases in PE plus PG with the accumulation of FFA (equation iii). The values calculated using equations ii and iii were similar for cells incubated in the presence of Ca^{++}, Mg^{++}, and Mn^{++}, suggesting that under these conditions both LPE and LPG are deacylated. Likewise, deacylation of both LPE and LPG appeared to be inhibited by Hg^{++}. In the presence of Fe^{++} and Zn^{++}, the values calculated using equation ii were much higher than those calculated using equation iii, suggesting that these metals may selectively inhibit deacylation of LPG.

Effect of pH and Temperature on Phospholipid Hydrolysis

The activities of phospholipase A and lysophospholipase varied as a function of pH (Fig. 1). In strain JW-31, PE hydrolysis exhibited a pH optimum of 8.5. In contrast, the hydrolysis of PG and LPE increased throughout the range of pH values studied (pH 6.0 to 9.0) and the pH optima may be greater than 9.0.

Figure 2 shows the effect of temperature on the relative activities of phospholipase and lysophospholipase. Labeled cells (strain JW-31) were suspended in growth medium and incubated for 10 min at the temperature indicated. The suspensions were then centrifuged, and the cells were resuspended in HEPES buffer, pH 8.5, containing Ca^{++} and incubated for 2 hr at

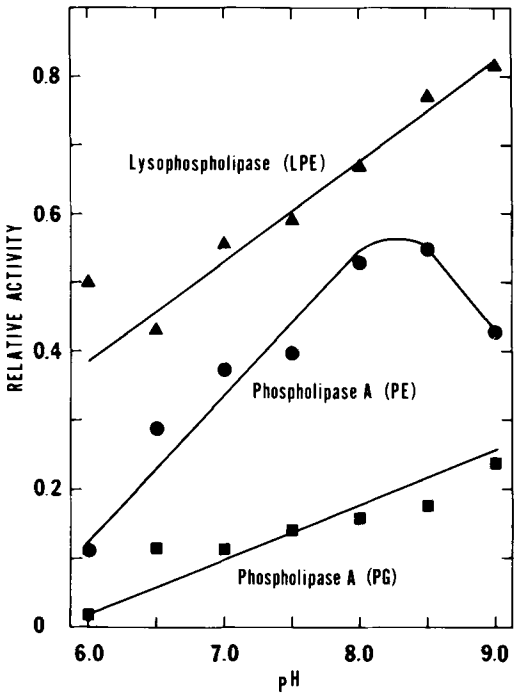

FIG. 1. Effect of pH on phospholipase activities of *N. gonorrhoeae* strain JW-31. Symbols: •, phospholipase A with PE as substrate; ▪, phospholipase A with PG as substrate; ▲, lysophospholipase with LPE as substrate. Activities were estimated as previously described.

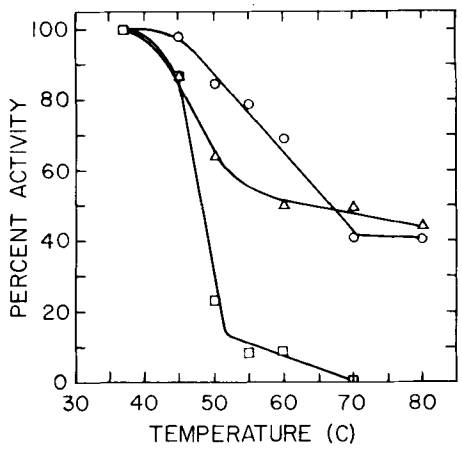

FIG. 2. Heat inactivation of phospholipase activities of *N. gonorrhoeae* strain JW-31. Symbols: ○, phospholipase A with PE as substrate; △, phospholipase A with PG as substrate; □, lysophospholipase with LPE as substrate. Activities were estimated as previously described.

37 C. The relative activities of phospholipase A and lysophospholipase were estimated as described in Table II, using equations i and ii. Lysophospholipase was more heat labile than phospholipase A. While lysophospholipase lost 80% of its activity at 50 C, phospholipase A with PE as substrate retained over 80% of its activity at the same temperature. At 70 C, lysophospholipase was completely inactivated, while ca. 40% of the phospholipase A activities with PE and PG remained. The fact that LPE accumulated in heat-inactivated cells in which the hydrolysis of PE to LPE was only partially inhibited indicates that the hydrolysis of LPE to GPE and FFA is enzymatically mediated. The presence of 40% of the phospholipase A activities at 70 C suggests the existence of both heat stable and heat labile forms of this enzyme in gonococci.

Effect of Alcohols on Phospholipid Hydrolysis in Buffer

The addition of 10% methanol (ca. 2.5 M) to cell envelope preparations of *N. gonorrhoeae* strain 2686 stimulated the in vitro activity of phospholipase A (3). The authors suggested that methanol might stimulate enzyme activity by increasing the solubility of the phospholipid substrates. Table III shows the effect of alcohols on the hydrolysis of endogenous phospholipid in labeled cells that were suspended in HEPES buffer (pH 8.5), with or without addition of Ca^{++} (20 mM). There was a marked increase in the hydrolysis of both PE and PG when cells were incubated in buffer containing Ca^{++} and either 1.25 M or 2.5 M methanol, relative to that observed in buffer with Ca^{++} alone. At a concentration of 0.62 M, methanol did not significantly stimulate phospholipid hydrolysis. Ca^{++} was required for stimulation of activity by 2.5 M methanol.

The effect of other alcohols on phospholipid hydrolysis was measured in the presence of Ca^{++}. Two concentrations of alcohol were tested: a lower concentration (1.25 M methanol, 0.7 M ethanol, 0.2 M propanol and 0.3 M isopropanol) that inhibited growth by 50%, and a higher concentration (2.5 M) that completely inhibited growth. At the lower concentration, methanol, ethanol, propanol, and isopropanol all stimulated phospholipid hydrolysis. At the higher concentration, methanol and ethanol further enhanced phospholipid hydrolysis, whereas propanol and tertiary butanol inhibited Ca^{++}-stimulated phospholipid hydrolysis. At a concentration of 2.5 M, isopropanol further stimulated the hydrolysis of PG but not PE. Other alcohols (e.g., butanol, isobutanol, isopentanol, and octanol), at a concentration (2.5 M) which completely inhibited growth, extracted more

TABLE III

Effect of Alcohols on Phospholipid Hydrolysis[a]

Conditions	^3H recovery (% of Time 0)	Percent of ^3H recovered as: PE	PG	LPE	FFA
Time 0	100.0	74.5	20.5	2.3	1.0
Ca^{++} (no alcohol)	93.0	36.2	17.4	7.3	36.9
Methanol (2.5 M) no Ca^{++}	76.5	62.4	20.8	3.4	6.8
Ca^{++} + methanol (0.62 M)	92.2	34.3	16.8	9.0	36.0
Ca^{++} + methanol (1.25 M)	86.0	25.6	14.9	9.9	47.8
Ca^{++} + methanol (2.5 M)	97.0	12.9	8.8	15.4	57.9
Ca^{++} + ethanol (0.7 M)	91.0	21.5	17.1	14.3	42.5
Ca^{++} + ethanol (2.5 M)	90.0	11.1	5.5	21.4	53.8
Ca^{++} + propanol (0.2 M)	97.0	21.1	16.8	13.4	44.0
Ca^{++} + propanol (2.5 M)	100.0	74.9	18.7	2.4	1.4
Ca^{++} + isopropanol (0.3 M)	100.0	18.0	14.8	14.5	48.1
Ca^{++} + isopropanol (2.5 M)	96.0	20.2	8.1	23.8	40.9
Ca^{++} + tert-butanol (2.5 M)	71.0	71.8	19.6	1.4	2.4

[a][^3H] Acetate-labeled cells of *N. gonorrhoeae* strain JW-31 were incubated for 2 hr in HEPES buffer, pH 8.5 with or without addition of 20 mM $CaCl_2$. Alcohols were added as indicated. Cells were extracted and lipids quantitated as previously described.

than 90% of the total phospholipids from cells (data not shown). Hydrolysis of PE or PG did not occur in the presence of these alcohols.

Table IV expresses the above data in the form of relative activities of phospholipase A and lysophospholipase. Enzyme activities were estimated as described in equations i - iii. At concentrations which inhibited growth by 50%, methanol, ethanol, propanol and isopropanol all stimulated the activity of phospholipase A with PE as substrate, whereas only methanol and isopropanol significantly stimulated the activity of phospholipase A with PG as substrate. At higher alcohol concentrations (2.5 M), the activity of phospholipase A with both PE and PG as substrate was increased. None of the alcohols tested stimulated lysophospholipase, but at high concentrations ethanol and isopropanol significantly inhibited lysophospholipase activity.

DISCUSSION

Gonococci undergo autolysis when suspended in various buffers at an alkaline pH in the absence of divalent cations (9). The strains employed here were selected because they differ in autolytic behavior. Strain JW-31 has a high rate of autolysis, whereas strain CS-7 exhibits minimal autolysis when suspended in HEPES buffer, pH 8.5 in the absence of divalent cations (6,9). Addition of divalent cations (Mg^{++}, Mn^{++} or Ca^{++}) to the buffer prevents autolysis (9). The present studies indicate that cell lysis is independent of phospholipid hydrolysis since both the autolytic strain (JW-31), and the nonautolytic strain (CS-7) possessed phospholipase and lysophospholipase activities. However, the activities of the enzymes were higher in strain JW-31. Moreover, hydrolysis of endogenous phospholipids was stimulated by divalent cations which inhibit cell lysis. Further studies (18) suggest that divalent cations may prevent autolysis by stabilizing the plasma membrane. Divalent cations do not, however, prevent loss of viability in either strain JW-31 or CS-7. The loss of viability which occurs in the absence of autolysis may result at least in part from hydrolysis of cell envelope components, including peptidoglycan (9) and phospholipids (6), and the toxic effects of accumulated FFA (19).

Autolysis can be prevented by suspension of gonococci in buffer containing sucrose (20) which osmotically stabilizes the cells. Under these conditions, both loss of viability (20) and the hydrolysis of peptidoglycan (9) occurred at an increased rate. Phospholipid hydrolysis also occurred in cells osmotically stabilized with sucrose (data not shown). Such conditions result in extensive disruption of the cell envelope as shown by the fact that cells which were incubated for 60 min in sucrose immediately lysed when centrifuged and resuspended in water or buffer. The addition of Ca^{++} to cells that were osmotically stabilized by suspension in buffer containing sucrose resulted in extensive release of [^3H] arginine-labeled protein with little or no release of [^3H] adenine-labeled nucleic acid (18). Further studies indicated that the proteins released into the buffer are associated with outer membrane or the periplasmic space. These data suggest that in osmotically stabilized cells, Ca^{++}-activated phospholipid hydrolysis may cause disruption of integrity of the outer membrane, while the integrity of the

TABLE IV

Effect of Alcohols on the Activities of Gonococcal Phospholipase A and Lysophospholipase[a]

Additions	Phospholipase A Net Hydrolysis of		Lysophospholipase Net Hydrolysis of	
	PE	PG	LPE	LPE + LPG
None	41.9	19.9	69.8	65.6
Methanol (1.25 M)	64.7	33.5	68.5	71.4
Ethanol (0.7 M)	71.4	18.2	52.2	46.7
Propanol (0.2 M)	71.9	19.6	55.9	49.4
Isopropanol (0.3 M)	65.2	24.4	57.9	51.9
Methanol (2.5 M)	88.1	63.5	64.3	65.5
Ethanol (2.5 M)	85.4	71.2	40.3	35.1
Isopropanol (2.5 M)	73.4	57.6	21.9	20.1

[a]Cells were assayed in HEPES buffer, pH 8.5 containing 20 mM Ca^{++}. Phospholipase and lysophospholipase activities (x 100) were estimated from the data in Table III and calculated as in Table II.

selective permeability barrier of the cell, the plasma membrane, is maintained.

The phospholipase activity of whole cells suspended in buffer was similar to that of envelope preparations (3) in that both activities: (i) exhibited a pH optimum at 8.5 with PE as substrate, and (ii) preferentially hydrolyzed PE as opposed to PG in the presence of Ca^{++}. In whole cells, Mn^{++} preferentially stimulated PG hydrolysis, while little phospholipid hydrolysis occurred in the presence of Mg^{++} or Zn^{++}. Phospholipase A activities from *Escherichia coli* (21), *Bacillus subtilis* (22), and *Mycobacterium phlei* (23) also exhibited alkaline pH optima and activation by Ca^{++}. In *E. Coli* there are at least two different phospholipase and three different lysophospholipase activities (24,25). Our data suggest that gonococci may also possess multiple activities of these enzymes.

The Ca^{++}-activated phospholipase A activity was further stimulated when gonococci were incubated in buffer containing methanol. Other alcohols either stimulated or inhibited phospholipid hydrolysis. Phospholipase A activity of envelope preparations (3) required 10% methanol for full activity but was totally inhibited in the presence of higher alcohols (propanol, isopropanol, butanol, and isobutanol) and various detergents including Triton X-100. Studies with whole cells indicate that 2.5 M butanol and isobutanol, as well as low concentrations of Triton X-100 (0.01%) (data not shown), solubilized >90% of the cellular phospholipids. In contrast, the phospholipase A of the outer membrane of *E. coli* (21) was activated by Trition X-100. The extraction of gonococcal phospholipids by Triton X-100 is in agreement with the observation that the growth of gonococci is inhibited by low concentrations of this detergent (26), whereas the growth of enteric bacteria is not. The stimulation of phospholipid hydrolysis by methanol may be due to increased accessibility of the enzyme to divalent cations and to fatty acid acyl chains of phospholipids. Methanol may also affect enzyme configuration.

It is significant that products of phospholipid hydrolysis remain cell-associated under nongrowth conditions and are not selectively released into the buffer. This is in contrast to reports that in gonococci, FFA accumulate in the medium during growth (1,27). The activity of phospholipase A in growing cells will be considered in a subsequent publication.

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Incorporation of *cis*- and *trans*-Octadecenoic Acids into the Membranes of Rat Liver Mitochondria[1]

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ABSTRACT

The incorporation of dietary isomeric fatty acids into the membranes of liver mitochondria was investigated. Three groups of rats were fed diets containing 3% sunflower seed oil plus 15%, 20%, or 25% partially hydrogenated arachis oil. A fourth group was fed 25% partially hydrogenated arachis oil, but no sunflower seed oil. All diets were given for 3, 6, or 10 weeks. After 10 weeks, the content of *trans* fatty acids in the lipids of the mitochondrial membranes was 15-19% of the total fatty acids. The composition of the *trans*- and the *cis*-octadecenoic acids in the lipids of the mitochondrial membranes was similar for all groups supplemented with sunflower seed oil (SO), irrespective of time and dietary level of partially hydrogenated arachis oil (HAO). The *cis* 18:1 (n-8), which was a major isomer of the partially hydrogenated arachis oil, was almost excluded from the mitochondrial fatty acids. Likewise, the content of *trans* 18:1(n-8) was considerably lower in the mitochondrial lipids than in the diet. On the contrary, the content of *trans* 18:1 (n-6) was higher in the mitochondrial lipids than in the diet. In the group fed without sunflower seed oil, isomers of linoleic acid and arachidonic acid were observed in the lipids of mitochondrial membranes.

INTRODUCTION

Partially hydrogenated oils are reported to contain a large range of positional isomeric fatty acids with *cis* or *trans* double bonds (1,2). These isomers are also present in commercially available margarines (3). So far, however, the incorporation of dietary positional isomers from partially hydrogenated oils has only been studied in lipids from whole organs, e.g., the liver (4,5). These studies have established that isomeric fatty acids in vivo are metabolized individually. The in vivo incorporation of dietary isomeric fatty acids into subcellular particles, such as mitochondria, has only been demonstrated with elaidic acid (6-8), but no attempts to study the fate of dietary *positional* isomeric acids have been published so far.

The present work describes a study of the incorporation of dietary positional isomeric *cis* and *trans* octadecenoic acids into liver mitochondrial lipids of rats fed 15%, 20%, or 25% of partially hydrogenated arachis oil (peanut oil), supplemented with sunflower seed oil for different experimental periods.

EXPERIMENTAL PROCEDURES

Animal Experiments

Seventy-two male Wistar rats (specific pathogen-free) were divided into 4 groups of 18 rats. Each of these groups was further divided into 3 subgroups of 6 rats each. The rats received a basal diet of the following composition (weight percentages): casein (A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark), 20%; sucrose, 74%; vitamin mixture, 0.5%; salt mixture (including trace elements), 5.0%; choline chloride, 0.5%. Composition of the vitamin mixture and the salt mixture has been published previously (9).

Groups 1, 2, and 3 received 15%, 20%, or 25% partially hydrogenated arachis oil (HAO) (Aarhus Oliefabrik A/S, Aarhus, Denmark). All 3 groups were given a supplement of 3% sunflower seed oil (SO) (Aarhus Oliefabrik A/S, Aarhus, Denmark). Group 4 was given 25% HAO, but no SO. The fats were incorporated instead of the corresponding weight of sucrose. Diets and water were given ad libitum. The rats were caged in pairs in a room with a temperature of 25 C and with automatic control of relative humidity (45%). The animals were weighed and examined weekly during the experimental period.

No differences in growth between the SO-supplemented groups were observed during the experiment, but the growth of the group fed 25% HAO with no SO was retarded from the third week of experiment, indicating essential fatty acid deficiency.

After 3, 6, and 10 weeks, respectively, one subgroup from each experimental group was anaesthetized with ether and, after removal of a blood sample for preparation of serum, killed by coronary puncture.

Preparation of Mitochondrial Membranes

The rat livers were quickly excised and placed in ice cold 0.25 M sucrose in 1 mM EDTA, pH 7.4. The livers from each subgroup were divided into 3 pools, each containing 2

[1]Presented in part at the ISF Congress, Marseille, September 1976.

TABLE I

Fatty Acid Composition of Dietary Fats. Weight Percentages Determined by Gas Chromatograpy

	Dietary fat	
	Partially hydrogenated arachis oil[a]	Sunflower seed oil
Fatty acids:	%	%
12:0	0.2	—
14:0	0.3	0.1
16:0	13.1	6.3
16:1	0.4	0.2
18:0	14.1	3.3
18:1	57.3	18.9
18:2 isomers	4.3[b]	—
18:2	1.1	68.5
20:0	2.1	1.3
20:1	1.7	0.3
20:2	—	0.1
22:0	3.7	0.7
24:0	1.5	0.3

[a]Melting point 41 C.

[b]Primarily *cis, trans* 18:2 or *trans,cis* 18:2.

livers. The livers were homogenized in 0.25 M sucrose in 1 mM EDTA, pH 7.4 (4.5 ml/g tissue) in a Potter-Elvehjem glass homogenizer with a teflon pestle. The homogenate was centrifuged for 10 min in a Sorvall centrifuge (Rotor SS-34) at 0-4 C and 900 x g_{max} to sediment nuclei and cell fragments. The supernatant fluid was recentrifuged for 10 min at 9000 x g_{max} at 0-4 C to sediment the mitochondria. The supernatant was decanted together with a fluffy medium layer. The bottom pellet was resuspended in the original volume of sucrose and centrifuged for 10 min at 9000 g_{max} at 0-4 C. The supernatant was discarded. The sediment containing the mitochondria was resuspended in the same volume of ice cold redistilled water to fracture the mitochondria osmotically. After 20 min at 0 C, the suspension was centrifuged for 10 min at 27,000 x g_{max} at 0-4 C to sediment the mitochondrial membranes. These were resuspended in redistilled water to an approximate volume of 5 ml.

Extraction of Lipids

The lipids of the mitochondrial membranes were extracted with chloroform/methanol (2:1, v/v) according to Folch et al. (10). The solvents were evaporated under nitrogen after which the lipids were dissolved in chloroform/methanol (2:1, v/v) and stored at -20 C. Thin layer chromatography (TLC) showed that the lipids of the mitochondrial membranes consisted of phospholipids (mainly phosphatidylcholines, phosphatidylethanolamines, and cardiolipins) and cholesterol. Only traces of free fatty acids and cholesterol esters were present.

TABLE II

Distribution of Isomeric *cis* Octadecenoic Acids in Partially Hydrogenated Arachis Oil and in the Total Lipids of Liver Mitochondrial Membranes from Rats Fed Diets Containing Partially Hydrogenated Arachis Oil

Groups and periods	Fatty acid percentages[a]										
	(n-13)	(n-12)	(n-11)	(n-10)	(n-9)	(n-8)	(n-7)	(n-6)	(n-5)	(n-4)	(n-3)
HAO[b]	0.2	1.0	3.6	14.5	29.7	23.8	15.0	8.2	3.0	0.5	0.5
Group 1:											
15% HAO + 3% SO:											
3 weeks				5.7	59.3	1.3	24.7	6.0	2.3	0.3	
6 weeks				3.3	60.3	1.0	26.3	4.0	2.3	1.3	2.0
10 weeks				3.7	61.3	1.7	23.3	5.3	1.7	1.3	1.3
Group 2:											
20% HAO + 3% SO:											
3 weeks				4.0	59.3	2.7	22.7	6.7	3.3	1.3	
6 weeks				3.0	64.3	2.0	21.0	6.7	2.0	0.7	0.3
10 weeks			0.3	4.3	62.7	2.0	21.0	5.3	2.0	1.0	1.3
Group 3:											
25% HAO + 3% SO:											
3 weeks				4.7	58.0	2.0	20.3	7.0	5.0	0.3	
6 weeks				6.0	69.0	2.3	15.3	5.0	1.7	0.3	0.3
10 weeks				4.3	59.3	2.0	22.7	7.3	2.0	1.0	1.0
Group 4:											
25% HAO:											
3 weeks				4.0	72.7	0.7	13.3	7.3	1.3		
6 weeks				4.3	76.0	1.0	11.0	6.0	1.0		0.7
10 weeks			0.3	4.3	74.3	2.0	11.0	4.7	1.0	1.3	0.3

[a]Averages for three different pools.

[b]Averages of two determinations.

TABLE III

Distribution of Isomeric *trans* Octadecenoic Acids in Partially Hydrogenated Arachis Oil and in the Total Lipids of Liver Mitochondrial Membranes from Rats Fed Diets Containing Partially Hydrogenated Arachis Oil

Groups and periods	Fatty acid percentages[a]								
	(n-11)	(n-10)	(n-9)	(n-8)	(n-7)	(n-6)	(n-5)	(n-4)	(n-3)
HAO[b,c]	4.0	12.4	25.5	22.9	18.9	9.9	3.5	1.5	1.4
Group 1:									
15% HAO + 3% SO:									
3 weeks	1.0	7.0	32.7	7.3	10.7	25.7	6.0	9.0	
6 weeks	1.0	4.3	33.3	5.7	11.7	31.3	5.7	5.7	1.0
10 weeks	0.7	8.7	35.7	8.3	11.3	23.0	4.7	6.0	1.0
Group 2:									
20% HAO + 3% SO:									
3 weeks	0.7	7.7	27.3	9.0	11.7	25.3	7.7	8.7	2.0
6 weeks	0.7	7.7	34.7	9.3	11.7	22.7	4.7	6.0	1.3
10 weeks	1.0	8.7	35.3	8.3	11.7	21.0	6.0	6.3	1.0
Group 3:									
25% HAO + 3% SO:									
3 weeks	1.0	7.0	30.0	7.7	11.7	25.7	6.0	9.7	1.7
6 weeks	0.7	6.7	32.7	10.7	11.7	22.7	5.3	6.7	1.7
10 weeks	1.0	7.3	35.0	7.3	13.6	21.3	5.7	7.3	1.0
Group 4:									
25% HAO:									
3 weeks	1.0	7.7	31.7	10.7	10.0	24.3	5.7	6.3	2.3
6 weeks	1.0	8.0	35.3	11.0	11.0	20.0	4.3	5.3	2.0
10 weeks	2.0	9.0	38.7	10.0	11.3	18.0	3.7	5.0	1.7

[a]Averages for three different pools.

[b]Averages of two determinations.

[c]HAO contained 54.3% *trans* fatty acids as determined by infrared spectroscopy.

Methylation

Lipids were *trans*-methylated as described by Stoffel et al. (11). Fifteen mg of total lipids of mitochondrial membranes were methylated in a mixture of 4 ml of 5% dry hydrogen chloride in dry methanol and 0.5 ml benzene. The mixture was refluxed for 2 hr at 80 C on a water bath. Methyl esters were extracted with hexane and dried with anhydrous $Na_2SO_4/NaHCO_3$ (4:1).

Gas Liquid Chromatography

Analytical gas liquid chromatography (GLC) of the methyl esters was performed on a Hewlett-Packard 5830A dual column instrument with FID detectors, using 1/8 in. o.d. x 6 ft columns of stainless steel packed with 15% DEGS on Chromosorb W (AW), 80/100 Mesh (Applied Sciences Lab., State College, PA); column temperature 180 C. Nitrogen was used as carrier gas at a flow rate of 60 ml/min. Air flow was 280 ml/min, and hydrogen flow was 60 ml/min. The linearity of the detector response was checked by chromatography of standard mixtures of known composition (Nu-Chek Prep, Inc. Elysian, MN).

Fractionation of Methyl Esters

Methyl esters were fractionated according to unsaturation by TLC on Silica Gel G plates containing 12.5% $AgNO_3$ (1). The *cis*-monoenes and the *trans*-monoenes were scraped off, and each of these fractions was further separated according to chain length by preparative scale gas chromatography on an Aerograph Autoprep 705 instrument with a single 1/4 in. o.d. x 6 ft stainless steel column packed with 15% DEGS on Chromosorb W; column temperature 175 C; detector temperature 225 C; injection temperature 225 C; split ratio 1:5. Nitrogen was used as carrier gas at a column flow of 140 ml/min. Octadecenoates were collected in chloroform in U-tubes at -20 C.

Ozonolysis

Preparations of *cis* and *trans* methyl octadecenoates were dissolved in hexane and ozonized at -70 C using a procedure of Beroza and Bierl (12). The ozonides were reduced with triphenylphosphine according to Stein and Nicolaides (13). A sample of the reaction mixture, containing aldehyde esters and aldehydes, was analyzed by gas chromatography on a Beckman GC 4 double column instrument using isothermal operation at 175 C. Column packing was 15% DEGS on Gas-Chrom W/AW, 100/120 Mesh (Applied Sciences Lab.) in 1/8 in. o.d. x 6 ft columns. Helium was used as

TABLE IV

Major Fatty Acids in the Total Lipids of Liver Mitochondrial Membranes from Rats Fed Diets Containing Partially Hydrogenated Arachis Oil

Groups and periods	Fatty acid percentages[a,b]											
	16:0	16:1	18:0	18:1	18:2	18:2 isomer	20:3	20:3	20:4	20:4 isomer	22:4	22:6
Group 1: 15% HAO + 3% SO:												
3 weeks	12.6	1.5	13.8	16.8	17.8		0.3	1.1	28.7		1.7	5.4
6 weeks	13.6	2.2	13.3	23.2	16.4		0.5	1.5	23.6		1.6	2.7
10 weeks	11.2	2.1	14.6	24.1	14.3		0.4	1.4	25.5		1.8	2.4
Group 2: 20% HAO + 3% SO:												
3 weeks	11.2	1.5	14.1	16.9	16.0		0.5	1.5	28.6		2.3	6.9
6 weeks	12.6	2.2	14.8	25.2	15.1		0.5	1.7	23.6		1.0	1.6
10 weeks	10.9	2.0	14.5	23.8	14.2		0.4	1.4	25.3		2.0	2.7
Group 3: 25% HAO + 3% SO:												
3 weeks	14.3	1.3	17.7	17.8	13.5		0.6	1.2	26.3		1.7	4.2
6 weeks	13.2	2.0	16.4	23.6	14.7		0.6	1.4	22.9		1.5	2.5
10 weeks	11.0	1.5	17.1	21.5	15.1		0.5	1.4	25.8		1.5	2.5
Group 4: 25% HAO:												
3 weeks	15.9	4.0	14.8	27.8	6.7	5.6	6.1	0.8	6.8	1.5	1.1	6.2
6 weeks	15.9	4.5	14.8	31.3	5.4	4.8	7.4	0.4	5.2	1.4	1.4	3.4
10 weeks	13.5	5.4	14.4	31.9	4.2	5.4	9.4	0.4	5.3	1.4	1.1	3.2

[a]Averages of three different pools.

[b]Minor components (< 0.5%) omitted.

carrier gas at 70 ml/min. Under these conditions, aldehydes were eluted in the solvent peak, whereas aldehyde esters were separated according to chain length. Aldehyde esters were identified by ozonolysis of appropriate standard methyl octadecenoates followed by GLC.

The distribution of positional isomeric fatty acids was calculated from the peak area of the aldehyde esters by the following formula: Percentage of fatty acid i = 100 x $A_i/MW_i/\Sigma$-(A_i/MW_i)%. A_i: peak area of aldehyde ester arising from fatty acid i. MW_i: molecular weight of aldehyde ester arising from fatty acid i.

Infrared Analyses

Infrared analyses of *trans* fatty acids in dietary fat as well as in mitochondrial lipids were performed as previously described (1).

RESULTS

Fatty Acid Composition of Dietary Fats

The partially hydrogenated arachis oil used in the experimental diets (Table I) contained primarily saturated fatty acids and octadecenoic acids, while the content of 18:2, mostly *trans* isomers, was low. Preliminary results had shown that addition of 3% sunflower seed oil to the diet provided enough linoleic acid to prevent the symptoms of essential fatty acid deficiency in the experimental animals. The *cis* 18:1 as well as the *trans* 18:1 fractions of HAO (Tables II and III) contained a variety of positional isomeric fatty acids, the 18:1 (n-7), (n-8), (n-9), and (n-10) being the predominant acids in both fractions. In the sunflower seed oil, only oleic acid was found in the 18:1 fraction and linoleic acid in the 18:2 fraction. The combination of partially hydrogenated arachis oil and sunflower seed oil made it possible to study the incorporation of isomeric octadecenoic acids under circumstances where the animals received only small amounts of isomeric 18:2 and 16:1 fatty acids.

Fatty Acids in the Total Lipids of Mitochondrial Membranes

In all groups, the percentages of 18:1 increase from 3 weeks to 6 weeks (Table IV), which probably reflects the contents of 18:1 fatty acids in partially hydrogenated arachis oil in the diet. After 6 weeks, a constant level of 18:1 is attained. This is similar for groups 1, 2, and 3. The increase in octadecenoic acids is accompanied by a decrease in the content of polyunsaturated fatty acids, especially the docosahexaenoic acid and the arachidonic acid, as well as the palmitic acid. Likewise, Hølmer and Høy (14) have reported that rats fed a diet containing 22% partially hydrogenated arachis oil + 6% arachis oil, which has the same 18:2

TABLE V

Trans Fatty Acids Determined by IR Spectroscopy in the Total Lipids of Liver Mitochondrial Membranes from Rats Fed Diets Containing Partially Hydrogenated Arachis Oil

Periods	Dietary fats			
	Group 1: 15% HAO + 3% SO %	Group 2: 20% HAO + 3% SO %	Group 3: 25% HAO + 3% SO %	Group 4: 25% HAO %
3 weeks	12.1 ± 0.3[a]	13.2 ± 0.3[a]	14.2 ± 2.3[b]	16.6 ± 3.4[b]
6 weeks	15.5 ± 2.8[b]	16.9 ± 1.2[b]	15.5 ± 0.1[a]	16.9 ± 0.2[a]
10 weeks	16.4 ± 0.1[a]	16.1 ± 0.6[a]	15.3 ± 0.1[a]	18.9 ± 0.7[a]

[a]Averages for two different pools ± deviations from average.

[b]Averages for three different pools ± standard deviations.

content as the diet for group 3, have decreased contents of 20:4, 22:6, 16:0, and 18:0 in liver mitochondrial phosphatidylcholines compared with rats fed 28% arachis oil for 16 weeks.

In group 4, fed partially hydrogenated arachis oil without a supplement of sunflower seed oil, the content of 18:1 in the mitochondria is much higher. Also, in this group, a constant level is reached after six weeks. After three weeks, changes in the fatty acid pattern typical of essential fatty acid deficiency are observed in this group, i.e., a decreased content of (n-6) fatty acids and increased content of (n-9) fatty acids. In addition, isomeric 18:2 and 20:4 fatty acids appear. The exact identity of these isomers has not been established. The quantity of lipid available from the mitochondrial membranes did not permit a detailed examination including infrared spectroscopic determination and ozonolysis. From their chromatographic characteristics, we assume that they both contain a *trans* double bond. The 18:2 might therefore be 5*c*,9*t*-18:2, as originally demonstrated by Lemarchal and Munsch (15) in rats fed elaidic acid, or it can be dietary 9*t*,12*c*-18:2, which is incorporated in liver lipids (16). The 20:4 might be identical to 5*c*,8*c*,11*c*,14*t*-20:4, which has been reported (17) to be found in essential fatty acid deficient rats fed 9*c*,12*t*-18:2. The incorporation of other positional isomers cannot, however, be excluded.

Trans Fatty Acids

The *trans* fatty acids as determined by IR (Table V), which includes polyenoic acids with *trans* double bonds, comprise 12-17% of the total mitochondrial fatty acids in accordance with previous reports that described the incorporation of dietary elaidic acid into liver mitochondria (6,7). The rats fed 25% HAO, group 4, appear to have a larger incorporation of *trans* fatty acids than the animals supplemented with sunflower seed oil, which is consistent with the higher content of total 18:1 fatty acids (Table IV) in the mitochondrial membranes.

Positional Isomeric Acids

The distribution of positional isomeric *cis* 18:1 fatty acids (Table II) in the total lipids of the liver mitochondrial membranes is very uniform in all groups, irrespective of dietary period, as is the distribution of positional isomeric *trans* 18:1 fatty acids (Table III). The *cis* 18:1 (n-9) and *cis* 18:1 (n-7), which can be formed endogenously, are the major components of the *cis* 18:1 fraction. Significant incorporation of *cis* 18:1 (n-10) and *cis* 18:1 (n-6), which are of dietary origin, is observed. The *cis* 18:1 (n-8), which is a major component of the *cis* 18:1 fraction of the HAO, is partially excluded from the mitochondrial lipids. The state of EFA deficiency, group 4, primarily affects the ratio between oleic and vaccenic acids but does not cause other changes in the distribution of positional isomeric *cis* octadecenoic acids. All the *trans* 18:1 fatty acids are of exogenous origin. The *trans* 18:1 (n-9) and *trans* (n-6) are incorporated preferentially, while there is a discrimination against the *trans* 18:1 (n-8) and the *trans* (n-7). Although EFA deficiency, group 4, does lead to an increased incorporation of *trans* fatty acids in the mitochondrial lipids (Table V), it does not appear to affect the distribution of positional isomeric *trans* octadecenoic acids in the mitochondrial lipids (Table III).

DISCUSSION

In previous experiments (18), it has been shown that rats fed 6% arachis oil or 6% olive oil + 4% sunflower seed oil only contain oleic acid and vaccenic acid in the octadecenoic fraction of liver mitochondrial phospholipids.

However, using mass spectroscopy, minor quantities of other positional isomeric octa-

decenoic acids have been detected in the liver lipids of rats raised on fat-free diets (19). Likewise, traces (< 0.1%) of elaidic acid have been detected in the total mitochondrial lipids of rats fed unhydrogenated olive oil (6). The origin of these isomeric fatty acids is not well established, but the overall contribution seems to be insignificant compared to the incorporation of isomeric fatty acids reported in feeding experiments with partially hydrogenated oils (4-8).

This experiment demonstrates that dietary isomeric octadecenoic acids are incorporated in the liver mitochondrial membranes. The incorporation is selective with respect to double bond position, but is neither affected by the dietary level of partially hydrogenated oil nor by the experimental period within the limits of this experiment. The patterns of incorporation of positional isomeric fatty acids are in good agreement with the results recently reported by Wood and Chumbler (5) for whole liver.

The intestinal absorption has been reported to be independent of double bond geometry (20) and of double bond position in *trans* octadecenoates (4). The observed patterns of incorporation may, therefore, reflect selectivity in the metabolism of fatty acids, such as activation and acylation.

The activation and acylation processes have been studied by others by in vitro experiments which by necessity are performed under simplified experimental conditions not strictly comparable to those of a feeding experiment. Lippel et al. (21,22) investigated the activation of octadecenoates by liver microsomal and mitochondrial fractions. At low concentrations (1 mM), all positional isomeric *cis* octadecenoates were activated at similar rates (21) as were all *trans* octadecenoates (22). At higher concentrations (3 or 4 mM) of fatty acids, the *cis* 18:1 (n-9) as well as the *trans* 18:1 (n-9) were activated at lower rates than all other octadecenoates, a fact which cannot be expected from our findings. Reitz et al. (23) studied the in vitro selectivity of rat liver microsomal acyl transferases with respect to the double bond position of *cis* octadecenoates. They found that oleic acid was primarily transferred to the 2-position of phosphatidylcholines, whereas all other *cis* octadecenoates were transferred to the 1-position at higher rates than to the 2-position. The transfer rates of *cis* 18:1 (n-10) and *cis* 18:1 (n-6) were high, in agreement with our data, but a high transfer rate of *cis* 18:1 (n-8) was also found.

Okuyama et al. (24) showed that *trans* octadecenoates were primarily transferred to the 1-position of phosphatidylcholine by rat liver microsomal acyl transferase in vitro. Their data did show a discrimination of *trans* 18:1 (n-8), but not of *trans* 18:1 (n-7). Likewise, they did not report any preference for the *trans* 18:1 (n-6), such as we have found. All isomeric *cis* 18:1 acids are readily incorporated into rat liver mitochondria in vitro upon incubation of isolated mitochondria with liver homogenate and fatty acid-albumin complexes (25). The in vitro investigations fail to explain the selective metabolism of isomeric fatty acids in feeding experiments observed by us for rat liver mitochondria and reported by Wood and Chumbler (5) for whole liver. The selectivity emphasizes the necessity of considering positional isomerism in addition to geometrical isomerism when the incorporation of dietary isomeric fatty acids is studied.

The incorporation of dietary isomeric acids reaches the limit at 15.3-16.9% *trans* fatty acids in the mitochondrial membrane, irrespecitve of time and dietary level of HAO. As other investigators using either partially hydrogenated oils (6,7) or trielaidin (26) and shorter feeding periods have observed the same incorporation, this might represent the limit incorporation of *trans* fatty acids into the mitochondrial membranes. Of all octadecenoic acids, oleic acid has the lowest melting point. It could, therefore, be speculated that incorporation of isomeric fatty acids into the mitochondrial membrane will decrease the membrane fluidity and thus affect the metabolite transport. However. Hølmer (27) has demonstrated that rats fed partially hydrogenated oils have reduced contents of saturated fatty acids in the mitochondrial phospholipids compared to rats fed unhydrogenated oils. Furthermore, Chapman et al. (28) have shown that replacement of stearic acid by elaidic acid in the 1-position of phosphatidylethanolamine does not affect the surface pressure of a monolayer film. Likewise, this substitution will not affect the interaction of the phospholipid with cholesterol. In contrast to this, phospholipids with elaidic acid in the 2-position form a condensed film and have reduced interaction with cholesterol. In vivo, the ratio between the incorporation of *trans* fatty acids into the 1-position and the 2-position is 15.5:1 for phosphatidylcholines and 7.0:1 for phosphatidylethanolamines (5). This will tend to oppose the effect on membrane fluidity caused by the observed incorporation of isomeric fatty acids into the mitochondrial membrane.

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Thermal Alteration of a Cyclic Fatty Acid Produced by a Flaxseed Extract

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ABSTRACT

Methyl 8-[2-(*cis*-pent-2'-enyl)-3-oxo-*cis*-cyclopent-4-enyl] octanoate (I) is the methyl ester of a cyclic fatty acid synthesized enzymically from an incubation of linolenic acid with an extract of flaxseed (*Linum usitatissimum* L.). A proposed trivial name for I is methyl 12-oxo-*cis*-10,15-phytodienoate (12-oxo-PDA). The evidence presented indicated that compound I has the *cis* configuration of the carbon chains with respect to the cyclopentenone ring. Treatment with acid, base, or heat isomerized I to a second product (II) that has the *trans* configuration of the carbon chains. Prolonged heat treatment of II yielded a third cyclic product, methyl 12-oxo-9(13),*cis*-15-phytodienoate (III).

Zimmerman and Feng (1) recently described the enzymic synthesis of a cyclic fatty acid that was structurally similar to prostaglandin A_1. The compound, characterized as 8-[2-(*cis*-pent-2'-enyl)-3-oxo-*cis*-cyclopent-4-enyl] octanoic acid, was derived from incubations of (9,12,15)-linolenic acid with extracts of flaxseed. The authors proposed the common name 12-oxo-*cis*-10,15-phytodienoic acid as the derivative of a parent compound, phytonoic acid (Fig. 1). Substrate specificity studies by Vick and Zimmerman (2) demonstrated that other polyunsaturated fatty acids were also active in the synthesis of cyclic fatty acids by extracts of flaxseed. Fatty acids with chain lengths of 18, 20, and 22 carbons could be converted to cyclic compounds provided they were unsaturated at the *n*-3,6,9 positions. Intermediate in the synthesis of cyclic fatty acids was an *n*-6 hydroperoxy fatty acid, formed by the action of lipoxygenase (EC 1.13.11.12).

The authors cited above reported that the cyclic fatty acids when purified by thin layer chromatography (TLC) and analyzed by gas chromatography (GC) yielded three components. The components were presumed to be diastereomers. We now present data intended to clarify the structure of each of the three compounds.

EXPERIMENTAL PROCEDURES

Materials

Linolenic acid was obtained from Nu Chek Prep, Inc., (Elysian, MN), platinum oxide (Adam's catalyst from Matheson, Coleman, and Bell (Norwood, OH), and 3% OV-210 on 100/120 mesh Gas-chrom Q from Applied Science Laboratories, Inc. (State College, PA). E. Merck Silica Gel 60 F-254 precoated glass plates were used for all TLC separations. Methoxyamine HCl in pyridine (MOX reagent) was obtained from Pierce Chemical Company (Rockford, IL).

Synthesis and Purification of 12-Oxo-cis-10,15-Phytodienoic Acid (12-Oxo-PDA)

12-Oxo-PDA was synthesized enzymically from (9,12,15)-linolenic acid by an extract of flaxseed (*Linum usitatissimum* L.), and isolated by TLC by the procedure of Zimmerman and Feng (1). The product was dissolved in ethyl ether and esterified by treatment with diazomethane. The methyl ester was chromatog-

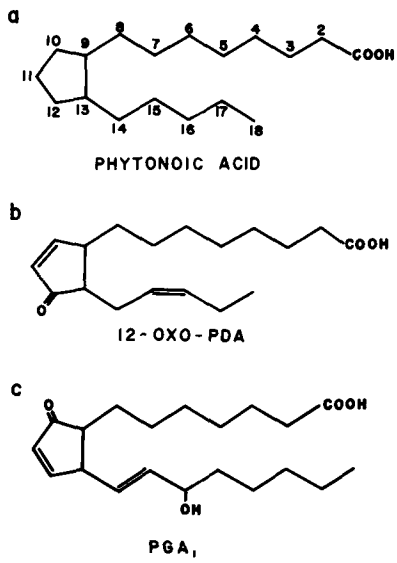

FIG. 1. a) Carbon skeleton and numbering system of phytonoic acid. b) 12-Oxo-*cis*-10,15-phytodienoic acid. c) Prostaglandin A_1.

raphed on a 2 m x 2 mm i.d. glass column containing 3% OV 210 on 100/120 mesh Gas-chrom Q. The column was temperature-programmed from 170 to 220 C at 2 C per min, and the flow rate of helium carrier gas was 20 ml per min.

Characterization of Products

Reductions. Carbonyl groups were reduced to hydroxyl groups by adding 2 mg of sodium borohydride to 1 ml of a solution containing 1-5 mg of the fatty acid in methanol. After 15 min at 24 C, 1 ml of 1 M citric acid was added, and the fatty acids were extracted twice into hexane. For hydrogenation, hydrogen was bubbled through 2 ml of a methanolic solution of the fatty acid for 15 min at 24 C with a platinum oxide catalyst (ca. 1 mg). The catalyst was removed by centrifugation.

Oxidations. Hydroxyl groups were oxidized to carbonyl groups with Jones' reagent prepared by dissolving 2.67 mg of chromium trioxide in 2.3 ml of concentrated sulfuric acid and diluting to 10 ml with water (3). The sample (ca. 1 mg) was dissolved in 1 ml of acetone, 0.1 ml of Jones' reagent was added, and the mixture was allowed to react for 30 min at 24 C. Water (15 ml) was added, the products were extracted twice with 5 ml hexane, and the hexane was evaporated under a stream of N_2.

Determination of Double Bond Position. The position of double bonds was determined by oxidative ozonolysis of the sample with boron trifluoride in methanol (14%, w/v) by the procedure of Ackman (4). The products were analyzed by gas chromatography-mass spectrometry (GC-MS).

Derivatives. Methoxime derivatives of carbonyl compounds were prepared by the reaction of the sample with 1 ml of 2% methoxyamine HCl in pyridine. After 3 hr at 24 C, 1.5 ml of water was added and the products were extracted into hexane. The hexane phase was concentrated under a stream of N_2.

Spectral Analysis. Infrared spectra were obtained with a Perkin-Elmer model 337 spectrophotometer. Micro-liquid cells with a 0.50 mm path length were used with carbon tetrachloride as solvent. UV spectra were recorded with a Beckman DK-2 spectrophotometer with ethanol solvent, and NMR spectra (^1H and ^{13}C) were recorded with a Bruker WH-90 instrument. Mass spectra were recorded with a Hewlett-Packard 5992A GC-MS system; the column was prepared and programmed as described above. The mass spectrometer was operated at 70 ev.

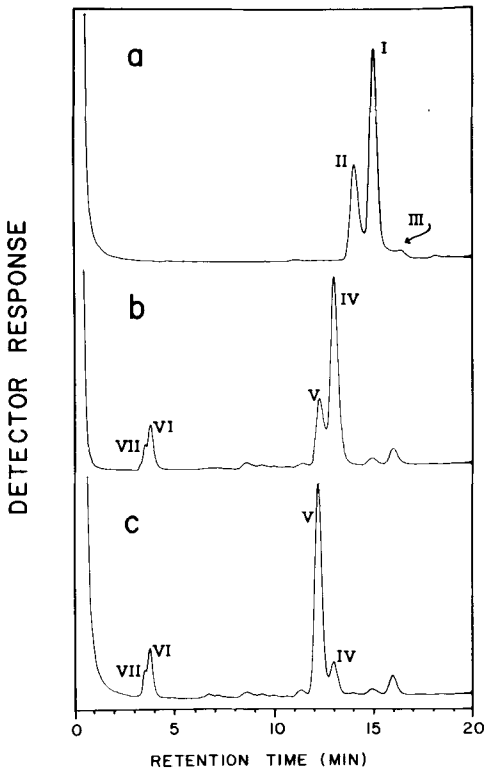

FIG. 2. a) Gas chromatographic analysis of a preparation of methyl 12-oxo-PDA (I) purified by TLC. b) Gas chromatogram of the preparation from *a* that had been hydrogenated using PtO_2 catalyst. c) Gas chromatogram of the preparation from *b* that had been heated for 10 min at 190 C.

RESULTS

Product Stability. 12-Oxo-PDA was enzymically synthesized from an incubation mixture of (9,12,15)-linolenic acid and flaxseed extract, and the product purified by TLC (chloroform/acetic acid, 100:2, v/v). Analysis of the purified product by two TLC solvent systems indicated that the preparation was homogeneous. Figure 2a shows a typical gas chromatographic analysis of the methyl ester of the purified cyclic fatty acid preparation. Three components were present, with compound I (methyl 12-oxo-PDA, Fig. 3) comprising ca. 70% of the total.

In earlier work we noted that ratios of the three compounds varied among preparations. Even preparations that had been stored under nitrogen at -20 C changed in composition over long times; the proportion of II increased and of I decreased. We were unable to isolate I in pure form by trapping the compound as it eluted from the gas chromatography column. Reinjection of the trapped sample into the gas

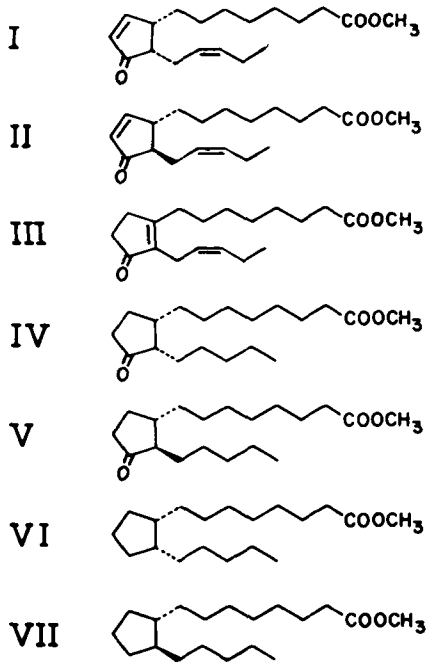

FIG. 3. Proposed structures for compounds derived from methyl 12-oxo-*cis*-10,15-phytodienoate (I). Compounds II and III were formed from I by heat treatment at 190 C. Compounds IV and V were derived from I and II, respectively, by hydrogenation. Compounds VI and VII are methyl phytonoate stereoisomers formed as minor products during the hydrogenation of I and II, respectively. The projections shown here represent the *cis* and *trans* configurations of the side chains with respect to the ring rather than the *R* or *S* configurations, which are not known.

chromatograph showed that it had been almost completely converted to II during the trapping procedure. Apparently heat treatment of I in the gas chromatograph converted compound I to compound II. Conversion also could be effected by heating a preparation of the cyclic fatty acid in a sealed vial under nitrogen atmosphere at 190 C. After 15 min most of I was converted to II, and after 2 hr about half of II was converted to III. The three compounds could be easily separated by TLC. We conducted a separate experiment to determine whether migration or geometric isomerization of double bonds was responsible for the conversion of I to II. The cyclic fatty acid preparation (Fig. 2a) was hydrogenated with platimun oxide as catalyst and analyzed by gas chromatography. Two saturated compounds, IV and V, were derived from I and II, respectively, and their proportions were not altered markedly during hydrogenation (Fig. 2b). Apparently the conversion of I to II did not involve the migration or isomerization of double bonds, because a single product would be expected from the hydrogenation of the mixture of I and II if the two compounds differed only by the position or geometry of double bonds. The two minor compounds (VI and VII) with short retention times were derived from I and II, respectively, during hydrogenation. This was shown by the observation that the hydrogenation of compound II, purified by TLC, gave two products, V and VII. Compounds VI and VII had identical mass spectra and were tentatively identified as methyl phytonoate stereoisomers based on molecular ions at m/e 296. Apparently the hydrogenation reaction removed the oxygen atom from the ring carbonyl in a small proportion of the molecules of I and II.

The hydrogenated mixture of 2b was then heated under a nitrogen atmosphere at 190 C for 10 min, and the products were reinjected into the gas chromatography column. Heat treatment converted compound IV to compound V, but the ratio of the methyl phytonoate isomers (VI and VII) was not altered (Fig. 2c). This demonstrated that the conversion of IV to V (and thus of I to II) was mediated by the ring carbonyl, because the methyl phytonoate isomers, which have no ring carbonyl, showed no conversion.

From the above experiments, we concluded that the conversion of I to II and of IV to V involved the reorientation of the side chains with respect to the ring. A mechanism involving an enol intermediate at carbons 12 and 13 was proposed for the conversion of IV to V. In this mechanism the *trans* configuration of the side chains with respect to the ring was presumed to be the most stable. Thus, IV probably represents the *cis* isomer and V the *trans* isomer of 12-oxo-phytonoic acid. If the above mechanism were correct, treatment with acid or base would also be expected to catalyze the tautomerization reaction. When I and IV were treated for 30 min with 0.1 N KOH in methanol or 0.1 N HCl in methanol at 24 C, there was nearly complete conversion to II and V, respectively.

Chemical Structures of I, II, and III. Compounds II and III were prepared by heating compound I at 190 C for 90 min, and purified by TLC with hexane/ethyl ether solvent (55:45, v/v) with three developments. The structures proposed for I, II, and III appear in Figure 3, and support for these assignments is discussed below.

Characterization of I. Some spectral data for compound I were reported earlier (1). In the present study, the IR spectrum demonstrated characteristic absorption at 3002 cm^{-1} due to

TABLE I

^{1}H Nuclear Magnetic Resonance Spectral Data[a]

Compound		Chemical shift (δ)	J (Hz)	Area	Proton
I	dd	7.74	6.0;2.8	1	10
	dd	6.18	6.0;1.7	1	11
	m	5.41		2	15,16
	s	3.67		3	$COOCH_3$
	m	2.98		1	13
	m	2.20-2.50		5	2,9,14
	m	2.04		2	17
	m	1.25-1.75		12	3-8
	t	0.97	7.4	3	18
II	dd	7.59	5.8;2.6	1	10
	dd	6.12	5.8;2.0	1	11
	m	5.36		2	15,16
	s	3.67		3	$COOCH_3$
	m	2.53		1	13
	m	2.20-2.40		5	2,9,14
	m	2.06		2	17
	m	1.25-1.75		12	3-8
	t	0.96	7.6	3	18
III	m	5.29		2	15,16
	s	3.67		3	$COOCH_3$
	d	2.93	5.6	2	14
	m	2.0-2.50		10	2,8,10,11,17
	m	1.25-1.75		10	3-7
	t	0.99	7.4	3	18

[a]All spectra were recorded with a Bruker WH-90 spectrometer. The samples were dissolved in $CDCl_3$ and were analyzed at ca. 24 C with tetramethylsilane added as an internal standard.

unsaturation, 1742 cm^{-1} from the ester carbonyl, and 1715 cm^{-1} from α,β unsaturation of a carbonyl in a five-membered ring (5). UV absorption at 220 nm also indicated carbonyl conjugation with a double bond.

The mass spectrum showed a molecular ion at m/e 306 and fragment ions at m/e 275 ($M-OCH_3$), m/e 238 ($M-C_5H_8$), m/e 206 ($238-CH_3OH$), m/e 149 [$M-(CH_2)_7COOCH_3$], and a base peak at m/e 95, attributed to the cyclopentenone ring plus a side chain methylene group and a proton. The fragments at m/e 238 and 149 indicated cyclization between carbons 9 and 13 of the fatty acid.

The proton absorptions in the ^{1}H NMR spectrum supported the proposed structure for I. The spectrum varied slightly from that of II, but the assignment of a *cis* or *trans* configuration of the side chains to either compound could not be made on the basis of the ^{1}H NMR spectra. Absorption for the protons of compound I at carbons 10, 11, 13, 15, and 16 appeared slightly downfield from the corresponding proton absorption of II. The absorptions and their assignments are shown in Table I.

The ^{13}C NMR spectral data for compounds I and II are given in Table II. Resonance signals were assigned with reference to published ^{13}C spectral data of polyunsaturated fatty acids (6) and prostaglandins (7). The ^{13}C NMR spectra differentiated between the *cis* and *trans* configurations of the side chains with respect to the ring in the two compounds. A *cis* orientation could be attributed to compound I on the basis of its upfield resonance signals for carbons 9 and 13 in relation to the signals for the same carbon atoms in compound II. This type of upfield resonance shift, called a "steric compression shift," occurs when a carbon atom becomes sterically crowded (8). A similar shift of resonance signal to higher field was observed for carbons 8 and 14 of compound I. Thus, the ^{13}C NMR data indicated that compound I had a *cis* orientation and compound II a *trans* orientation of the side chains with respect to the ring.

Characterization of II. The IR spectrum of II was identical to that for I. UV absorption at 221 nm was similar to that for compound I and indicated a carbonyl in conjugation with a double bond. The mass spectrum of II was indistinguishable from the mass spectrum of I. The ^{1}H and ^{13}C NMR spectra of II were discussed above.

The position of the exocyclic double bond

TABLE II

^{13}C Nuclear Magnetic Resonance Data[a]

Carbon	Chemical Shift (δ)[b] I	II	Carbon	Chemical Shift (δ)[b] I	II
OCH_3	51.5	51.5	10	167.0	167.3
1	174.2	174.2	11	133.0[d]	133.9[d]
2	34.1	34.1	12	210.7	211.6
3	25.0	25.0	13	49.9	51.5
4	29.1[c]	29.1[c]	14	23.9	28.3
5	29.1[c]	29.1[c]	15	127.1	125.2
6	29.6[c]	29.6[c]	16	132.6[d]	133.0[d]
7	27.6[c]	27.4[c]	17	20.9	20.6
8	30.8	34.5	18	14.0	14.1
9	44.4	47.1			

[a]Spectra were recorded with a Bruker WH-90 spectrometer. The samples were dissolved in $CDCl_3$ and analyzed at ca. 24 C.

[b]The chemical shift (δ) is reported as ppm downfield from tetramethylsilane.

[c]Tentative assignments.

[d]These assignments within either vertical column may be reversed.

at carbon 15 was confirmed by oxidative ozonolysis. Prior to ozonolysis, however, the ring double bond was saturated by treatment of the compound with sodium borohydride, which reduced the double bond of the cyclopentenone ring and converted the carbonyl to a hydroxyl (9). The hydroxyl was converted back to a carbonyl by treatment with Jones' reagent. The exocyclic double bond that remained was cleaved by oxidative ozonolysis, and a methoxime derivative of the compound was prepared. Analysis by GC-MS showed a molecular ion at m/e 341 and other fragments at m/e 310 ($M-OCH_3$), m/e 184 [base peak; $M-(CH_2)_7-COOCH_3$] and m/e 152 ($184-CH_3OH$). The structure was proposed to be methyl 8-(2-carbomethoxymethyl-3-methoxime-cyclopentyl)-octanoate. Characterization of this product confirmed the position of the exocyclic double bond at carbon 15 of compound II.

Characterization of III. The spectral data of III differed from those of I and II. UV absorption at 236 nm suggested a 2,3-substituted cyclopent-2-enone structure (10). Absorbance in the IR region occurred at 3000 cm^{-1} due to unsaturation, 1741 cm^{-1} from the ester carbonyl, and 1703 cm^{-1} due to a C-C double bond conjugated to a carbonyl. The carbonyl absorption at 1703 cm^{-1} was similar to that reported for jasmone, 1702 cm^{-1} (11), and 2-ethyl-3-methyl-cyclopent-2-enone, 1701 cm^{-1} (12), each of which is a 2,3-disubstituted cyclopent-2-enone compound.

The mass spectrum of III varied slightly from the spectra of I and II. The base peak occurred at m/e 149 indicating cleavage between carbons 8 and 9. Another large fragment at m/e 177 indicated cleavage between carbons 6 and 7. Samuelsson and Stallberg (13) observed a similar strong cleavage γ to a 2,3-substituted cyclopentenone ring in the mass spectrum of 2-(6-carboxyhexyl)-3-octylcyclopent-2-enone. Other characteristic fragments occurred at m/e 306 (M), m/e 275 ($M-OCH_3$), and m/e 95. The fragments m/e 238 and 206 that were observed with I and II were absent in the spectrum of III.

Compound III was subjected to oxidative ozonolysis, and the methoxime derivatives of the products were prepared. Analysis by GC-MS showed that one major product of the reaction was methyl 4-methoxime-dodecanedioate (Fig. 4a). Characteristic fragment ions in the mass spectrum were m/e 301 (M), m/e 270 ($M-OCH_3$), m/e 159 [$M-(CH_2)_6\ COOCH_3+H^+$] (base peak; β-cleavage to the methoxime), m/e 127 ($159-CH_3OH$), and m/e 214 [$M-(CH_2)_2-COOCH_3$] (α-cleavage to the methoxime). The identification of this product confirmed that the position of the ring carbonyl in compound III was carbon 12 (Fig. 4). Because compound III was derived from compounds I and II, and because there was no evidence for the movement of the carbonyl, we concluded that the ring carbonyl in those compounds also was at carbon 12.

A second major product of the above reaction resulted from partial ozonolysis of compound III. The product was identified by GC-MS as methyl 8-(2-carbomethoxymethyl-3-methoxime-cyclopent-1-enyl)octanoate (Fig. 4b). The mass spectrum showed fragments at m/e 339 (M), m/e 308 ($M-OCH_3$), m/e 210 [base peak; $M-(CH_2)_5COOCH_3$], m/e 178

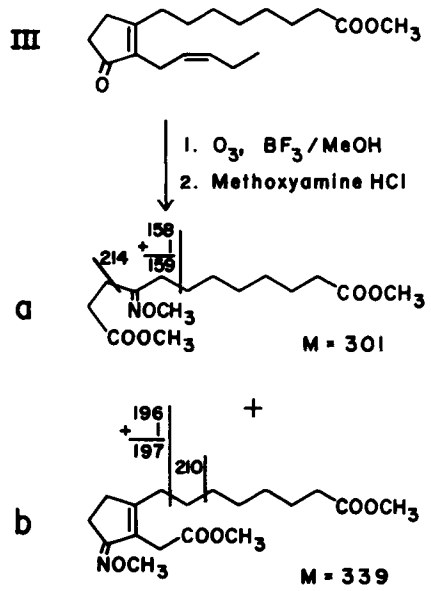

FIG. 4. Methoxime derivatives of the major cleavage products obtained from a) complete or b) partial oxidative ozonolysis of compound III [methyl 12-oxo-9(13)-*cis*-15-phytodienoate] in 14% boron trifluoride in methanol.

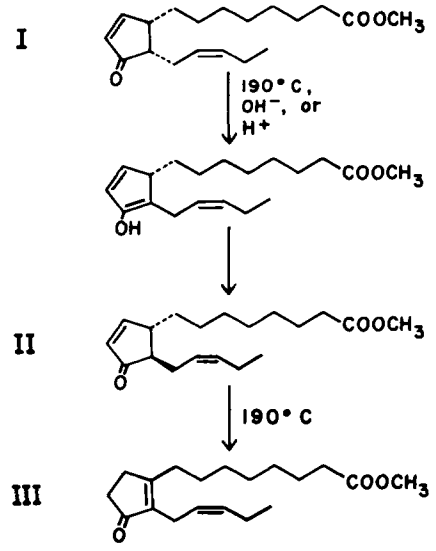

FIG. 5. Proposed reactions for the conversion of compound I to compounds II and III. The projections shown here represent the *cis* and *trans* configurations of the side chains with respect to the ring rather than the *R* or *S* configurations, which are not known.

(210-CH_3OH), and m/e 197 [M-$(CH_2)_6$-$COOCH_3$+H^+]. On the basis of molecular weight, we concluded that the position of the exocyclic double bond was unchanged from that in I and II and remained at carbon 15 in compound III.

The proton absorptions of III (Table I) clearly showed the loss of the ring olefinic protons. A marked downfield shift (δ 2.05 to δ 2.93) of the absorption due to the protons attached to carbon 14 supported the presence of a 1,4-pentadiene structure in the molecule (-C=CC$\underline{H}_2$CH=CH-).

DISCUSSION

The procedure for the enzymic synthesis and purification of methyl 12-oxo-*cis*-10,15-phytodienoate (I) described by Zimmerman and Feng (1) gave a preparation containing two minor products (II and III) in addition to I. Our data indicated that compound II was derived from I by an acid-, base-, or heat-promoted mechanism involving a cyclopentadienol intermediate (Fig. 5). Reversion of the enol to the keto structure resulted in a reorientation of the carbon chains from a *cis* configuration in I to a *trans* configuration in II. Thus, the rapid conversion of I to II in the presence of acid, base, or heat underscored the necessity of minimizing such conditions during the isolation and analysis of I.

Prolonged heat treatment of II caused further isomerization, characterized by migration of the ring double bond to give product III. A similar isomerization was reported by Stork, et al. (14), who noted that 2*S*-(*cis*-pent-2′-enyl)-3*R*-methyl-cyclopent-4-enone could be converted to 2-(*cis*-pent-2′-enyl)-3-methyl-cyclopent-2-enone (jasmone) in the presence of aqueous base, by heating with acid, or heating at 220 C for one hour. Analogous rearrangements have also been observed in the formation of prostaglandin B.

Although the data indicated the *cis* orientation of the two carbon chains with respect to the ring in methyl 12-oxo-PDA (I), the absolute configuration (9*S*,13*S* or 9*R*,13*R*) was not established. Cucurbic acid, a plant growth inhibitor with related structure, has been shown to have the *S,S* configuration (15). The projections for compounds I and II (Figs. 3,5) represent the *cis* and *trans* configurations of the side chains rather than the *R* or *S* configurations, which are not known. The function of 12-oxo-PDA in plant metabolism is not known but is under investigation in our laboratory.

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Experiment Station, Paper No. 983. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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The Effects of the Hypocholesteremic Compound 3β-(β-Dimethylaminoethoxy)-androst-5-en-17-one on the Sterol and Steryl Ester Composition of *Saccharomyces cerevisiae*[1]

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ABSTRACT

When yeast was grown in the presence of 10^{-4} M 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one (DMAE-DHA), the compound 2,3;22,23-dioxidosqualene (DOS) accumulated. Total free sterol was reduced by about 30%, whereas almost no steryl esters were found. The same drug at lower concentration (3×10^{-6} M) caused a slight increase in steryl ester production, and a 24% reduction in free sterol content. The marked accumulation of ergostra-5,7,22,24(28)-tetraen-3β-ol with 3×10^{-6} M DMAE-DHA indicated that the C_{24-28} reductase is especially sensitive to the action of the drug.

INTRODUCTION

The hypocholesteremic compound, 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one, (DMAE-DHA), inhibits the cyclization of 2,3 oxidosqualene to lanosterol by yeast (1,2) and by rat liver homogenates (3). Cyclization of squalene to tetrahymanol (3) and the synthesis of diplopterol (4) by *Tetrahymena pyriformis* are also inhibited by DMAE-DHA.

Gordon et al. (5) found that feeding DMAE-DHA to rats resulted in lowered blood and liver levels of cholesterol and in the appearance of desmosterol as the major sterol in the liver. Sipe and Holmlund (3) also found that DMAE-DHA inhibited the conversion of C-30 to C-27 sterols by rat liver homogenates. Studies on the metabolism of silkworm larvae by Hikino et al. (6,7) revealed that DMAE-DHA inhibited both the dealkylation of β-sitosterol and the conversion of desmosterol to cholesterol.

This paper describes the appearance of ergosta-5,7,22,24(28)-tetraen-3β-ol as the major sterol when yeast is treated for 24 hr with a low concentration of DMAE-DHA, while 2,3;22,23-dioxosqualene (DOS) is the major component formed when a higher concentration of DMAE-DHA is employed. In addition, ergosterol and its sterol precursors in free and esterified form were isolated and quantified in both normal and treated yeast cultures. The isolation and characterization of DOS from yeast has been described earlier (2).

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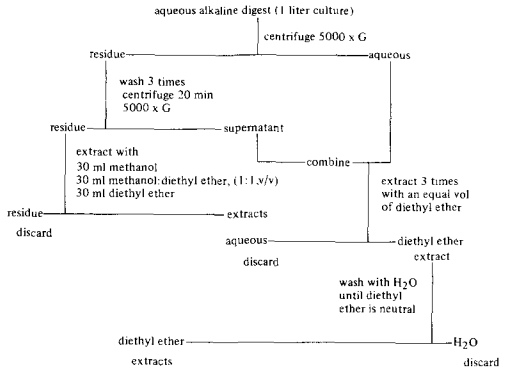
MATERIALS AND METHODS

Saccharomyces cerevisiae, strain MY306, obtained through the courtesty of Dr. Eugene Dulaney, Research Laboratories, Merck and Co., Rahway, NJ, was maintained and cultured with and without DMAE-DHA as previously described (2). However, cultures employed for isolation of sterols were grown on a slightly modified medium: 2% glucose, 0.5% NH_4Cl, 1.1% K_2HPO_4, 1.05% KH_2PO_4, and 0.5% Difco yeast extract (Difco Laboratories). DMAE-DHA was provided by S. Bernstein, Lederle Laboratories Division, American Cyanamide Co., Pearle River, NY. Biosil A was obtained from Bio-Rad Laboratories. Redistilled bulk hexane (68-70 C) was used for extractions and thin layer chromatography (TLC). Spectranalyzed (Fisher Scientific Co.) hexane was used for high performance liquid chromatography (HPLC) and gas chromatography (GC). Squalene (98% pure, Eastman organic chemicals), lanosterol (Sigma), ergosterol (Eastman Kodak), 2,3-oxidosqualene and DOS—prepared as described in (2)-and TLC No. 1, containing oleic acid, triolein, methyl oleate, and cholesteryl oleate (Applied Science Laboratories, Inc.) served as TLC standards.

Extraction of Lipids

Cells were collected and washed as described (2) and then heated at 60 C for 1 hr in 30 ml of 20% aqueous KOH containing 0.5% (w/v) pyrogallol (8). After the digestion, lipids were extracted from the washed residue and from the supernatant by a modification of the

method of Gendleman (9) as follows:



During the ether extraction of the aqueous supernatant, a foamy layer occurred between the ether and aqueous layers. This foam dissipated on standing. Extracts of the aqueous phase and the residue were combined and dried over sodium sulfate and then taken to dryness in a rotary evaporator prior to column chromatographic separation of sterols, steryl esters, and fatty acids.

Separation of Sterols, Steryl Esters and Fatty Acids

This separation was carried out by the method of Gendelman (9). The lipid extracts from a 1 liter culture were slurried with 1 g Biosil A and dried in a rotary evaporator. The mixture was transferred to a filter paper on a Buchner funnel. The funnel was then inserted in a flask containing NH_4OH. The NH_4OH was heated so that NH_3 vapors permeated the sample-coated Biosil A. The treated sample was then layered on a column slurry-packed with 15 g Biosil A that had been treated similarly with NH_3. For a 1 liter culture, hydrocarbons were eluted with 100 ml hexane, steryl esters with 450 ml hexane/diethyl ether (98:2, v/v), and sterols with 300 ml diethyl ether. The fatty acid remained on the column. The composition of the eluates was determined by TLC employing the method described by Sobus and Holmlund (8) in which the spotted plates were saturated with NH_3 prior to development.

Saponification of Steryl Esters

Ten ml of 20% KOH in 80% ethanol, containing 0.5% (w/v) pyrogallol, was added to the residues of the steryl ester eluates and refluxed for 1 hr. To extract the sterols, 20 ml water was added to the reflux mixture, which was then extracted 3 times with hexane. The combined hexane extracts were washed 2 times with 10 ml water.

Isolation of the Sterol Components of the Free Sterol Fractions and of the Sterols Derived from Steryl Esters Fractions

The residues of the eluates (free sterol fractions and sterols derived from steryl ester fractions) were dissolved in hexane/ether (85:15, v/v). Aliquots were injected into a high pressure liquid chromatograph (Model ALC-GPC 20, Waters Assoc., Inc.) containing two μPorasil columns, 4 mm i.d. x 30 cm (Waters Assoc., Inc.). Sterols were eluted with hexane/ether (85:15, v/v). The column effluent was monitored by uv absorption at 220 nm (Schoeffel Electroflow Monitor SF 770 coupled with a Schoeffel GM 770 monochromator), and fractions were collected with a Gilson microfractionator (Model FC-80H, Gilson Medical Electronics, Inc.). All solvents and samples were filtered through a Millipore filter FGLPO4700 (Millipore Corp.). The hexane and diethyl ether were sonicated to remove dissolved air. The eluted fractions that contained a mixture of sterols were recycled on μPorasil with hexane/diethyl ether (85:15, v/v) until maximum resolution was achieved.

Identification and Quantification of the Isolated Sterols

The components that were isolated by HPLC were analyzed by GC. A Hewlett-Packard Gas Chromatograph model 5830A equipped with an 18850A GC terminal and an Automatic Sampler model 7671A was employed. The analysis was carried out on glass columns (6 ft x 1/8 in) packed with 1% QF-1, 3% SE 30, or 3% HI-EFF-8BP on Gas Chrom Q (100-120 mesh). Nitrogen was the carrier gas, flow rate 20 ml/min, and the column temperature for the QF-1 column was 230 C, while for the SE-30 column it was 250 C and 240 C for the HI-EFF-8BP column. The injection temperature was 275 C, and the temperature of the flame ionization detector was 300 C. Analysis with HI-EFF-8BP for one of the fractions (free sterol fraction of the control culture) was performed with a Perkin-Elmer GC 900 equipped with a glass column (180 cm x 2 mm) containing 3% HI-EFF-8BP on Chromosorb W (100-120 mesh) with nitrogen as the carrier gas, flow rate approximately 35 ml/min. The column temperature was 240 C, and the injection temperature and the manifold temperature were both 275 C.

The areas that were printed out by the GC terminal of the Hewlett-Packard GC were quantified by comparing them to the area of a known quantity of cholesterol; 534965 ± 5334 (S.E. for N = 17) area units are equivalent

TABLE I

Effect of DMAE-DHA on Production of DOS, Free Sterols and Esterified Sterols by *S. cerevisiae*

Culture[a]	DOS μg/g protein	Free sterols μg/g protein	Esterified sterols μg/g protein
control	0	3160	6480
3×10^{-6} M DMAE-DHA	0.417	2410	6980
10^{-4} M DMAE-DHA	5100	2100	262

[a]A 1 liter culture of yeast was incubated for 24 hr, without DMAE-DHA, or with 3×10^{-6} M or 10^{-4} M DMAE-DHA. The cells were collected, lipids extracted from the alkali-digested cells, sterols, steryl esters and fatty acids separated, and the steryl esters saponified as described in Materials and Methods. The free sterol fraction (which also contains DOS) and the sterols obtained by saponification of steryl esters were separated by HPLC. DOS and the sterols were quantitatively determined by measurement of peak areas on a gas liquid chromatogram. A similar chromatogram of the reference standard, cholesterol, (1 μg equivalent to 534965 ± 5334 area units) was included in the analysis of each sample. No corrections have been applied for any losses which may have been incurred during isolation.

to 1 μg. Identification of the sterols was made by comparing the retention times relative to cholesterol on three columns with those of authentic samples, or with values determined by Patterson (10). Further identification was made by determining the molecular weight of the samples by gas chromatography-mass spectrometry (GC-MS). A Finnigan model 1015D gas chromatograph-mass spectrometer equipped with a model 6000 data system was employed. Helium was the carrier gas, and columns containing 3% SE-30 or 3% HI-EFF-8BP on Gas Chrom Q (100-120 mesh) were used to separate the components. Reagent grade ammonia or methane gas was mixed with carrier gas from the chromatograph prior to entrance into the mass spectrometer. The ionizing voltage was 100 eV, the source pressure was 1 torr, and the source temperature < 100 C. In most cases the base peak was M-18. For characterization of ergosta-5,7,22-24(28)-tetraen-3β-ol, electron impact ionization was also employed.

A Perkin-Elmer Model 225 grating infrared spectrophotometer was employed to determine the IR spectrum of ergosta-5,7,22,24(28)-tetraen-3β-ol; spectral grade CS_2 was the solvent. Spectral grade hexane was the solvent for obtaining UV spectra with a Beckman DBG spectrophotometer.

RESULTS

The effect of DMAE-DHA (3×10^{-6} M and 10^{-4} M) on the growth of 24 hr cultures was minimal, as determined from the absorbance of the cultures and by measurement of protein by the method of Lowry et al. (11) as adapted to yeast by Sobus and Holmlund (8). The absorbance at 640 nm (Spectronic 20, Bausch and Lomb) was 5.1, 4.8 and 4.3 for the control, 3×10^{-6} M DMAE-DHA, and 10^{-4} M DMAE-DHA treated cultures, respectively, and the protein content (g/l culture) was 1.41 ± 0.06 (S.E.) for the control, and 1.42 ± 0.038 and 1.48 ± 0.11 for the 3×10^{-6} M DMAE-DHA and 10^{-4} M DMAE-DHA treated cultures, respectively.

From Table I it may be seen that the lower concentration of DMAE-DHA (3×10^{-6} M) resulted in ca. 24% reduction of free sterols (μg/g protein), while the content of esterified sterols was slightly elevated. At the higher concentration of DMAE-DHA (10^{-4} M), DOS was the single major nonsaponifiable compound to accumulate. Esterified sterols were almost nonexistent, whereas free sterols represented about 2/3 of the control value.

Table II shows the tentative identification of the isolated sterols by determining GC relative retention times on three columns and comparing these values to either authentic standards, to synthesized DOS and 24,25-oxidolanosterol, to values reported by Patterson (10), or to calculated values that were obtained from Patterson's separation factors (10). In addition to the sterols shown in Table II, nine more sterols were isolated but not identified. The characterization of ergosta-5,7,22,24(28)-tetraen-3β-ol is based on several considerations. Relative retention times on three GC systems correspond to values calculated for this compound (Patterson, G.W., personal communication), and chemical ionization mass spectra revealed a molecular ion of 394, whereas spectra produced by electron impact ionization provide an m/e peak of 123 (57% of base peak), corresponding to the di-unsaturated side chain of the tetraenol. Moreover, the uv spectrum (λ_{max} 230 nm; other maxima at 272, 281 and 294 nm) was analogous to those reported by Breivik et al. (12), by Barton et al. (13), and by

TABLE II

The Molecular Weights and GC-Relative Retention Times of Sterols and a Sterol Precursor (DOS) from *S. cerevisiae*[a]

Compound	M.W.	Relative retention time		
		SE-30[b]	QF-1[c]	HI-EFF-8BP[d]
Zymosterol	384	1.12	1.09	1.29
Ergosta-5,7,22,24(28)-tetraen-3β-ol	394	1.27	1.32	1.92
Ergosterol	396	1.20	1.20	1.44
Ergosta-8,22,24(28)-trien-3β-ol	396	1.22	1.29	1.54
Ergosta-7,14,22-trien-3β-ol	---	1.13	1.07	1.18
Ergosta-5,7,24(28)-trien-3β-ol	---	1.32	1.37	1.73
Ergosta-7,22-dien-3β-ol	---	1.22	1.20	1.34
4α-Methyl-5α-cholest-7-en-3β-ol	---	1.32	1.19	1.36
Ergosta-8,24(28)-dien-3β-ol	398	1.32	1.29	1.43
Ergosta-5,7-dien-3β-ol	398	1.42	1.44	1.70
Ergosta-8,24-dien-3β-ol	398	1.47	1.37	---
14α-Methylergost-7-en-3β-ol	412	1.53	1.53	1.52
4,4-Dimethylzymosterol	412	1.63	1.50	1.70
Lanosterol	426	1.63	1.50	1.51
Dihydrolanosterol	428	1.50	1.46	1.14
2,3;22,23-Dioxidosqualene	442	0.964	1.18	0.606
24,25-Oxidolanosterol	442	2.22	3.71	3.70

[a]The molecular weights were determined by GC-MS and the retention time is related to cholesterol.
[b]3% SE-30; column temperature = 250 C; cholesterol retention time = 12.73 min.
[c]1% QF-1; column temperature = 230 C; cholesterol retention time = 3.73 min.
[d]3% HI-EFF-8BP; column temperature = 240 C; cholesterol retention time = 20.63 min.

Petzoldt et al. (14). The fingerprint region of the ir spectrum in CS_2 (ν_{max} 1060, 1040, 967, 885, 857, 835 and 799 cm^{-1}) compared well with that reported by Breivik et al. (12). The above UV, IR and mass spectral data also agree with those reported for ergosta-5,7,22,24(28)-tetraen-3β-ol as reported by Nes et al. (15).

Table III shows the effect that the two concentrations of DMAE-DHA had on the free sterol and esterified sterol composition. Ergosterol, which is the major component of the control both in the free sterol and esterified sterol fractions, was decreased dramatically in the free sterol fraction in the presence of increased concentration of DMAE-DHA. The ergosterol percentage of the esterified sterols was greatly reduced by 3 x 10^{-6} M DMAE-DHA, but only slightly reduced by 10^{-4} M DMAE-DHA. However, the quantity of sterol ester is reduced to ca. 1/25 of the control in the culture treated with 10^{-4} M DMAE-DHA. The actual quantity of ergosterol in the esterified sterol fraction was reduced by DMAE-DHA from 2580 μg/g protein to 545 and 94 μg/g protein for the 3 x 10^{-6} M and 10^{-4} M treated cultures, respectively.

The presence of DMAE-DHA resulted in increased percentages of lanosterol and 4,4-dimethyl zymosterol in the free sterol fraction. The relative quantity of zymosterol was slightly increased with 3 x 10^{-6} M DMAE-DHA, but zymosterol was not detected with 10^{-4} DMAE-DHA. However, in the esterified steryl fraction, the relative amounts of lanosterol and 4,4-dimethyl zymosterol decreased, while the relative amount of zymosterol increased when the drug concentration was elevated.

The most abundant free sterol components of the control were: ergosterol (82%), zymosterol (6.7%), and lanosterol (3.6%). The most abundant free sterol components of the culture treated with 3 x 10^{-6} M DMAE-DHA were ergosterol (64%), zymosterol (10.7%), ergosta-5,7,22,24(28)-tetraen-3β-ol (7.1%), lanosterol (4.9%), and 24,25-oxidolanosterol (2.3%). The most abundant free sterol components of the culture treated with 10^{-4} M DMAE-DHA were ergosterol (33%), ergosta-5,7,22,24(28)-tetraen-3β-ol (30%), lanosterol (13%), and 24,25-oxidolanosterol (13%).

The sterol composition of the esterified fraction was different from the sterol composition of the free sterol fraction in the control and treated cultures. In the control, the most abundant sterol components from the steryl ester fraction were ergosterol (40%), zymosterol (25%), ergosta-8,24(28)-dien-3β-ol (11%), lanosterol (6.7%), 4,4-dimethyl zymosterol (6.5%), and ergosta-5,7,24(28)-trien-3β-ol (2.3%). In the culture treated with 3 x 10^{-6} M DMAE-DHA, the most abundant sterol components from the steryl ester fraction were ergosta-5,7,22,24(28)-tetraen-3β-ol (39%), zymosterol (28%), ergosterol (7.8%), ergosta-

TABLE III

The Effects of DMAE-DHA on the Composition of Free and Esterified Sterols of *S. cerevisiae*

Compound (M.W.)[a]	Free sterols			Sterols derived from steryl esters		
	Control %[b]	3×10^{-6} M DMAE-DHA %[b]	10^{-4} M DMAE-DHA %[b]	Control %[c]	3×10^{-6} M DMAE-DHA %[c]	10^{-4} M DMAE-DHA %[c]
Ergosterol (396)	82	64	33	40	7.8	34
Zymosterol (384)	6.7	10.7	0	25	28	34
Lanosterol (426)	3.6	4.9	13	6.7	3.3	3.4
Ergosta-5,7-dien-3β-ol (398)	1.7	1.4	0.14	0.58	2.0	1.6
Dihydrolanosterol (428)	1.6	0	0	0	0	0
4,4-Dimethylzymosterol (412)	0.51	1.9	1.4	6.5	2.5	1.9
Ergosta-8,24-dien-3β-ol (398)	0.42	0	0	0	0	0
14α-Methylergost-7-en-3β-ol (412)	0.16	0	0	0	0	0
Ergosta-8,22,24(28)-trien-3β-ol (396)	0.098	1.5	0.11	0	4.4	0
Ergosta-5,7,22,24(28)-tetraen-3β-ol (394)	0	7.1	30	0	39	21
24,25-Oxidolanosterol (442)	0	2.3	13	0	0	0
4α-Methyl-5α-cholest-7-en-3β-ol (398)	0	1.5	0	0	0	0
Ergosta-5,7,24(28)-trien-3β-ol (396)	0	0.90	0.43	2.3	3.9	1.3
Ergosta-7,14,22-trien-3β-ol (396)	0	0.11	0	0.34	0.69	0
Ergosta-7,22-dien-3β-ol (398)	0	0.11	0	0	0	0
Ergosta-8,24(28)-dien-3β-ol (398)	0	0	0	11	4.9	0
Unidentified mixture	3.8	3.2	7.8	7.7	4.1	2.5

[a]M.W. of each compound is cited. See Table II for M.W. determined by GC-MS.
[b]% of total free sterols.
[c]% of total sterols derived from the saponification of steryl esters.

TABLE IV

The Effects of DMAE-DHA on the Relative Amounts of the Sterol Components That Are Esterified

Sterol	Total sterol			% Esterified		
	Control μg/g protein	3×10^{-6} M DMAE-DHA μg/g protein	10^{-4} M DMAE-DHA μg/g protein	Control	3×10^{-6} M DMAE-DHA	10^{-4} M DMAE-DHA
Ergosterol	5170	2096	780	50	26	12
Zymosterol	1840	2194	88.2	88	88	100
Lanosterol	546	351	278	79	66	3.2
Ergosta-8,24(28)-dien-3β-ol	695	197	0	100	100	0
4,4,-Dimethyl zymosterol	435	221	33.8	96	80	15
Ergosta-5,7,24(28)-trien-3β-ol	149	294	12.5	100	93	27
Ergosta-5,7-dien-3β-ol	91.0	171	7.18	42	80	59
Ergosta-8,22,24(28)-trien-3β-ol	3.09	343	2.31	0	89	0
Ergosta-5,7,22,24(28)-tetraen-3β-ol	0	2870	690	0	94	8.1

8,24(28)-dien-3β-ol (4.9%), ergosta-8,22,24-(28)-trien-3β-ol (4.4%), ergosta-5,7,24(28)-trien-3β-ol (3.9%), lanosterol (3.3%), 4,4-dimethyl zymosterol (2.5%), and ergosta-5,7-dien-3β-ol (2.0%). In the culture treated with 10^{-4} M DMAE-DHA, the most abundant sterol components from the steryl ester fraction were ergosterol (34%), zymosterol (34%), ergosta-5,7,22,24(28)-tetraen-3β-ol (21%), lanosterol (3.4%), and 4,4-dimethyl zymosterol (1.9%).

Table IV shows the effect of the two concentrations of drug on the amount of each sterol that was esterified. The amount of ergosterol, lanosterol, 4,4-dimethyl zymosterol, and ergosta-5,7,24(28)-trien-3β-ol that was esterified decreased in the treated cultures with increasing concentration of the drug, but the percentage of total zymosterol that was esterified was the same as the control in the culture treated with 3 x 10^{-6} M DMAE-DHA and increased to 100% in the culture treated with 10^{-4} M DMAE-DHA. Ergosta-8,24(28)-dien-3β-ol was detected in the control and the 3 x 10^{-6} M DMAE-DHA treated cultures only, and in both cases was 100% esterified. Ergosta-5,7,22,24(28)-tetraen-3β-ol occurred only in the treated cultures, but 94% of it was esterified in the culture treated with 3 x 10^{-6} M DMAE-DHA, while only 8.1% was esterified in the culture treated with 10^{-4} M DMAE-DHA.

DISCUSSION

Fung and Holmlund (1) observed that DMAE-DHA had little effect on the growth of yeast. These findings were corroborated in the present studies with 24 hr cultures. These workers also found that DMAE-DHA caused an accumulation of 2,3-oxidosqualene, presumably by inhibition of 2,3-oxidosqualene cyclase. In prolonged incubation, the level of 2,3-oxidosqualene decreased, apparently by conversion to an unidentified more polar product. Field and Holmlund (2) repeated these observations and characterized the product formed from 2,3-oxidosqualene as DOS. Removal of DMAE-DHA then permitted cyclization of DOS to 24,25-oxidolanosterol.

In the current investigation, DMAE-DHA was also found to promote accumulation of ergosta-5,7,22,24(28)-tetraen-3β-ol, apparently by inhibiting the reduction of the 24(28) double bond. Ergosta-5,7,22,24(28)-tetraen-3β-ol was found to be 7.1 and 30% of the free sterol fraction, and 39 and 21% of the esterified sterol fraction from the 3 x 10^{-6} M and 10^{-4} M treated cultures, respectively, while no tetraene was detected in the control culture.

Gordon et al. (5) found that DMAE-DHA interfered with the reduction of the Δ^{24} in the side chain in cholesterol biosynthesis. Avigan et al. (16) found that dihydrolanosterol could not be formed from lanosterol in the presence of 3β-(β-diethylaminoethoxy)-androst-5-en-17-one due to the inability of the enzyme to reduce Δ^{24}. These authors concluded that the inhibited enzyme is the same side chain reductase that converts desmosterol to cholesterol. Failure to observe the presence of dihydrolanosterol in the DMAE-DHA treated cultures is in keeping with this conclusion.

Corroborating the findings that DMAE-DHA inhibited the reduction of $\Delta^{24(28)}$ is the observation that two other $\Delta^{24(28)}$ sterols were found in the 3 x 10^{-6} M treated culture in quantities greater than found in the control. Only 3.09 μg/g protein of ergosta-8,22,24(28)-trien-3β-ol was detected in the control (in the free sterol fraction only), while the culture treated with 3 x 10^{-6} M contained 343 μg/g protein (89% esterified). The control also contained only 149 μg/g protein of ergosta-5,7,24(28)-trien-3β-ol (100% esterified) whereas the 3 x 10^{-6} M treated culture contained 294 μg of the $\Delta^{5,7,24(28)}$ sterol per g protein (93% esterified).

The differences in the quantity of sterol and in sterol composition caused by the two different concentrations of DMAE-DHA may reflect differences in the degree of sensitivity of various enzymes to the drug. When the culture was treated with 3 x 10^{-6} M DMAE-DHA, ergosta-5,7,22,24(28)-tetraen-3β-ol was the major product, but when 10^{-4} M DMAE-DHA was employed DOS (the product of the accumulation of OS) was the major product of the 24 hr culture. It can be inferred from these observations that the $\Delta^{24(28)}$ reductase is more readily inhibited by DMAE-DHA than is the 2,3-oxidosqualene cyclase. Since tetraene is regarded (17,18) as the most likely immediate precursor to ergosterol in yeast, and since the $\Delta^{24(28)}$ reductase appears to be inhibited by DMAE-DHA, the fact that the ratio of total ergosterol to total tetraene is higher in the presence of 10^{-4} M DMAE-DHA than in the presence of 3 x 10^{-6} M is at first surprising. However, examination of the distribution of free and esterified sterol (Table IV) reveals that in the presence of the lower drug concentration almost all of the accumulated tetraene is in the esterified form, whereas at the higher drug concentration less than 10% is esterified. One important factor involved in the conversion of tetraene to ergosterol is the rate at which both sterols are made available and used in other metabolic reactions. Bailey and Parks (19) have shown that once yeast sterols are esterified they

do not readily undergo additional metabolism. Therefore, any tetraene which becomes esterified is probably no longer available for reduction to ergosterol. Thus, the altered degree of esterification of tetraene could account for what appears to be a greater extent of reduction of tetraene at the higher drug concentration.

The effect of DMAE-DHA concentration on the extent of total sterol esterification is also of interest. Earlier work indicated that exposure of yeast to either trifluperidol (20) or triparanol (21) resulted in the production of at least twice as much esterified sterol as in control cultures. The increased sterol esterification occurring in the presence of these drugs may be due to the accumulation of Δ^8 sterols, since they appear to be better substrates than ergosterol for esterification (22). Quite different results were obtained with DMAE-DHA. At the low concentration (3×10^{-6} M), a slight increase in sterol esterification was observed relative to the control (74% vs. 67% of the total sterols were esterified). However, with the higher concentration of DMAE-DHA (10^{-4} M), only about one-fourth as much total sterol is produced, relative to the control, and only 11% of the total sterol is present in esterified form. This marked reduction in the extent of sterol esterification, especially of ergosterol, was observed with all of the sterols except zymosterol, and may reflect the cell's requirement for a minimum amount of free sterol. Large amounts of steryl ester only accumulate in normal cultures after entering stationary phase (19); therefore, cellular need for steryl esters is probably not as great as that for free sterols. In the face of a waning ability by the cell to produce sterol because of the presence of DMAE-DHA, the acyl CoA-sterol acyl transferase may compete unfavorably with membrane-forming systems for available sterol, especially for ergosterol. Alternatively, there may be less enzyme produced under these circumstances, or the drug may inhibit the enzyme. Further study is required to distinguish between these alternatives.

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METHODS

A Specific Spectrophotometric Determination of Long Chain Nitro Compounds and its Application to NO_2-Initiated Lipid Autoxidation

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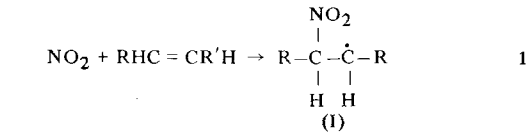
ABSTRACT

A specific spectrophotometric method is described for the determination of nitro-β-hydroperoxides produced during the initiation of lipid autoxidation by ppm NO_2; this determination provides a measure of the rate of initiation by addition of NO_2 to lipids. The nitro group is reduced to an amine using polymethylhydrosilane (PMHS) and Pd on charcoal, and the amine is then determined spectrophotometrically as a neutral methyl orange salt. Although this method was developed for the determination of micromolar quantities of nitro-β-hydroperoxides in lipid samples, it can be used to determine any long chain, nonpolar nitro compound. The method also can be adapted to allow the determination of polar or short chain nitro compounds by using a different amine determination.

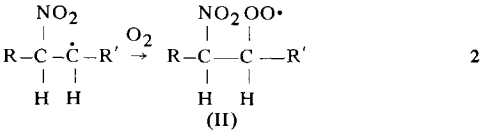
INTRODUCTION

In the investigation of the nitrogen dioxide-initiated free radical autoxidation of polyunsaturated fatty acid esters (PUFA) (1,2), it is necessary to have a means of determining the rate of initiation by addition. Although initiation also occurs by abstraction of an allylic hydrogen by nitrogen dioxide (1), this methanism does not produce carbon-bound nitrogen as does the addition mechanism. Addition of NO_2 to one of the double bonds of a PUFA molecule produces a nitro-alkyl radical, I, eq. 1,



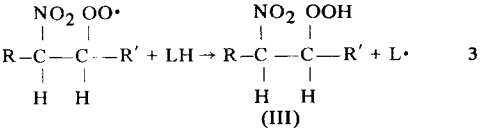
(I) 1

which then reacts with oxygen to form a nitro-peroxy radical, II, eq. 2.



(II) 2

This nitro-peroxy radical can then abstract a hydrogen atom from another PUFA molecule, LH, to produce a stable nitro-β-hydroperoxide, III, eq. 3, and a lipid radical, L•.



(III) 3

The lipid radical created then enters the propagation steps, eq. 4 and 5, and several molecules of hydroperoxide are produced before the chain is terminated.

$$L\bullet + O_2 \rightarrow LOO\bullet \quad 4$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad 5$$

Since one nitro-β-hydroperoxide is formed for each addition of NO_2, a measurement of the concentration of nitro-β-hydroperoxide product provides a means of determining the rate of initiation by the addition mechanism.

Determination of the nitro-β-hydroperoxide concentration in the autoxidation system requires a method that is sensitive, unaffected by the presence of hydroperoxides, and independent of the position of the nitro group along the carbon chain. Although many methods have been reported for determining both aromatic and aliphatic nitro compounds (3-7), none were suited to this system. Therefore, a new method was developed; it relies upon reduction of the nitro group followed by spectrophotometric determination of the amine formed.

[1] To whom correspondence and reprint requests should be sent.

EXPERIMENTAL PROCEDURES

Materials

Pd on charcoal-5% (Alfa Division of the Ventron Corp.), polymethylhydrosilane-PMHS and 1-dodecyl amine-98% (Aldrich Chemical Co.), and 1-iodohexadecane (Eastman Chemicals) were obtained commercially.

1-Nitrohexadecane is synthesized according to the procedure of Kornblum and Powers (8), and the product mixture, 47.5 g (70% 1-nitrohexadecane by NMR comparison of the methine and methyl ester protons), is purified in the following manner. Add the product mixture to 150 ml of methanol containing an equivalent amount, 3.3 g, of sodium metal, mix to precipitate the nitro-carbanion salt, and add 500 ml of diethyl ether. Collect the nitro-carbanion salt in a fritted glass filter, wash with 300 ml of additional diethyl ether, and partially dry the salt by pulling air through the filter. Add the salt to a separatory funnel containing 300 ml of chloroform and 200 ml of 0.5 N hydrochloric acid, mix, and add additional acid, in small portions, until the aqueous layer remains at a pH of about 1. Collect the chloroform layer, dry over anhydrous magnesium sulfate, and remove the solvent by rotary evaporation. The 1-nitrohexadecane obtained (64.5% yield) is more than 98% pure, as determined by GLPC.

All other chemicals used were reagent grade or better.

Determination Method

Add the sample (20-200 μl of lipid) to a test tube containing 5.0 ml of the ethanol/hydrochloric acid solution (1.0 ml concentrated HCl in 400 ml of 95% ethanol) and a spatula tip (ca. 0.025 g) of 5% Pd on charcoal. Add 0.3 ml of PMHS, and, after mixing, place the tube in a 40-60 C bath for 30 min. After heating, remove the catalyst by filtration, and place the filtrate, along with a 2.0 ml chloroform wash of the test tube, in a 125 ml separatory funnel. Add 50 ml of water, 4.0 ml buffer (125 g KCl, 42.19 g CH_3COONa, and 300 ml of glacial acetic acid, dilute to 1 L), and 2.0 ml of the methyl orange solution (0.05%) to the separatory funnel. Extract the neutral amine-methyl orange salt into the chloroform layer, allow the layers to separate for 3 min, and collect the chloroform layer in a 10 ml volumetric flask. Make two additional chloroform extractions, and add these to the volumetric. Finally, add 2.0 ml of absolute ethanol to clarify the solution, dilute the contents of the flask to the mark with chloroform, and determine the absorbance at 485 nm vs. a chloroform blank. Exercise caution to prevent the inclusion of any of the aqueous layer in the volumetric, since the ethanol added will make it miscible and cause the methyl orange contained in it to interfere.

RESULTS AND DISCUSSION

None of the literature methods for the determination of nitro compounds are satisfactory for the determination of the nitro-β-hydroperoxides produced in the NO_2-initiated autoxidation of PUFA. Many of the literature methods rely upon a reductive quantification of the nitro group (3,9), and hence are unacceptable due to interference from hydroperoxides (which are present in much higher concentration in autoxidation systems and which react readily with reducing agents). The remainder of the methods are either too insensitive, subject to interference from hydroperoxides, or unsatisfactory for the determination of long chain nitro compounds (4-7, 9).

A method described by Sweet et al. (5) appeared well suited to the autoxidation system. It utilizes the oxidation of the nitro compound to form free nitrite ion, which is then determined spectrophotometrically by the very sensitive Griess-Ilosvay method – first used as a nitro compound determination by Bose (10). The Sweet method is sensitive, nonselective (with respect to the location of the nitro group along a carbon chain), and free from interference by hydroperoxides. Sweet found a decrease in the yield of nitrite ion from medium chain nitro compounds such as 1-nitrodecane for his method, but he was able to obtain acceptable yields of nitrite ion from 1-nitrodecane by using higher reagent concentrations. Studies in our laboratories, however, show that neither these conditions nor harsher ones give a reasonable yield of nitrite ion from long chain nitro compounds (9).

Therefore, a new nitro determination method was developed. The method relies upon the reduction of the nitro group to an amine according to the procedure developed by Lipowitz and Bowman for reduction of aromatic nitro compounds (11). In the method, PMHS and Pd on charcoal are reacted with nitro-β-hydroperoxide in a protic solvent, ethanol, and the nitro group is reduced to an amine; hydroperoxides present in PUFA systems are also reduced by these conditions, forming alcohols. The alcohol-amine produced is then determined by an adaption of the dye method developed by Silverstein for the determination of long chain amines (12). The amine is protonated at pH 3.5, reacted with the

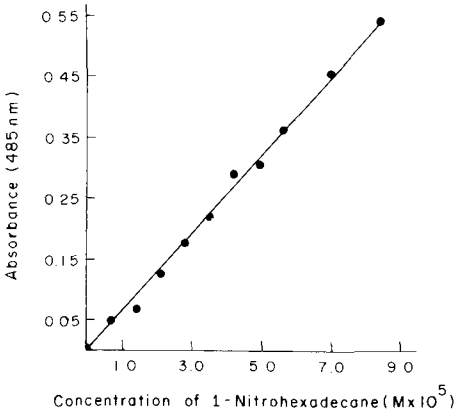


FIG. 1. Absorbance of the methyl orange-amine salt at 485 nm vs. the concentration of 1-nitrohexadecane analyzed.

sulfonic acid anion of methyl orange to form a neutral salt which is extracted into chloroform leaving behind unreacted methyl orange, and the extracted amine-methyl orange salt is determined spectrophotometrically. The nitro-lipid method is sensitive, free from interference by hydroperoxides, and independent of the position of the nitro group along the carbon chain. The latter advantage is particularly important for PUFA systems because, for example, there can be six different nitro-β-hydroperoxides formed from the addition of NO_2 to methyl linolenate. Since the same chromophore is produced for any amine in the nitro-lipid method, the extinction coefficient is merely a measure of the basicity and polarity of the amine. All of the lipid alcohol-amines are essentially identical in these respects, and so give the same extinction coefficient.

Determination of 1-Nitrohexadecane by the Nitro-Lipid Procedure

When samples of 1-nitrohexadecane were quantified by the nitro-lipid procedure, a linear Beer-Lambert plot was obtained (Fig. 1) with positive deviation at high concentrations. Silverstein also found this deviation for all of the amines that he determined (12). A least squares treatment of the linear portion of the plot gave an extinction coefficient of 6,340 M^{-1} cm^{-1} (r = 0.998).

Standardization of the Nitro-Lipid Procedure for the Determination of Nitro-β-hydroperoxides in Lipid Autoxidation Systems

Originally, the nitro-lipid method was to have been standardized for use in the PUFA autoxidation systems by the synthesis of an authentic nitro-β-hydroperoxide or some model compound which would behave identically in the test procedure. This ultimately proved impractical, and standardization was carried out in the following manner. Samples of methyl oleate and methyl linoleate were exposed to very high (70 μg/g) levels of NO_2 until the lipid was ca. 15% reacted. (The samples were reacted to such a great extent to allow determination by the total nitrogen method, which is too insensitive for the determination of normal PUFA autoxidation samples (9)). These samples were then analyzed both by the nitro-lipid procedure and by the total nitrogen method. Total nitrogen analyses were performed by Galbraith laboratories; reported values were stated to be accurate to within 10% of the value given. Each of the samples, although comprised of a different lipid and reacted to a different extent, gave the same extinction coefficient, within experimental error, for the nitro-lipid method (Table I).

Determination of Nitro-β-Hydroperoxide in Authentic PUFA Autoxidation Samples by the Nitro-Lipid Method

The nitro-lipid method was tested using a typical sample of autoxidized PUFA, which was prepared by exposing methyl linoleate to 2 μg/g NO_2 until ca. 5% had reacted. The nitro-lipid in this sample was then determined by our procedure. A plot of these data (Fig. 2) shows that the method is linear over a 10-fold range of

TABLE I

Molar Extinction Coefficients for the Nitro-β-Hydroperoxides Produced from the Autoxidation of Methyl Oleate and Linoleate

Lipid	Nitro-lipid (μmol/ml)[a]	ε (M^{-1} cm^{-1})[b]
Methyl oleate	37.7	2,150
Methyl linoleate	16.8	2,080
		Average = 2,120 ± 80

[a]μmol nitro-β-hydroperoxide/ml lipid, calculated from the total nitrogen analyses carried out by Galbraith laboratories.

[b]Mole extinction coefficient for the nitro-β-hydroperoxide, calculated from the absorbance for each sample and the concentrations determined by Galbraith.

concentration, giving an average extinction coefficient of 2,100 M^{-1} cm^{-1} (r = 0.997) the same value as was obtained in Table I.

Interferences

The nitro-lipid method is subject to the interferences found for the original amine determination, (12) and to interference by any substance reduced to an amine under the test conditions. Interference by amines already present in samples before reduction can be accounted for, however, by subtracting a blank obtained by eliminating the reduction step.

Determination of Other Nitro Compounds

The nitro-lipid method, as described, can only be used for the determination of long chain, nonpolar nitro compounds, since the amine determination used is only satisfactory for long chain compounds. This is because the methyl orange amine determination relies upon the formation of a neutral methyl orange-amine salt that is sufficiently nonpolar to be extracted into chloroform. Short chain and polar amines do form neutral salts, but these are too polar for extraction into chloroform and cannot be determined. The nitro-lipid method can easily be adapted for the determination of short chain and polar nitro compounds, however, simply by using a different amine determination method. For example, the fluorescamine method of Undenfriend et al. (13) could be used to determine a variety of short chain and polar amines at the picomole level.

Conclusion

The nitro-lipid procedure accurately determines micromolar quantities of nitro-β-hydroperoxide produced from the initiation of lipid autoxidation by ppm levels of NO_2 in air, thus providing a measure of the rate of initiation by addition of NO_2. The method can be used for the determination of other long chain, nonpolar nitro compounds, and can be adapted for short chain or polar nitro compounds.

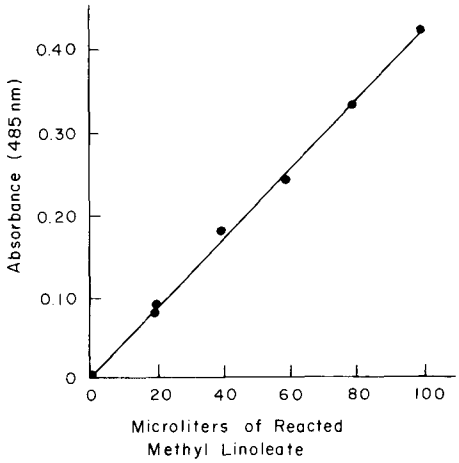


FIG. 2. Determination of nitro-β-hydroperoxide produced from the NO_2 initiated autoxidation of methyl linoleate showing that the response in our test is linear over a 10-fold concentration range.

ACKNOWLEDGMENT

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COMMUNICATIONS

Lipid Composition of SV40-Induced Transplantable Hamster Tumor

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ABSTRACT

The analysis of the lipid composition of the SV40-induced transplantable hamster tumor has shown that: (a) the total phospholipid content in the tumor is lower than that usually found in normal tissues and similar to that reported for other neoplasms; (b) except for a lower percentage of diphosphatidylglycerol, the phospholipid composition is comparable with that reported for normal tissues; (c) the fatty acid profiles of the various glycerophospholipid classes are characterized by high proportions of oleic acid; and (d) phosphatidylethanolamine contains proportions of alkenyl side chains which are consistent with the amounts of plasmalogenic phospholipids found in other neoplasms.

INTRODUCTION

Interest in the chemistry and metabolism of tumor lipids – see Wood for a review (1) – is based on the desire to find correlations between changes in lipid components and biological properties of malignant cells. Among several changes found in tumor lipids by different laboratories, the more consistent ones are: a lowered amount of phospholipids (2-13), the occurrence of appreciable amounts of ether-linked lipids (5,6,10,12,13), and an increase of monoenoic acids associated with a decrease of C_{20} and C_{22} polyunsaturated fatty acids (2,6,8,14-22). It is still uncertain, however, whether these changes represent general trends characteristic of cancer cells or whether they reflect cell-specific differences. Peculiarities of tumor lipids have hitherto been studied largely in hepatomas (2,3,6-8,10,14,16-20), ascites tumors (1,3-5,10,14,17), mammary carcinomas (15), brain tumors (11,21) and virally transformed cell lines (9,12,22). It was, therefore, of interest to study the lipid composition of the transplantable hamster tumor induced by SV40 virus, because, while there is no obvious control tissue for this kind of tumor, it represents a very interesting neoplastic system and one very different from those studied up to now owing to the species of animals used and to the specific promoter of the tumor.

MATERIALS AND METHODS

Tumors, originally induced in newborn Golden Syrian hamsters by subcutaneous inoculation of SV40-infected kidney cell cultures of *Cercopithecus aethiops*, were transplanted for 5 to 10 passages into adult hamsters by Dr. D. Balducci (Itallevamenti, Pianella di Siena, Italy) before being supplied to our laboratory. In our laboratory, tumors were serially passed at intervals of 8-10 days into adult male Golden Syrian hamster weighing between 150-170 g who were fed a commercial stock diet whose lipid composition was reported in a previous paper (14). These animals had free access to food up to the time of the experiment when they were killed by exsanguination under light

TABLE I

Lipid Class Content of SV40 Hamster Tumor[a]

Lipid class	SV40 Hamster tumor	
Esterified cholesterol	3.5 ± 0.24	(1.2)
Free cholesterol	14.2 ± 0.64	(5.0)
Triglycerides[b]	175.0 ± 37.10	(61.2)
Diglycerides[b]	2.4 ± 0.31	(0.8)
Monoglycerides[b]	0.3 ± 0.04	(0.1)
Free fatty acids[c]	7.1 ± 1.60	(2.5)
Total phospholipids[d]	83.6 ± 2.88	(29.2)

[a]The values are means ± SE of seven successive experiments and are expressed as mg/g lipid-free dry weight. Lipid-free dry weight was calculated by difference between dry weight and total lipids determined gravimetrically. Water content represents 83% of wet weight. Figures in parentheses are percentages of total lipids.

[b]The amounts of tri-, di- and monoglycerides were evaluated from the glycerol determination (25) and using molecular weights of 830,546 and 270, respectively.

[c]Determined colorimetrically (26) and using an average molecular weight of 270.

[d]Phospholipid-phosphorus x 25 (27).

TABLE II

Phospholipid Composition of SV40 Hamster Tumor

Phospholipid class	Percent of total lipid-phosphorus[a]
Diphosphatidylglycerol	2.0 ± 0.96
Phosphatidylethanolamine[b]	28.4 ± 4.35
Phosphatidylserine[b]	2.8 ± 0.29
Phosphatidylinositol	3.3 ± 1.28
Phosphatidylcholine	53.8 ± 3.68
Sphingomyelin	8.3 ± 2.18
Lysolecithin	1.4 ± 0.13

[a]Values are means ± SE of four experiments.
[b]Phosphatidylethanolamine and Phosphatidylserine were eluted together from Unisil columns, and then separated by silicic acid-ammonia silicate column chromatography (33).

diethyl ether anaesthesia.

At each passage, from the first to the seventh, tumors deriving from 2-3 hamsters were pooled, freed from necrotic tissue, and homogenized in $CaCl_2$ 0.04%. Lipids were extracted from the homogenates by chloroform/methanol (2:1, v/v) (23) and fractionated into individual lipid classes following chromatographic procedures described previously (10).

Colorimetric methods were used to assay cholesterol (24), glyceride glycerol (25), free fatty acids (26) and phospholipid phosphorus (27). Fatty acid methyl esters of individual lipid classes were prepared and analyzed by gas liquid chromatography as described previously (10).

All the SV40 hamster tumors used throughout these experiments were examined histologically. In our opinion, as well as in that of Dr. Balducci, who provided the original tumor, the histological appearence was that of an undifferentiated carcinoma, with stroma fat scattered throughout the tumorous tissue. This is at variance with the findings of other laboratories which reported that tumors produced by subcutaneous inoculations of SV40-infected monkey kidney cell cultures were undifferentiated sarcomas (28).

RESULTS AND DISCUSSION

Table I shows that the lipids of SV40-induced transplantable hamster tumors were mainly accounted for by the triglycerides and phospholipids; lipids of tumors also contained appreciable proportions of free cholesterol and free fatty acids and small percentages of esterified cholesterol, di- and monoglycerides. The absolute amounts of phospholipids found in SV40 hamster tumors were much lower than in most normal tissues (e.g., liver, kidney, lung) of different animal species (29). The concentrations of free and esterified cholesterol, and free fatty acids of SV40 hamster tumors were of the same order of magnitude as those found in several ascites tumors (5,10) and transplantable solid hepatomas (6,7). Due to the heavy contamination of stroma fat, the large quantities of tumor triglycerides found in the present study are not comparable with the triglyceride concentrations reported in other neoplasms (6,7,10).

The phospholipid composition of SV40 hamster tumors reported in Table II is similar to that found in most normal tissues (29), the only exception being that SV40 hamster tumors showed a diphosphatidylglycerol proportion of 2%, which is lower than that found in normal tissues (29).

Table III lists the fatty acid composition of individual lipid classes from SV40 hamster tumors. In cholesteryl esters, oleic acid represents the major fatty acid, followed by palmitic and linoleic acids, while stearic, palmitoleic and arachidonic acids were minor components. Triglycerides showed a fatty acid pattern similar to that of cholesteryl esters, with the exception of a lower level of arachidonic acid, and free fatty acids contained more stearic acid than triglycerides and cholesteryl esters. In phosphatidylethanolamine there was a higher proportion of stearic acid than of palmitic acid, and there were appreciable amounts of C_{20} and C_{22} polyunsaturated acids. In phosphatidylcholine, there was more palmitic than stearic acid, while stearic acid prevailed in phosphatidylinositol and phosphatidylserine; in sphingomyelin, there were the typical 24:0 and 24:1 fatty acids, and in diphosphatidylglycerol the most prevalent fatty acid was linoleic acid.

The aforementioned characteristics of fatty acid profiles of individual phospholipid classes are similar to those found in normal tissues (e.g., liver) of different animal species, including hamster (30-32). SV40 hamster tumors, however, showed elevated proportions of oleic acid in phosphatidylethanolamine, phospha-

TABLE III

Fatty Acid Composition of the Major Lipid Classes of SV40 Hamster Tumor.
(Values, Expressed as Weight Percent of Total Fatty Acids, Are Means ± SE of Seven Experiments)

Fatty acid[a]	CE[b]	TG	FFA	PE	PC	LPC	PS	PI	DPG	SP
14:0	1.1 ± 0.2	1.5 ± 0.5	1.7 ± 0.4	0.7 ± 0.3	2.2 ± 0.8	1.0 ± 0.3	0.5 ± 0.2	0.3 ± 0.2	1.3 ± 0.4	0.6 ± 0.2
16:0 ald.	---	---	---	6.1 ± 0.4	0.2 ± 0.1	---	---	0.8 ± 0.4	---	---
16:0	15.3 ± 0.6	20.9 ± 1.0	35.4 ± 1.3	9.0 ± 0.7	32.2 ± 1.5	38.2 ± 2.0	8.2 ± 0.5	13.3 ± 2.0	13.9 ± 1.4	69.3 ± 3.8
16:1	5.8 ± 0.7	5.8 ± 0.8	4.8 ± 0.3	3.0 ± 0.2	5.5 ± 0.9	2.7 ± 0.5	2.5 ± 0.3	2.1 ± 0.5	11.8 ± 1.4	0.8 ± 0.2
18:0 ald.	---	---	---	1.1 ± 0.3	0.1 ± 0.1	---	---	---	---	---
18:0	6.7 ± 0.7	3.2 ± 0.8	13.7 ± 1.4	16.3 ± 1.3	7.3 ± 0.5	25.0 ± 1.9	36.3 ± 1.2	32.9 ± 3.1	9.8 ± 0.9	6.9 ± 0.9
18:1[c]	50.8 ± 1.5	44.9 ± 1.4	31.6 ± 2.4	33.5 ± 2.0	28.3 ± 1.3	21.8 ± 2.0	28.7 ± 0.7	24.9 ± 2.5	31.8 ± 1.0	5.8 ± 0.6
18:2	14.6 ± 0.9	20.5 ± 2.8	10.9 ± 1.2	6.0 ± 0.8	12.2 ± 0.9	7.7 ± 1.2	6.4 ± 0.5	7.1 ± 0.7	28.8 ± 1.7	1.8 ± 0.3
18:3	2.7 ± 0.8	2.7 ± 0.2	---	1.4 ± 0.4	2.0 ± 0.3	---	1.1 ± 0.4	1.3 ± 0.4	0.7 ± 0.3	---
20:0	---	---	---	---	---	---	---	0.8 ± 0.3	---	1.3 ± 0.2
20:3	0.4 ± 0.3	---	---	0.8 ± 0.3	0.5 ± 0.2	0.6 ± 0.4	1.9 ± 0.4	1.4 ± 0.6	---	---
20:4	2.6 ± 0.5	0.5 ± 0.2	1.9 ± 0.4	8.4 ± 1.0	4.1 ± 0.6	3.0 ± 1.0	6.9 ± 0.7	9.0 ± 2.3	1.9 ± 0.8	---
20:5	---	---	---	2.1 ± 0.2	0.5 ± 0.1	---	0.8 ± 0.2	1.2 ± 0.6	---	---
22:0	---	---	---	---	---	---	---	---	---	2.5 ± 0.6
22:1	---	---	---	---	---	---	---	---	---	1.5 ± 0.3
22:5	---	---	---	3.6 ± 0.4	0.7 ± 0.3	---	2.3 ± 0.3	0.6 ± 0.2	---	---
22:6	---	---	---	7.4 ± 0.4	2.4 ± 0.7	---	3.5 ± 0.5	1.7 ± 0.1	---	---
24:0	---	---	---	0.6 ± 0.1	1.4 ± 0.3	---	0.4 ± 0.1	0.8 ± 0.5	---	7.5 ± 2.6
24:1	---	---	---	---	0.4 ± 0.2	---	0.5 ± 0.2	1.8 ± 1.6	---	2.0 ± 0.8

[a]Chain length:number of double bonds.

[b]Abbreviations are: CE, cholesteryl esters; TG, triglycerides; FFA, free fatty acids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PS, phosphatidylserine; PI, phsphatidylinositol; DPG, diphosphatidylglycerol; SP, sphingomyelin.

[c]Although the location of double bonds was not determined, octadecenoic acid was reported throughout the text under the name of the more common isomer, oleic acid.

tidylcholine, phosphatidylinositol, phosphatidylserine and diphosphatidylglycerol; these relatively high oleic acid levels are rather unusual in the phospholipid classes of normal tissues with the exception of nervous tissue (30-32). It is worth noting that the percentage of oleic acid in phosphatidylethanolamine and in phosphatidylcholine from SV40 hamster tumors was similar to that found in several poorly differentiated hepatomas (6,8,10). In addition, appreciable proportions of 16:0 and 18:0 alkenyl side chains were detected as dimethylacetals mainly in phosphatidylethanolamine, indicating the presence of plasmalogenic phospholipids in SV40 hamster tumors. From the percentage of alkenyl side chains and the total amount of hydrocarbon chains predicted on the basis of phosphatidylethanolamine-phosphorus content, we calculated the contribution of the plasmalogenic forms of phosphatidylethanolamine from SV40 hamster tumors. According to this calculation, ca. 14% of the phosphatidylethanolamine is accounted for by alkenyl species. This value is comparable to the percentage of alkenyl-phosphatidylethanolamine found in Yoshida hepatoma (10) and Ehrlich carcinoma (13) but higher than that reported in 7794A and 7777 Morris hepatoma (6).

In conclusion, the peculiarities of the lipid composition of the SV40-induced transplantable hamster tumor (i.e., low phospholipid content, high level of oleic acid in glycerophospholipids, and presence of ether-linked lipids) are analogous to major lipid variations found in tumors (e.g., hepatomas) which can be compared to an appropriate control tissue, suggesting that tumor cells exhibit a common change in their lipid make up regardless of the transforming agent or of the tissue of origin.

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Fatty Acid and Molecular Species Composition of Rat Brain Phosphatidylcholine and -Ethanolamine from Birth to Weaning[1]

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ABSTRACT

The fatty acid composition of diacyl phosphatidylcholine and phosphatidylethanolamine from the brain of rats 3, 6, 9, 12, 15, 18, and 21 days old were determined. In phosphatidylcholine, the relative amounts of stearic and oleic acid increased from 25% to 33% while the relative amounts of myristic, palmitic, and palmitoleic decreased from 65% to 50% during this time period. The same pattern was seen in phosphatidylethanolamine with stearic and oleic increasing from 38% to 49% and the shorter chain acids decreasing from 17% to 13%. The polyunsaturated fatty content of phosphatidylcholine was approximately 10% and increased slightly during the first 3 weeks, while the polyunsaturated content of phosphatidylethanolamine decreased from 44% to 37%. The molecular species composition of phosphatidylcholine and phosphatidylethanolamine was determined in brains of rats 3, 6, and 9 days old. The relative amounts of the molecular species remained nearly constant during this time period with phosphatidylcholine containing 35% saturated, 40% monoenoic, 6% dienoic, 11% tetraenoic, 2% pentaenoic, and 5% hexaenoic. Phosphatidylethanolamine contained 1% saturated, 8% monoenoic, 3% dienoic, 40% tetraenoic, 9% pentaenoic, and 37% hexaenoic species. Analysis of the fatty acid composition of the molecular species reveals that in phosphatidylcholine the polyunsaturated fatty acids 20:4 and 22:6 are predominately paired with 16:0, while in the phosphatidylethanolamine these two unsaturated fatty acids are paired with 18:0. Furthermore, dipalmitoyl phosphatidylcholine accounts for approximately 25% of the total molecular species of that lipid.

INTRODUCTION

Previous studies on rat brain lipids have shown that the amount (μmoles/g) of diacyl phosphatidylcholine and phosphatidylethanolamine double during the first three weeks of postnatal development (1), and during that time there are two distinct morphological periods (2). These are characterized by the increase in brain size and outgrowth of axons and dendrites during the first 10 days, while from day 10 to weaning, myelination begins and continues at a rapid rate.

Our interest in phospholipid metabolism in developing rat brain led us to determine the changes in fatty acid composition of diacyl phosphatidylcholine and phosphatidylethanolamine from birth to weaning and determine the molecular species of these lipids prior to the onset of myelination. We were particularly interested in determining whether the fatty acid and/or molecular species composition of phosphatidylcholine and phosphatidylethanolamine changed during the period of rapid growth of the brain prior to myelination. In this period the brain increases in weight at a rate of .065 g/day while the amount of phosphatidylcholine increases at a rate of 1.8 μmoles/day/brain, and phosphatidylethanolamine at a rate of 0.7 μmoles/day/brain (1).

There are reports on the changes in the fatty acid composition of rat brain phosphatidylcholine (3-5), but there are no reports on the composition of diacyl phosphatidylethanolamine or the composition of the molecular species in rat brain before weaning.

REAGENTS AND MATERIALS

All solvents were reagent grade and were mixed in proportion by volume. (^3H) and (^{14}C) acetic anhydride, and Aquasol liquid scintillation fluid were purchased from New England Nuclear (Boston, MA). Labeled compounds were obtained from the supplier at greater than 98% purity and were used without further purification. Fatty acid standards were obtained from the Hormel Institute (Austin, MN).

Experimental Animals and Lipid Extraction

Sprague-Dawley rats from a colony maintained at the University of Arizona College of Medicine, Division of Animal Resources, were maintained on Formulab Chow 5008, Ralston Purina (St. Louis, MO). Brains were removed immediately upon decapitation of unanesthetized animals, frozen on dry ice, and lipid extraction was begun within 30 min of tissue removal.

Extraction of lipids was carried out by the methods of Wells and Dittmer (6), omitting the acidified chloroform/methanol extraction, and nonlipid material was removed by partitioning with a salt solution (7). All lipids were stored in chloroform under nitrogen at -18 C. Phospholipid phosphorus was determined by the

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TABLE I

Fatty Acid Composition in Mole % of Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) and the Resulting 1,2 Diacylglycerol Acetates (DAGA) Produced by Phospholipase C Hydrolysis and Acetylation (Samples from Six-day-old rats)

Fatty acid	PC	DAGA	PE	DAGA
14:0	4.2	3.9	1.4	0.9
16:0	51.1	51.6	14.5	14.5
16:1	7.1	7.0	1.8	1.6
18:0	5.2	5.4	24.7	25.0
18:1	18.1	18.6	9.5	9.9
18:2	1.9	1.8	1.1	1.0
20:4	6.3	6.3	19.0	19.9
22:4[a]	0.9	0.8	4.7	4.7
22:5	---	---	3.0	2.9
22:6	2.8	2.7	18.1	18.2

[a]Tentative identification – fatty acids comprising less than 0.5 mole percent omitted. Values are the mean of at least four determinations.

method of Bartlett (8) after digestion with 70% perchloric acid.

Purification of Diacyl Phosphatidylcholine and Diacyl Phosphatidylethanolamine

Aliquots of the total brain extract were chromatographed on DEAE (Reeve Angel, Clifton, NJ) as described by Rouser et al. (9) to separate choline-containing phospholipids from ethanolamine-containing phospholipids.

The column was eluted in succession with chloroform, chloroform/methanol 12:1, chloroform/methanol 9:1, and chloroform/methanol 4:3. Separation was monitored by thin layer chromatography (TLC) on Silica Gel G using the solvent system chloroform/methanol/H_2O 95:35:4. The chloroform/methanol 12:1 fractions which contained phosphatidylcholine and sphingomyelin were combined and applied to a silicic acid column (CC-4 Mallinkrodt, St. Louis, MO) and the column eluted in succession with chloroform/methanol 6:1, 3:1, and 1:1. Phosphatidylcholine was eluted in the 3:1 fraction and chromatographed as a single spot on TLC.

The chloroform/methanol 4:3 fractions from the DEAE column which contained the ethanolamine phospholipids were combined and treated by a modification of a method described by Wells and Dittmer (10) to convert ethanolamine plasmalogen to 2-acyl phosphatidylethanolamine. Approximately 100 μmoles of lipid were taken to dryness under nitrogen then incubated 30 min at 37 C with 8.0 ml chloroform/methanol/0.025 M aqueous $HgCl_2$ (5:11:4). After cooling to room temperature, 2.0 ml isobutanol, 6.8 ml chloroform, and 1.0 ml H_2O were added. This solution was mixed vigorously then centrifuged at 1800 x g, the upper phase removed, and the lower phase was washed with 4.0 ml methanol/water (1:1). After centrifugation, the lower phase was taken to dryness in vacuum. The residue was dissolved in chloroform and applied to a silicic acid column (CC-7 Mallinkrodt, St. Louis, MO) which was eluted with chloroform, chloroform/methanol (8:1) and (4:1). Those fractions of the 8:1 eluate which contained diacyl phosphatidylethanolamine were combined and chromatographed as a single spot on a TLC plate developed in chloroform/methanol/water (95:35:4). The recovery of diacyl phosphatidylcholine and diacyl phosphatidylethanolamine was 88-92% of the amount of the lipid in the original lipid extract as determined by the method of Wells and Dittmer (10).

Methyl Ester Formation and Gas Liquid Chromatography

The fatty acids of esterified lipids were transmethylated by 0.5 M KOH in MeOH (11), and gas liquid chromatography (GLC) was performed on a Hewlett Packard 102 equipped with a Hewlett Packard 3370A integrator. The instrument was operated isothermally at 180 C using 0.6 x 100 cm columns packed with 15% diethylene glycol succinate on 100/200 mesh Gas Chrom P (Alltech Associated, Arlington Heights, IL).

Formation of Diacylglycerol Acetates

Phospholipids were converted to diacylglycerols by hydrolysis with phospholipase C. Ten to 20 μmoles of the purified lipid were dried under nitrogen and mixed with 2.0 ml diethyl ether and 2.0 ml 0.02 M HEPES (pH 7.3). To this was added 10 I.U. *Bacillus cereus* phospholipase C (Calbiochem, La Jolla, CA) and 10 μl 0.1 M $ZnCl_2$. The reaction mixture was incubated at 37 C for 1 to 2 hr, with frequent mixing and was then extracted twice with petroleum ether.

The crude diacylglycerols were acetylated essentially as described by Kuksis and Marai (12). Labeled acetic anhydride was diluted to a specific activity of 0.2 to 5 μCi/μmole with unlabeled acetic anhydride and a 10-100 fold molar excess added to the diacylglycerol in anhydrous pyridine. The reaction was incubated 1-2 hr at room temperature, then treated with methanol to destroy the excess acetic anhydride. After addition of water, the diacylglycerol acetates were extracted with petroleum ether and purified on thin layer chromatographic plates developed in petroleum ether/diethyl ether (4:1). The 1,2-diacylglycerol

acetates were extracted from the silica gel by the method of Arvidson (13).

Argentation Thin Layer Chromatography of Diacylglycerol Acetates

Separation of the diacylglycerol acetates into molecular species was carried out using a modification of the procedures described by Renkonen (14). Fifty grams of Silica Gel G were slurried into 100 ml H_2O containing 5.0 g $AgNO_3$. The mixture was degassed and spread 250 μ thick on 20 x 20 cm plates. After air drying 30 min in the dark, the plates were dried for 1.5 hr at 120 C. The cooled plates were stored for up to two weeks in the dark over dessicant. Five to 10 μmoles of diacylglycerol acetate were applied to the plate about 1 cm from the bottom, then the plate was developed 10 cm in chloroform/methanol (97:3). The plate wad dried under vacuum 15 min, then developed the full length in benzene/chloroform (9:1). The individual bands of lipids were visualized with ultraviolet light after spraying the plate with 0.02% DCF in methanol and aqueous 0.1% ANS, then scraped from the plate and the lipids eluted from the gel as described above.

Analysis of Diacylglycerol Acetate Fractions

The relative mole percentage of each band was determined by drying an aliquot of the extract in a scintillation vial, then adding 10 ml Aquasol and counting in a Beckman LS-230 or LS-250 liquid scintillation spectrometer. GLC of hydrogenated diacylglycerol acetates from each band was performed following the procedure of Kuksis and Ludwig (15) with a temperature program of 2°/min from 275 C to 315 C using ¼" x 2' columns packed with 2.25% SE-30 on 60/80 mesh Chromabsorb W AW-PMCS (Alltech Associates, Arlington Heights, IL). Carbon numbers were determined by calibration with dipalmitoyl and distearoyl glycerol acetates prepared in the laboratory by K. Yabusaki. The fatty acid composition of each band was carried out as described above.

RESULTS

The validity of the results depends on three factors: 1) the recovery of the diacylphospholipids during purification; 2) the purity of the diacylphospholipids; and 3) conversion of the diacylphospholipids to diacylglycerol acetates without alteration in fatty acid composition. The basis for estimation of recovery was an analysis of the amount of diacylphosphatidylcholine and diacylphosphatidylethanolamine in the original lipid extract (10). At all ages studied, the recovery was 88-92%. The diacylphosphatidylcholine was judged to be pure on the basis of a single spot on TLC and quantitative conversion to glycerophosphorylcholine by mild alkaline hydrolysis (10). The diacylphosphatidylethanolamine also gave a single spot on TLC, contained less than 1% vinyl ether as judged by iodine titration, and was converted in 95-98% yield to glycerophosphorylethanolamine by mild alkaline hydrolysis. Based on these data, diacylphosphatidylethanolamine contained less than 1% plasmalogen and 2-5% of the 1-0-alkyl ether analog.

During phospholipase C treatment of both phospholipids, more than 95% of the phosphorous became water-soluble, suggesting nearly quantitative hydrolysis. As shown in Table I, conversion of both phosphatidylcholine and phosphatidylethanolamine to diacylglycerolacetates proceeds without significant alteration in fatty acid composition. These data show that there is no selectivity in the phospholipase C hydrolysis and no loss of polyunsaturated fatty acids during the acetylation reaction and diacylglycerolacetate purification.

Fatty Acid Composition of Diacyl Phosphatidylcholine and Phosphatidylethanolamine

Tables II and III show the fatty acid composition of diacyl phosphatidylcholine and phosphatidylethanolamine from rat brain during the first 3 weeks of development. These analyses represent quadruplicate determinations. The standard deviation was ± 1% for major components and 0.2% for minor components. Considering the complex nature of changes occurring in brain during this period, it is surprising that the fatty acid composition changes so little. The most striking data in Tables II and III relate to the different amounts of 16:0 and 18:0 in phosphatidylcholine and phosphatidylethanolamine, and to the much higher content of polyunsaturated fatty acids in phosphatidylethanolamine compared to phosphatidylcholine. These differences are maintained throughout development. There is a tendency for the average chain length of both saturated and unsaturated fatty acids to increase during this period.

Molecular Species Analysis

The diacylglycerol acetates derived from phosphatidylcholine and phosphatidylethanolamine were fractionated on the basis of unsaturation to determine the molecular species and fatty acid composition of each species in 6 day old animals. These data are presented in Table IV. Calculation of the fatty acid compo-

TABLE II

Mole Percent Fatty Acid Composition of Diacyl Phosphatidylcholine Isolated from Rat Brain

Fatty acid	Days after birth						
	3	6	9	12	15	18	21
14:0	4.2	4.0	3.9	3.2	2.6	1.6	1.7
16:0	50.5	51.3	53.8	51.7	51.0	50.2	46.7
16:1	9.0	7.7	6.5	5.2	4.8	2.9	3.1
18:0	5.1	5.2	6.0	6.5	7.2	9.5	10.1
18:1	19.8	18.0	18.4	18.7	20.7	22.7	23.0
18:2	1.5	1.9	1.9	2.3	1.9	1.7	2.0
20:3	0.3	0.7	0.4	0.6	0.3	0.6	0.9
20:4	5.6	6.1	6.0	7.0	7.4	6.1	7.0
22:4[a]	0.4	0.8	0.4	0.6	0.7	0.6	0.8
22:6	2.1	2.0	1.7	2.3	2.4	2.3	2.6
% Saturated	59.8	60.5	63.8	61.4	60.8	61.3	58.5
Average chain length	16.03	16.06	16.04	16.11	16.15	16.26	16.29
% Monounsaturated	28.8	25.7	24.9	23.9	25.5	25.6	26.1
Average chain length	17.38	17.40	17.48	17.56	17.62	17.77	17.76
% Polyunsaturated	9.9	11.5	10.4	12.8	12.7	11.3	13.3

[a]Tentative identification – fatty acids comprising less than 0.5 mole percent omitted. Values are the mean of at least four determinations.

TABLE III

Mole Percent Fatty Acid Composition of Diacyl Phosphatidylethanolamine Isolated from Rat Brain

Fatty acid	Days after birth						
	3	6	9	12	15	18	21
14:0	0.9	1.2	0.9	1.0	1.0	1.1	1.0
16:0	14.8	14.5	14.5	13.9	12.8	10.6	11.2
16:1	1.8	1.8	1.6	0.9	0.8	0.7	0.5
18:0	27.1	28.5	29.2	32.5	32.5	31.5	33.9
18:1	10.9	10.6	10.6	11.0	11.5	14.4	14.1
18:2	1.0	1.1	1.3	0.7	1.1	1.4	0.8
20:3	0.5	0.4	0.6	0.4	0.5	1.1	0.4
20:4	18.2	19.5	20.0	19.2	18.6	16.4	15.7
22:4[a]	4.4	4.1	4.1	4.0	4.2	4.6	4.2
22:5	2.8	1.7	1.4	1.1	1.1	1.1	0.9
22:6	16.8	15.6	15.2	15.0	15.0	15.1	15.0
% Saturated	42.8	44.2	44.6	47.4	46.3	43.2	46.1
Average chain length	17.22	17.24	17.27	17.33	17.36	17.41	17.43
% Monounsaturated	12.7	12.4	12.2	11.9	12.3	15.1	14.6
Average chain length	17.72	17.71	17.74	17.85	17.87	17.91	17.93
% Polyunsaturated	44.5	43.4	43.2	40.7	41.4	41.7	39.3

[a]Tentative identification – fatty acids comprising less than 0.5 mole percent omitted. Values are the mean of at least four determinations.

sition of phosphatidylcholine and phosphatidylethanolamine in the unfractionated sample based on the data of Table IV gave values which agreed with those of Tables II and III within 10%; thus, no preferential loss of any fatty acids occurred during molecular species analysis. The same analysis was carried out on the lipids from 3- and 9-day-old animals, but the data are essentially identical to that obtained for the 6 day rats and are not presented.

The fatty acid composition of the various bands do not always correspond to an integral number of double bands. This is especially true in bands 2 and 5 for phosphatidylcholine and band 2 for phosphatidylethanolamine. These are minor constituents and may not be pure, or the particular pairing of fatty acids may lead to the observed separation. However, the separation was quite reproducible with all samples.

The molecular species composition of phosphatidylcholine and phosphatidylethanolamine reflect the overall fatty acid composition

of the lipid. Thus, the species of phosphatidylcholine which contained 0 or 1 double bond account for over 75% of the total and the polyunsaturated species of phosphatidylethanolamine containing 4, 5, or 6 double bonds account for over 85%. A striking feature of the molecular species analysis is the high palmitate content of the saturated species of phosphatidylcholine indicating that the major saturated species must be dipalmitoyl phosphatidylcholine.

Further information on the molecular species composition are given in Table V where the intact diglyceride acetates from each band are fractionated after hydrogenation on the basis of the number of carbons in the acyl chains. The major species of band 4 of phosphatidylcholine contains 36 carbon atoms showing that 20:4 is paired with 16:0, and in band 6 the major species has 38 acyl carbons showing 22:6 to also be paired with 16:0. These same bands from phosphatidylethanolamine show the major species of 20:4 to have 38 carbon atoms while 22:6 has 40 carbon atoms showing that these acids are paired with 18:0. Over 70% of the saturated species contain 32 acyl chain carbon atoms and, from the fatty acid composition of that band shown in Table IV, the only molecular species which can have 32 carbon atoms is palmitoyl-palmitoyl. Therefore, one can calculate that ca. 25% of the total molecular species of phosphatidylcholine is dipalmitoyl phosphatidylcholine.

DISCUSSION

Although the brain undergoes gross morphological changes in the cellular structures which contain lipids, the fatty acid composition of two major lipid components do not show a striking change. Our data on the fatty acid composition and changes in acyl content of phosphatidylcholine from whole rat brain are comparable to previous reports on whole brain (3), cerebrum (4,5) and brain microsomes (16). The high content of polyunsaturated fatty acids in diacylphosphatidylethanolamine is similar to other reports on the total ethanolamine phosphoglycerides of brain.

The high amount of dipalmitoyl phosphatidylcholine is of interest because appreciable quantities of this molecular species have been found only in erythrocytes (17), brain (18), and lung (18,19). In lung, dipalmitoyl phosphatidylcholine is the major lipid in the lung surfactant (2) while in brain a species containing 32 acyl carbon atoms has been found in nerve endings and 15,000 x g supernatant (30%), mitochondria (20%), and myelin (10%)

TABLE IV

Fatty Acid Composition in Mole % of the Molecular Species of Phosphatidylcholine and Phosphatidylethanolamine from Developing Rat Brain at 6 Days after Birth

	Band[a]	Mole%	14:0	16:0	16:1	18:0	18:1	18:2	20:4	22:4	22:5	22:6	Average No. double bonds
Phosphatidylcholine	0	36.1	12.4	81.3		6.3							0
	1	40.8	2.8	43.4	13.9	3.7	36.2						1.0
	2	5.8	6.8	25.0	14.3	6.2	28.9	18.8					1.6
	4	10.3	1.8	35.7		13.8	2.3		46.4				3.8
	5	2.2	4.4	20.9	6.6	4.7	22.1		18.3	6.5	16.5		4.2
	6	4.8	3.0	31.1		8.6	6.3		3.6			45.0	5.9
Phosphatidylethanolamine	0	1.1	24.6	45.1		30.2							0
	1	8.6	5.3	29.3	9.6	17.2	38.5						1.0
	2	3.8	7.6	13.7	9.5	10.5	45.5	13.2					1.6
	4	41.0	1.3	9.6	1.5	34.1	1.9		37.6	14.0			4.2
	5	8.9	2.6	11.7	2.9	13.7	19.5		19.4	9.6	20.5		5.0
	6	36.6	1.4	17.7	1.9	20.9	6.5		3.7	2.5	1.9	42.7	6.0

[a]Band number refers to the number of double bonds found in the predominate species.

TABLE V

Mass Distribution by Carbon Number[a] of the Molecular Species of Phosphatidylcholine and Phosphatidylethanolamine from Developing Rat Brain at 6 Days after Birth

	Band[b]	Mole[b]	% 28	% 30	% 32	% 34	% 36	% 38	% 40	% 42	% 44
Phosphatidylcholine	0	36.1	1.8	15.4	72.4	10.4					
	1	40.8		2.0	25.1	65.9	7.0				
	2	5.8		3.3	14.9	48.4	33.4				
	4	10.3				3.3	55.1	36.7	4.9		
	5	2.2					19.6	46.1	29.1	5.2	
	6	4.8					5.9	60.6	28.7	4.8	
Phosphatidylethanolamine	0	1.1		16.1	28.7	55.2					
	1	8.6		10.1	10.5	79.4					
	2	3.8		4.5	20.2	70.3	5.0				
	4	40.9				1.4	16.0	72.5	8.6	1.5	
	5	8.9					6.8	58.1	32.0	3.1	
	6	36.5					0.8	37.0	54.9	5.3	2.0

[a]Carbon number refers to the number of fatty acyl carbon atoms.

[b]Band number refers to the number of double bonds found in the predominant species.

(20). Although there is no information concerning the role of dipalmitoyl phosphatidylcholine in the brain, it is interesting to note that the tissues in which appreciable amounts of this species are found are involved in oxygen uptake, oxygen transport, and high oxygen utilization.

During the period from 3 to 9 days, the fatty acid and molecular species composition of phosphatidylcholine and phosphatidylethanolamine do not change significantly. There is a net synthesis of 11 μmoles/brain of phosphatidylcholine and 4 μmoles/brain of phosphatidylethanolamine in this time period, yet the striking difference between the molecular species composition of these lipids are maintained. These results raise some interesting questions about the biosynthesis of these compounds in brain. The predominant pathway involves a common intermediate, diacylglycerol. Whether the CDP-choline or CDP-ethanolamine transferases have the required specificity to generate such differences in molecular species composition or whether some form of compartmentation exists are unknown. Other reactions such as deacylation-reacylation could also be involved but have not been studied in detail in brain. Based on the results reported here, it would appear that a detailed investigation of the de novo synthesis of phosphatidylcholine and phosphatidylethanolamine in developing rat brain would provide some new insights into the control of the fatty acid composition of phospholipids.

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Differential Alteration of Δ9 and Δ6 Desaturation of Fatty Acids in Rat Brain Preparations in vitro

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ABSTRACT

Developing rat brain has the capacity for either Δ9 or Δ6 desaturation of fatty acids. In liver, evidence supports the existence of separate enzymes for each reaction, but it is not known whether in brain Δ9 or Δ6 desaturation of saturated fatty acids involves distinct enzymes. We have used fatty acids, including the cyclopropene fatty acid, sterculic acid, to alter desaturation activities with substrates that are desaturated predominantly in the Δ9 position or in the Δ6 position. In addition, differential alteration of desaturation of palmitic acid, a substrate that can be desaturated in either the Δ9 or Δ6 positions by brain preparations from neonatal rats, was examined. Sterculate reduced Δ9 desaturation of palmitate 80-90% but reduced Δ6 desaturation only 35%. In contrast, linoleic acid preferentially reduced Δ6 desaturation of palmitate. Thus, Δ9 desaturation of saturated fatty acids appears to be catalyzed by an enzyme or enzyme site distinct from that for Δ6 desaturation. Accordingly, these activities may be independently regulated during crucial stages of brain development.

INTRODUCTION

The developing brain has a significant capacity, particularly in the neonatal period, for desaturation of the essential fatty acids, linoleic (18:2(n-6)) and linolenic (18:3(n-3)) acids (1) and of the saturated fatty acids, palmitic (16:0) and stearic (18:0) acids (2,3). Analyses of the positions of the inserted double bond have shown that with 18:2(n-6) and 18:3(n-3) as substrates, desaturation occurs almost exclusively at the Δ6 position; with saturated fatty acids as substrate, desaturation at the Δ9 position generally predominates but with fetal and neonatal rat brain preparations, as much as 60% of the observed monoene formation from 16:0 may be from Δ6 desaturation. On the basis of observed developmental changes, we postulated (3) that Δ6 desaturation activity with 16:0 may be due to the enzyme system that primarily desaturates essential fatty acids but which also has activity with saturated acids.

In liver, evidence from several experimental approaches supports the existence of separate enzymes for Δ9 and Δ6 desaturation. A number of dietary, hormonal, physical and chemical manipulations alter the activities of Δ9 and Δ6 desaturations differently. For example, high carbohydrate stimulates Δ9 but decreases Δ6 desaturation, whereas high protein stimulates Δ6 and has relatively little effect on Δ9 desaturation (4). However, to compare Δ9 and Δ6 activities of brain using similar approaches is not practicable as lipid synthesizing enzymes of developing and mature brain are not altered significantly by such dietary and hormonal changes that markedly alter liver activity (5,6). Accordingly, an alternate approach is necessary to permit comparison of these enzymes in brain.

Alteration of desaturation activities with both saturated and polyunsaturated fatty acids by other fatty acids that compete for or interfere with desaturation processes has been demonstrated with preparations from liver (7-10) but not with brain preparations in vitro. The cyclopropene fatty acid, sterculic acid, has been shown to be a highly specific inhibitor of Δ9 desaturation in numerous systems in vitro (11-14) and in vivo (15-17). In the present study, we have tested sterculate and other fatty acids as modulators of Δ9 and Δ6 desaturation by rat brain in vitro to determine whether differential alteration of activities could provide further evidence that distinct enzyme sites exist for these two reactions in the central nervous system.

MATERIALS AND METHODS

Materials

[1-^{14}C] Stearoyl-CoA, and linoleic acid (50-60 mCi/mmol) were obtained from NEN Canada, Lachine, Canada and [1-^{14}C] palmitic and linolenic acids (50-55 mCi/mmole) from Amersham, Don Mills, Ontario, Canada. Radiopurity determined by argentation thin layer chromatography (TLC) and radio gas liquid chromatography (GLC) of the methyl esters (3) was more than 98% for all acids. Sterculate methyl ester and dihydroxysterculic acid were obtained from Supelco, Inc., Bellefonte, PA. The sterculate methyl ester was converted to the free acid by saponification and acidification observing the precautions outlined by James et al. (11). Other fatty acids and lipid standards were obtained from Serdary Research laboratories, London, Ontario, Canada; Supelco, Inc.,

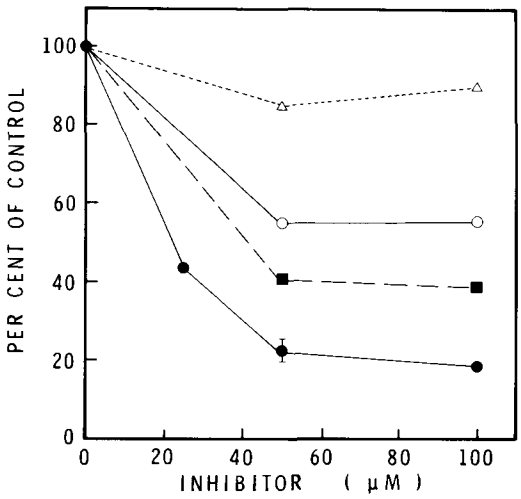

FIG. 1. Alteration of desaturation in brain homogenates by sterculic and dihydrosterculic acids. Various concentrations of the test acids were preincubated for 5 min with cofactors and rat brain preparation before initiation of the desaturation assay by addition of substrate: Δ------Δ sterculic acid with 25 μM [1-^{14}C]-18:2(n-6); o———o dihydrosterculic acid with 50 μM [1-^{14}C] stearoyl-CoA; ■———■ sterculic acid with 50 μM [1-^{14}C] palmitic acid; ●———● sterculic acid with 50 μM [1-^{14}C] stearoyl-CoA. The bar represents standard deviation for 9 separate determinations. All other values are the average of duplicate determinations.

Bellefonte, PA; or Applied Science Laboratories Inc., State College, PA. CoA and pyridine nucleotides were obtained from Sigma Chemical Company, St. Louis, MO.

Wistar rats of either sex from our own inbred colony were used. Homogenates and microsomes were prepared as described previously (1). Protein content was determined by the method of Lowry et al. (18).

Methods

Unless otherwise specified, the inhibitory fatty acids were suspended in 0.05% Triton WR1339 and were preincubated with 0.2 mM Coenzyme A, 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM NADH, 0.1 M phosphate buffer at pH 7.4 and 1 to 3.5 mg of homogenate or microsomal protein for 5 min at 37 C. Control tubes, without the test fatty acid present but with equivalent amounts of all other components, were preincubated similarly. The desaturation assay was then initiated by addition of [1-^{14}C]-fatty acid substrate (50 μM for stearoyl-CoA and palmitate and 25 μM for all other substrates) suspended in 0.05% Triton WR1339. After 10 min, the reaction was stopped with KOH in methanol, the reaction mixture was saponified, acidified and extracted, and the fatty acids were methylated with diazomethane. Fatty acid methyl esters were separated on argentation TLC, and the product was quantitated by liquid scintillation counting. Details of the assay have been published previously (1).

For quantitation of monoenoic isomers formed from [1-^{14}C] palmitic acids, incubations were for 30 min. The monoene fraction was isolated by argentation TLC, checked for purity by radio GLC, and subjected to periodate-permanganate oxidation. Propyl esters of the [^{14}C] dicarboxylic acids formed as oxidation products were quantitated by radio GLC. This procedure was described in detail elsewhere (3).

TABLE I

Alteration of Desaturation of Various Substrates by Sterculic Acid

Substrate	Substrate concentration (μM)	Reduction[a] %
18:0 CoA	25	95.1
	50	87.3 ± 10.2[b]
16:0[c]	25	62.0
	50	58.7
16:0[d]	25	46.3
	50	27.5
18:2(n-6)	12	24.4
	25	15.2
18:3(n-3)	30	5.2
20:3(n-6)	25	35.6
	50	7.7

[a] $100 - \left[\left(\frac{\text{rate with 50 } \mu M \text{ sterculic acid present}}{\text{rate with no inhibitor acid present}}\right) \times 100\right]$.

[b] Mean ± S.D. for 9 determinations. Other values are means of duplicate determinations.

[c] Incubated with brain homogenates from 10-day-old rats.

[d] Incubated with brain homogenates from newborn rats (less than 36 hr).

RESULTS

Under the conditions used for the assay of desaturation activity, the amount of product formed was directly proportional to time and protein concentration for all substrates except palmitate; for the latter, 30 min incubations were used to ensure adequate generation of monoene for further isomer analysis. Coenzyme A, ATP, $MgCl_2$ and NADH were required for desaturation of the fatty acid substrates. Using stearoyl-CoA as substrate, it was shown that all of these cofactors were necessary for modulation of desaturation activity by the competitor

fatty acids, even when the substrate was in the activated form. Whole brain homogenates were used as the preferred source of desaturase enzyme since microsomes contained only 30% of total homogenate activity with neonatal brain (1); however, in cases where microsomes were tested, results obtained were similar to those observed with crude homogeantes.

Inhibition by Sterculic and Dihydrosterculic Acids

Desaturation of stearoyl-CoA and palmitic acid by rat brain homogenate was markedly reduced by 50 μM sterculic acid (Fig. 1). In contrast, desaturation of linoleic acid was lowered less than 25% at all concentrations of sterculate tested. Dihydrosterculate, the cyclopropane analogue of sterculate, was approximately half as effective as modulator of stearoyl-CoA desaturation producing less than 45% reduction in activity, even at 100 μM. Sterculate inhibition of desaturation of stearoyl-CoA and palmitic acid was greater at lower concentrations of substrate (Table I). Reduction of palmitate desaturation activity was appreciably greater with brain preparations from 10-day-old rats than with those from neonatal rats. Inhibition of desaturation of linoleic, linolenic or icosatrienoic acids by sterculic acid was much less than inhibition of desaturation of the saturated acids, even at low concentrations of substrate.

Modulation by Linoleic Acid

As sterculate and dihydrosterculate were not effective in altering the activity of desaturation of linoleic or linolenic acids, we investigated whether other acids might be more useful with these substrates. Linoleic acid was an effective inhibitor of desaturation of linolenic acid (Fig. 2A). Preincubation of the enzyme and cofactors in the absence of 18:2 depressed overall desaturation activity by as much as 50% at 37 C and 40% at 30 C, but it also appreciably enhanced the observed reduction of desaturation in the presence of 18:2 (Fig. 2B).

To ensure that the alteration of desaturation observed with unsaturated fatty acids as substrates was not due to impurities such as peroxides or other oxidation products, two suspensions of 18:2(n–6) from different suppliers were freshly prepared. In all cases, precautions were taken to avoid oxidation. Results with all preparations were similar to those shown in Figure 2.

Differential Alteration of Palmitic Acid Desaturation

When palmitic acid was incubated with brain homogenates from newborn to 3-day-old rats, 35-40% of the monoenoic product formed had the double bond in the Δ6 position (Table II). Sterculic acid specifically reduced Δ9 desaturation of palmitate by 80-90%, whereas Δ6 desaturation was lowered less than 35% under these conditions. In distinct contrast, when linoleic acid was used as modulator, Δ6 desaturation of palmitate was preferentially lowered, although a significant reduction of Δ9 desaturation also occurred.

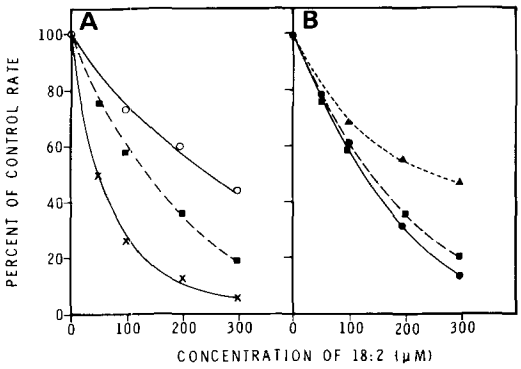

FIG. 2. Effect of substrate concentration and preincubation on the alteration of [1-^{14}C]18:3(n-3) desaturation by 18:2(n-6). A. Various concentrations of the test acid 18:2(n-6) were preincubated at 30 C for 5 min with cofactors and brain homogenate from 3-4-day-old rats. Desaturation assays were initiated by addition of: X———X, 4 μM [1-^{14}C]18:3(n-3); ■———■ 25 μM [1-^{14}C]18:3(n-3); o———o 50 μM [1-^{14}C]18:3(n-3). B. 18:2(n-6) was preincubated with brain preparation and cofactors for 5 min at 37 C (●———●) or 5 min at 30 C (■——■) before initiation of the assay by addition of 25 μM [1-^{14}C]-18:3(n-3). For ▲- - -▲, there was no preincubation of enzyme and test acid; i.e., reaction was initiated by addition of the enzyme preparation to a mixture of the cofactors, test acid and substrate acid.

DISCUSSION

Desaturation of fatty acid substrates in vitro involves both activation of the fatty acid to fatty acyl-CoA derivative and an oxygen- and reduced pyridine nucleotide-dependend desaturation. Although slight reduction of activation may occur in our assay system, the alterations measured in this study occur primarily at the desaturation step. This clearly must be the case with stearoyl-CoA as substrate and must also be responsible for the shift in monoenoic products formed from palmitate in the presence of sterculate and linoleate. Several previous studies in our laboratory have indicated that acyl activation is a relatively rapid process in developing brain and is not rate limiting to desaturation under most circumstances (1,19).

TABLE II

Differential Alteration of Desaturation of Palmitic Acid in the Δ6- or Δ9-Position

Test acid	Concentration (μM)	Total product pmoles/mg[a]	Δ6-Desaturation pmoles/mg[a]	Δ6-Desaturation Reduction %	Δ9-Desaturation pmoles/mg[a]	Δ9-Desaturation Reduction %
None	0	357 ± 64[b] (14)	131 ± 20 (14)	0	226 ± 55 (14)	0
Sterculic acid	50[c]	37	30	37	7	91
	100	152	96	27	56	75
	200	140	87	34	50	78
18:2(n-6)	50	150	33	75	117	48
	100	114	17	87	97	57
	200	95 ± 7 (8)	11 ± 2 (8)	92	84 ± 12 (8)	63

[a]In each case, incubation of substrate in the presence of enzyme, cofactors and the test acid was continued for 30 min to allow optimal accumulation of product for further analysis. Values are expressed as product formed in 30 min.

[b]Mean ± S.D. for the number of independent determinations indicated in parentheses. All others are averages of duplicate determinations.

[c]For this experiment only, brain homogenate was preincubated with inhibitor for 5 min. Total product with preincubated control was 126 pmoles/mg. In all other cases, test acid, substrate, and cofactors were premixed and reaction was initiated by addition of the enzyme preparation.

On the other hand, it is evident that activation of the competitor fatty acids was required for them to be effective as modulators. Preincubation of the test acid, enzyme preparation, and cofactors before initiation of the desaturation assay apparently allowed sufficient activation of the competitor fatty acid and possibly permitted interaction of the fatty acyl-CoA with the desaturation site of the enzyme before addition of [1-^{14}C] substrate.

The cyclopropene fatty acid, sterculic acid, is known from many studies to be an effective inhibitor of Δ9 desaturation and to increase stearic acid and reduce oleic acid levels in tissues of many species (11-17). Clearly, the Δ9 desaturation activity of brain is strongly reduced by sterculate, apparently by competitive inhibition, whereas Δ6 desaturation is reduced only slightly. The slight inhibition of Δ6 desaturation that we observed may be due to nonspecific or detergent action of sterculate or its CoA thioester (13). It appears that sterculate does not cross the blood-brain-barrier at detectible levels (17). However, despite the possibility that inhibition of Δ9 desaturation may be of minor significance for brain in vivo, sterculate is a valuable probe for differential alteration of desaturation in vitro as demonstrated in this study.

The formation of monounsaturated fatty acids by oxidative desaturation in brain involves enzymatic reactions that introduce double bonds at the Δ9 and Δ6 positions (3,20). These desaturation reactions coupled with chain elongation and β-oxidation can account for all observed monoenoic isomers in developing and mature brain. In an attempt to determine whether the processes of Δ9 and Δ6 desaturation involved in monoene formation in brain might be regulated independently, we have demonstrated that Δ9 and Δ6 desaturation can be differentially altered. The cyclopropene acid, sterculate, reduced Δ9 desaturation to a much greater extent than Δ6 desaturation; with linoleic acid as a modulator, total monoene formation was similarly reduced, but in contrast, Δ6 desaturation was preferentially reduced. Thus, it appears that Δ9 desaturation of saturated fatty acids is catalyzed by an enzyme or enzyme site distinct from that for Δ6 desaturation.

The relative importance of formation of different monoenoic isomers as the brain develops is not clear. Isomer analyses of the fatty acids of brain at various ages have shown that, in addition to monoenes formed by Δ9-desaturation and chain elongation (the (n-9) and (n-7) families), large quantities of monoenes formed by Δ6-desaturation and elongation (the (n-10) and (n-12) families) accumulate in fetal and newborn brain and even in adult animals account for ca. 12% of the 16-carbon monoene (19). How isomer composition affects the structure and function of brain membranes remains obscure, but it seems probable that appropriate balance of these isomers may be required for normal development and maintenance of function in the brain. The fact that the two desaturation systems involved in their formation can be differentially altered in vitro, supports the possibility that in vivo these enzyme activities may be independently regu-

lated during crucial stages of brain development to modulate the types of monoenoic isomers available for membrane synthesis.

Whether Δ6 activity observed with saturates is due to the same enzyme system that acts on essential fatty acids, as previously suggested (3), must await further clarification. However, evidence does indicate that this may be so. For example, Δ6 activity with either saturated (3) or unsaturated (1) fatty acid substrates is greatest in the fetal and newborn period and the decline in activity with both classes of substrate after 5 days of age is much greater than that observed for the Δ9 system (5). In addition, the data from the present study are compatible with this since the response of Δ6 desaturation of palmitate to sterculate and linoleate was as predicted from the investigations with linolenate as substrate for desaturation. Thus, it seems probable that these activities may reside in the same enzyme, but more direct investigations are required to establish this conclusively.

To summarize, using in vitro measurement of fatty acid desaturation by developing rat brain, we have shown that several fatty acids can be used to alter Δ9 and Δ6 desaturation differentially. This suggests that Δ9 desaturation of saturated fatty acids may be catalyzed by an enzyme or enzyme site distinct from that for Δ6 desaturation. Accordingly, these activities may be independently regulated during crucial stages of brain development.

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Lipid Oxidation Products and Chick Nutritional Encephalopathy

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ABSTRACT

Safflower oil and its distilled methyl esters were thermally oxidized and fed to young chicks in a vitamin E deficient diet. At a dietary level of 10%, the oxidized lipids caused more severe nutritional encephalopathy (NE) than the unoxidized methyl esters, indicating that factors other than dietary linoleic acid and vitamin E affect the development of NE. A polar lipid extract from oxidized methyl esters accelerated the induction of NE, as did the synthetic methyl esters of keto-octadecenoic and keto-octadecadienoic acids. Dicumarol exerted a protective action against NE. The possibility is discussed that conjugated keto-polyenoic fatty acids, provided by oxidized oils or formed endogenously in vitamin E deficiency, may play a role in causing NE.

INTRODUCTION

Nutritional encephalopathy (NE), better known as encephalomalacia, is induced in young chicks by diets deficient in α-tocopherol and containing polyunsaturated fatty acids (1). The disease is characterized by degenerative changes mainly in the cerebellum, accompanied by ataxia, prostration, and death. Ultrastructural changes in the cerebella of chicks affected with NE have been described by various authors (for a brief review, see ref. 2). The dietary lipid causing NE is linoleic acid or its esters, whereas derivatives of linolenic acid are inactive (3-5).

Autoxidized polyunsaturated oils have occasionally been used to induce NE in chicks (4,5), and we found thermally oxidized safflower oil to be very effective for that purpose (2,6,7). The α-tocopherol level of such oil is greatly reduced while the linoleic acid content is still high. Since the treatment of the oil results in the accumulation of oxidation products, the question arises whether or not some of these products may play an active role in causing NE. The evidence presented in this report points in this direction.

MATERIALS AND METHODS

Animals and Feeds

Day-old crossbred New Hampshire X White Leghorn male chicks were housed in thermostatically heated battery brooders with raised wire floors and had free access to water and feed.

The compositions of the two vitamin E deficient diets are presented in Table I. The diets contained 4 or 10% lipids, and the composition was adjusted so as to ensure a constant ratio of metabolizable energy to protein. The linoleic acid in these diets was provided by distilled safflower methyl esters or by thermally oxidized safflower oil or methyl esters, prepared as described below. The diets contained 0.005%, 2,6-di-*tert.*-butyl-4-methylphenol (BHT), a level of antioxidant which ensured the stability of the dietary linoleic acid and α-tocopherol for over a week at room temperature. During the experiments, the diets were kept at -18 C and dispensed daily.

Dietary Lipids

Refined edible safflower oil was purchased

TABLE I

Composition of Diets

Ingredient	Percent	
Lipid[a]	4.00	10.00
Extracted soybean meal	52.00	56.00
Cellulose	1.00	3.00
D,L-methionine	0.14	0.15
Mineral mix[b]	4.00	4.00
Vitamin mix[c]	0.50	0.50
BHT[d]	0.005	0.005
Glucose monohydrate	38.36	26.35

[a]Safflower oil or safflower methyl esters.

[b]Supplying per kg feed: dicalcium phosphate 28 g; limestone 7 g; NaCl 3.5 g; $MnSO_4 \cdot H_2O$ 370 mg; $ZnCO_3$ 145 mg; ferric citrate 165 mg; $CuSO_4 \cdot 5H_2O$ 11.8 mg; KI 2.35 mg; and $CoCl_2 \cdot 6H_2O$ 1.21 mg.

[c]Supplying per kg feed: vitamin A 3000 U; vitamin D_3 200 U; menadione sodium bisulfite 1 mg; thiamine 3.6 mg; riboflavin 7.2 mg; Ca pantothenate 20 mg; niacin 55 mg; pyridoxine 6 mg; biotin 0.2 mg; folic acid 2.4 mg; vitamin B_{12} 0.02 mg-choline chloride 1.3 g. These amounts were premixed with 3.6 g glucose monohydrate.

[d]2,6-di-*tert.* butyl-4-methylphenol.

[1]Mention of firm names or trade products does not imply endorsement or recommendation by the Department of Agriculture over other firms or similar products not mentioned.

TABLE II

Effect of Thermally Oxidized Safflower Oil (SO-24)[a] and Freshly Distilled Safflower Methyl Esters (ME-0) on Nutritional Encephalopathy[b]

Dietary lipids	Analysis of lipids		Incidence per 20 chicks	
	α-Tocopherol	Linoleic acid	Ataxia	Mortality
	μg/g	%		
			At 3 weeks	
SO-24 4%	10-20	60-65	7	5
ME-SO 4%	5-12	70-76	15	9
			At 2 weeks	
SO-24 10%			12	11
ME-0 10%			8	1

[a]Safflower oil aerated at 145 C for 24 hr.

[b]The lipids were fed from hatching at the levels indicated.

from Shemen Ltd., Haifa and from Teth-Beth Ltd., Petah-Tikva. Different batches contained from 71 to 76% linoleic acid and ca. 350 μg α-tocopherol/g. Methyl esters were prepared from the oil by a modification of the transmethylation procedure of Hartman (8), followed by vacuum distillation.

Thermal oxidation of safflower oil and distilled methyl esters was done by heating batches of 1-2 kg to 145 C ± 2 C under a stream of air (0.5 l/min). The length of the thermal treatment was 24 hr for the oil and 3 hr for the esters. The following abbreviations were used: SO-24 for the oxidized oil; ME-3 for the oxidized methyl esters; and ME-0 for the fresh methyl esters.

A crude extract of polar lipids was prepared from thermally oxidized methyl esters by repeated partition between hexane and 90% (v/v) ethanol, using six separatory funnels arranged in countercurrent fashion. The final ethanolic extract was concentrated under reduced pressure and extracted with ethyl ether. The yield of polar lipids averaged 6%.

Methyl esters of conjugated keto-octadecenoic acid and keto-octadecadienoic acid were prepared from methyl oleate and linoleate, respectively, as described elsewhere (9). The oleate-derived product contained 96% conjugated keto esters consisting of an isomeric mixture of methyl 8-, 9-, 10-, and 11-oxo-octadecenoate. The linoleate-derived product contained 91% keto-dienes consisting mainly of methyl 13-oxo-9,11- and 9-oxo-10,12-octadecadienoate.

For testing in chicks, lipid fractions or synthetic products were dissolved in safflower methyl esters in the amounts indicated.

Quantitative Expression of NE

Chicks were inspected twice daily and the times at which the first signs of ataxia were observed and when death occurred, were recorded. Inspection of the cerebella always confirmed that the affected chicks were stricken with NE. Results were expressed as number of chicks affected per total number of chicks per treatment at the age indicated. Alternatively, curves representing the cumulative incidence of ataxia or death have been plotted.

Analytical Determinations

α-Tocopherol in the dietary lipids was determined by saponification, fractionation of the unsaponifiables by thin layer chromatography on Silica Gel G with hexane/ethyl ether (8:2), and colorimetric reaction of the α-tocopherol fraction with ferric chloride and bathophenanthroline (10).

The fatty acid composition of the lipids was determined after transmethylation of the samples with 3% (w/v) H_2SO_4 in methanol at reflux temperature for 1 hr and extraction of the methyl esters with hexane. The esters were submitted to isothermal gas liquid chromatography at 180 C on Gas Chrom W coated with 15% DEGS. All materials were obtained from Packard Ltd., Jerusalem. Methyl esters prepared for feeding experiments were injected directly into the chromatograph. Glyceryl triheptadecanoate and methyl heptadecanoate were added as internal standards to the oil and methyl ester samples, respectively, for calculation of the true linoleic acid content of the oxidized samples.

RESULTS

In a first trial, two encephalopathogenic diets were compared: thermally oxidized safflower oil, SO-24, and freshly distilled

TABLE III

Effect of Fresh and Thermally Oxidized Safflower Methyl Esters on Nutritional Encephalopathy

Dietary lipids[b]	Analysis of lipids		Vitamin E[a] added to feed	Incidence per 20 chicks at 19 days	
	α-Tocopherol	Linoleic acid		Ataxia	Mortality
	μg/g	%	μg/g		
ME-0	5.0	75.9	0	14	6
			1	13	4
ME-3	0.35	68.5	0	18	14
			1	19	14

[a]D,L-α-Tocopheryl acetate.

[b]ME-0, freshly distilled safflower methyl esters; ME-3, methyl esters aerated at 145 C for 3 hr. The lipids were fed as 10% of the diet from the 8th day, after the chicks received 4% ME-0 during the first week.

safflower methyl esters, ME-0. The oxidized oil had less linoleic acid but no less α-tocopherol than the fresh esters (Table II). When these lipids were fed as 4% of the diet, ME-0 caused a greater incidence of ataxia and mortality than did SO-24. However, at the 10% level, the oxidized oil was more active than the fresh methyl esters.

A similar comparison was made between fresh and oxidized methyl esters, ME-0 and ME-3. Table III shows that ME-3, with less linoleic acid, nevertheless produced a more severe incidence of NE, compared to ME-0. This greater activity of ME-3 was not due to the lower α-tocopherol content of the oxidized vs. the fresh esters, since the difference in tocopherol content between the two diets was no more than 0.5 μg tocopherol/g diet, whereas even the addition of 1 μg DL-α-tocopheryl acetate/g diet had virtually no effect on NE (Table III).

Extracts of polar lipids were prepared from ME-3 by repeated partition between hexane and 90% ethanol. The polar lipids were added to encephalopathogenic diets and their influence on NE was studied. The results of one such experiment, illustrated in Figure 1, show that the polar lipids increased the incidence of NE.

Two synthetic fatty acid oxidation products were tested in the same chick model: a keto monoene prepared from methyl oleate and a keto diene obtained from methyl linoleate. Both compounds accelerated the induction of NE (Figs. 2 and 3).

Fibrin clots have previously been observed in cerebellar capillaries of chicks affected with NE (2). The present chick model was used to study the effect of dicumarol on the incidence of NE. Table IV shows that the anticoagulant exerted a protective effect which increased in direct relation to its concentration in the diet. In this experiment, the diet contained 4% ME-0, but similar results were obtained with diets containing 10% oxidized safflower oil.

DISCUSSION

Several authors (4,5) have reported that the incidence of NE in vitamin E deficient chicks is directly related to the amount of linoleic acid consumed by the chicks. This is seen to be the case in the first experiment in which SO-24 and ME-0 were fed at the 4% level (Table II). However, the reversal of activities of the two lipids at the 10% level does not agree with this concept. One possible explanation for the apparently contradictory results is that SO-24 contains oxidation products which are encephalopathogenic and which, at the higher dietary level, are absorbed in sufficient amounts to overcome the opposite reaction expected from the lower linoleic acid content of SO-24 vs. ME-0.

The above explanation receives support from the observation that the oxidized esters, in spite of their lower linoleic acid content, caused a more severe incidence of NE than the fresh esters (Table III), while more direct evidence for the involvement of lipid oxidation products is provided by results obtained with polar lipids extracted from oxidized methyl esters (Fig. 1).

The effects of the two synthetic ketoenoic fatty acid esters (Figs. 2 and 3) are of interest for the following reasons: 12-oxo-9-*cis*-octadecenoic acid was previously reported to increase the severity of NE when given orally with stripped corn oil (11), but not after injection (12). Subsequently, this compound was shown to possess strong prooxidant activity in vitro (13) and eventually its activity on NE was ascribed, according to a communication from the same laboratory (14), to destruction of residual tocopherol in the diet during the

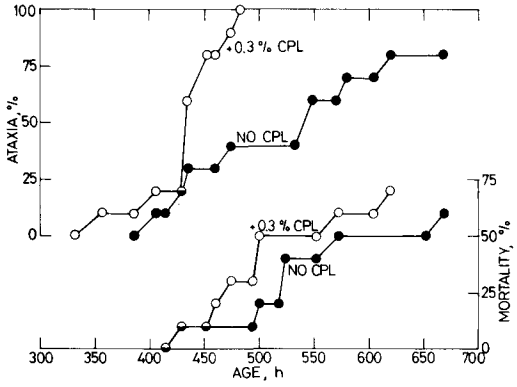

FIG. 1. Effect of crude polar lipids (CPL) on cumulative incidence of encephalopathy induced by 4% safflower methyl esters. The polar lipids were obtained from thermally oxidized safflower methyl esters and were fed with 3.7% fresh safflower methyl esters from the 8th day. During the first week, the chicks received 4% fresh safflower methyl esters. The control chicks received 4% fresh esters throughout the entire period. There were 20 chicks per treatment.

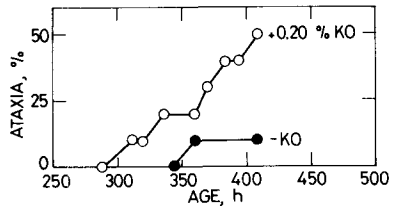

FIG. 2. Effect of conjugated methyl keto-octadecenoate (KO) on cumulative incidence of ataxia induced by 4% safflower oil methyl esters. KO was fed with 3.8% fresh safflower methyl esters from the 8th day. During the first week, the chicks received 4% safflower methyl esters. The control chicks received 4% safflower methyl esters throughout the entire period. There were 10 chicks per treatment.

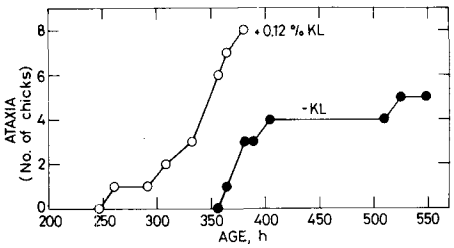

FIG. 3. Effect of conjugated methyl keto-octadecadienoate (KL) on cumulative incidence of ataxia induced by 4% safflower methyl esters. The safflower methyl esters were fed from the first day and KL was added from the 8th day. There were eight chicks per treatment.

tests. The same keto oleate has recently been reinvestigated by Fukuzawa and Sato (15) who reported that it had definite anti-vitamin E activity in the rat and that it specifically combined with bovine serum albumin to form a strongly fluorescent compound (16). These authors postulated that the keto oleate undergoes isomerization to the conjugated 12-oxo-10-*trans*-ene and that the conjugated enone then condenses with albumin amino groups to form fluorescent Schiff base compounds (16). That keto oleate is absorbed by rats when administered by stomach tube was mentioned in a recent review by Perkins (17).

Keto oleate is of little nutritional or pathological significance, because it is unlikely to be found in autoxidized polyunsaturated oils, nor would it be expected to form in vivo. On the other hand, allylic ketoenes have been found in autoxidized methyl oleate (18), and conjugated keto-dienes have been found in autoxidized methyl linoleate (19). Thus, the results of this study with synthetic conjugated ketoenes indicate that this type of compound could be one of the active species contributing to the NE-accelerating effect of oxidized oils rich in linoleic acid.

Moreover, conjugated keto-dienes could be expected to form in vivo, even in the absence of any exogenous supply. In vitamin E deficiency, for instance, polyunsaturated membrane lipids are believed to undergo peroxidation (20,21), and the hydroperoxides formed could yield conjugated keto compounds by several enzymic and nonenzymic reactions, such as those discussed by Gardner (22) for linoleic acid hydroperoxide. In fact, an increase in monocarbonyls has been reported in adipose tissue of vitamin E deficient rats (23). Lipid hydroperoxides are also readily reduced to the corresponding allylic hydroxy compounds by the ubiquitous glutathione peroxidase (EC 1.11.1.9) (24,25); this reaction raises the intriguing question of the possible biological activity on NE of these compounds, or their desaturation to active keto-dienes.

The mechanism by which the synthetic ketoenes enhance the severity of NE is not known. One possibility is suggested by the reaction of 12-oxo-9-octadecenoic acid with albumin, referred to above (16). A similar reaction of conjugated enones or dienones with amino lipids or proteins of cell membranes must result in impaired membrane function, a result usually ascribed to peroxidation of membrane lipids in vitamin E deficiency. The additional possibility that such a condensation reaction might lead to fluorescent products deserves to be investigated, although the fluorophore formed in this case would differ from the 1-amino-3-imino group derived from

TABLE IV

Effect of Dietary Dicumarol on Nutritional Encephalopathy[a]

Dicumarol concentration	Incidence per 20 chicks at 3 weeks	
	Ataxia	Mortality
μg/g		
0	12	8
200	8	7
400	1	1

[a]Chicks received 4% freshly distilled safflower methyl esters from hatching.

malondialdehyde and postulated to form in vitamin E deficiency (26).

The results on the protective effect of dicumarol (Table IV) indicate that the blood coagulation system plays a role in the etiology of NE, in agreement with the histological observation on the presence of fibrin clots in the cerebral vessels (2). Whether or not the process is triggered by thrombocyte aggregation remains to be clarified, but the inability of linolenic acid to induce NE (3-5) would indicate that the cyclo-oxygenase system is involved. For instance, linolenic acid and especially its long chain metabolites are strong inhibitors of prostaglandin formation from arachidonic acid (27), and all *cis*-5,8,11,14,17-eicosapentaenoic acid has recently been reported to be a precursor of a powerful anti-aggregating substance (28). Also, among brain tissues of the rat and guinea pig, the cerebellum has the greatest capacity for PGE_2 formation (29).

The possibility that conjugated keto-enoic fatty acids play a role in the etiology of NE and other syndromes of vitamin E deficiency deserves further study. From a nutritional point of view, attention should be given to the formation and concentrations of conjugated keto-dienes in artificially and commercially heated polyunsaturated oils.

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Cardiopathogenicity of Soybean Oil and Tower Rapeseed Oil Triglycerides when Fed to Male Rats[1]

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ABSTRACT

The triglycerides of soybean oil were purified by molecular distillation and those of Tower rapeseed oil by molecular distillation and adsorption chromatography. The original oils and the purified triglycerides were incorporated in semisynthetic diets at 20% by weight and fed for 16 weeks to weanling male Sprague-Dawley rats to compare the nutritional and pathological effects of the oils and their triglyceride fractions on rats. The study was carried out at two independent laboratories. No significant differences were observed between the results of the two establishments. The incidence of myocardial lesions was significantly higher in rats fed Tower rapeseed oil than in those fed soybean oil. Purification of the triglycerides by molecular distillation and adsorption chromatography appeared to have no major effect on the incidence of myocardial lesions. This supports our previous findings that the cardiopathogenicity of the test oils to rats resides in the triglycerides of these oils.

INTRODUCTION

It has been well documented that male Sprague-Dawley rats fed for at least 16 weeks low erucic acid rapeseed (LEAR) oils develop a higher incidence of myocardial lesions than those fed other vegetable oils (1,2). The increased incidence of lesions in this strain of rat has been attributed to cardiotoxic contaminants (3,4), residual erucic (Δ^{13}-cis-docosenoic) acid (22:1) (5), or a fatty acid imbalance (2,6-9) of LEAR oils. We considered the possibility of cardiotoxic contaminants in LEAR oils and, therefore, fractionated Span rapeseed oil (RSO) containing 4.8% 22:1 by molecular distillation and adsorption chromatography to prepare pure triglycerides and fractions enriched in nontriglyceride components (10). The results of feeding these fractions to rats suggested that the cardiopathogenic properties of Span RSO were associated with the triglycerides of the oil, and not with nontriglyceride contaminants present in the fully refined oil (8).

We decided to repeat the fractionation to further examine the hypothesis that LEAR oil triglycerides are responsible for the increased incidence of cardiac lesions in male Sprague-Dawley rats. With the availability of a LEAR oil which contained only 0.2% 22:1, a fractionation was possible to practically eliminate complicating effects, if any, of 22:1 on myocardial lesions. Soybean oil was similarly fractionated by molecular distillation to evaluate possible effects of the molecular distillation procedure itself. To increase the purity of the triglycerides of the LEAR oil, the purest triglyceride fraction from molecular distillation was further purified by column chromatography, instead of using the two methods independently as performed previously (10). Finally, a protocol was set up to conduct the feeding trial in two independent laboratories (Agriculture Canada, Ottawa and Department of Veterinary Pathology, University of Saskatchewan) with an exchange of histological sections of myocardium before decoding and evaluation of the data.

The results of the fractionation of soybean oil and Tower RSO, the feeding of the oils and their fractions to male Sprague-Dawley rats for 16 weeks, and the cardiopathological findings are presented in this communication.

MATERIALS AND METHODS

Fully refined soybean oil and *Brassica napus* cv. Tower (Tower RSO) were obtained from Canada Packers Ltd., Toronto, Ontario and Cooperative Vegetable Oil Ltd., Altona, Manitoba, respectively.

Molecular Distillation

The distillation was performed by Distillation Products Industries, Rochester, NY, using a CMS-36 molecular still. Prior to distillation, the molecular still was flushed with 50 kg of soybean oil and then by 40 kg of oil to be distilled. A total of 509 kg of soybean oil and 950 kg of Tower RSO were distilled in a similar

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TABLE I

Sterol Content and Composition of Soybean Oil, Tower RSO and Its Fractions[a]

Oils	Sterol content (μg/g oil)	Sterol composition (wt%)[b]			
		I	II	III	IV
Free sterols					
Soybean oil	0.20		27.4	19.5	53.1
Soybean oil MD7	0.005		12.0	32.2	55.8
Tower RSO	0.26	14.0	30.8	trace	55.2
Tower RSO MD7	0.004	10.2	31.8	trace	57.9
Tower RSO AC A	ND				
Tower RSO AC B	ND				
Sterol esters					
Soybean oil	0.08		21.5	4.0	74.5
Soybean oil MD7	0.015		14.6	3.7	81.6
Tower RSO	0.44	6.8	39.6	trace	55.6
Tower RSO MD7	0.031	3.3	29.8	trace	66.9
Tower RSO AC A	0.001	4.2	30.1	trace	65.7
Tower RSO AC B	0.002	4.4	29.8	trace	65.7

[a]Abbreviations: RSO, rapeseed oil; MD7, molecular distillate 7; AC, adsorption chromatography fractions A and B; ND, not detected.

[b]Sterol: I, brassicasterol; II, campesterol; III, stigmasterol; IV, β-sitosterol.

manner. The oil was placed in a holding tank and passed through the CMS-36 under conditions yielding a distillate of ca. 10%. Five further cycles yielding distillate amounts of similar to the first distillate (i.e., 10% of original charge) from each successive residue were performed. Distillate 7 was obtained by recycling the residue. A still pressure of 5 to 9 x 10^{-4} Pa was obtained for both distillations. The distillation temperature was 260-275 C for distillates 1 to 4 and 241-255 C for distillates 5 to 7. Distillate 7 of soybean oil weighed 164 kg and of Tower RSO 313 kg. The residues of soybean oil and Tower RSO weighed 34 kg and 57 kg, respectively. For details regarding collection of distillates and addition of antioxidant, see Kramer et al. (10).

Adsorption Chromatography

Molecular distillate 7 of Tower RSO (90 kg) was selected to be fractionated by adsorption chromatography at Applied Science Laboratories Inc., State College, PA, as described previously (10). The purified triglycerides obtained from several chromatographic columns were arbitrarily combined to give two fractions labeled A (21.7 kg) and B (21.3 kg). The two highly purified fractions provided enough material to feed two groups of rats at each trial establishment.

Analysis of Oils and Tissues

Methyl esters and sterols were analyzed as described previously (10), except that sterol esters were transesterified with $NaOCH_3$-CH_3OH (11). Rat tissue lipids were prepared and analyzed according to Kramer and Hulan (12).

Animals and Diets

Weanling 3-week-old male Sprague-Dawley rats (Bio-Breeding Laboratories, Ottawa, Ontario) weighing between 40-50 g were delivered at the same time to both trial establishments. The rats were housed 2 per cage and fed a stock diet (Ralston-Purina) and water ad libitum for one week before commencing the feeding trial. The rats (78 ± 3 g, mean of all rats at Ottawa), randomly assigned to 6 treatment groups, were given access to one of 6 semisynthetic diets, and water, ad libitum for 16 weeks. There were 20 rats per group in Saskatoon and 30 rats per group in Ottawa. Four of the rats in each group at Ottawa were used for lipid analysis. All rats at Ottawa were weighed at the beginning and at 2 week intervals throughout the experiment; only the final weight at 16 weeks was recorded at Saskatoon. The total feed consumption (kg) per group during the 16 week period was calculated by subtracting the feed remaining for each group at the end of the experiment from that allocated to the group at the start of the trial. Feed wastage was negligible and approximately the same for all groups.

Each semisynthetic diet (8), to which a test oil was added at 20% by weight, was prepared in one batch, then proportioned and color-coded differently for each establishment. The test oils were: soybean oil, molecular

TABLE II

Fatty Acid Composition of the Dietary Oils

Fatty acid	Soybean	Soybean MD7	Tower RSO	Tower RSO MD7	Tower RSO AC A	Tower RSO AC B
14:0	0.1	0.1	0.1	0.1	0.1	0.1
16:0	10.5	8.8	5.7	4.9	4.3	4.2
16:1	0.2	0.2	0.1	0.1	0.3	0.3
18:0	3.2	3.8	2.1	2.4	2.3	2.3
18:1	24.9	26.9	57.7	59.9	58.6	58.6
18:2	51.5	51.0	24.6	22.7	23.0	22.9
18:3	8.4	7.9	7.9	7.2	6.9	6.9
20:0	1.0	0.7	0.2	0.6	0.9	0.9
20:1	0.6	0.5	1.0	1.3	2.0	2.1
22:0	---	---	0.2	0.4	0.5	0.5
22:1	---	---	0.2	0.2	0.3	0.3
24:0	---	---	0.1	0.1	0.2	0.2
24:1	---	---	0.1	0.2	0.2	0.2

distillate 7 of soybean oil (soybean MD7), Tower RSO, molecular distillate 7 of Tower RSO (Tower RSO MD7), and 2 fractions from adsorption chromatography of Tower RSO (Tower RSO AC A and B).

At the end of the 16 week feeding trial, 4 rats from each group at Ottawa only were used for cardiac and hepatic lipid analysis; the hearts of the remaining rats at both establishments were examined histologically as described earlier (13). All histological slides were numbered and exchanged between the two establishments. A meeting was then held at which the assessment of cardiac lesions was standardized. Findings were tabulated according to the number of rats affected and the number of observed lesions per rat heart.

Analyses of variance were carried out on all methyl ester data, and the least significant differences (LSD) were calculated based on pooled error estimates. Approximate chi-square (χ^2) statistics obtained, following the approach of Fienberg (14), were used to examine the incidence of myocardial lesion data for differences among dietary treatments and establishments.

RESULTS

Analysis of Oils and Their Fractions

Large amounts of purified soybean oil and Tower RSO triglycerides were prepared by molecular distillation. Many nontriglyceride components were removed by this method (15), including the free sterols (Table I). The sterol esters, however, because of their similarity in volatility to triglycerides (15), were most difficult to remove. For this reason, the sterol ester content was determined to indicate the probable minimum purification of the oil triglycerides. Six successive strip cuts of about 10% of the original charge were necessary to yield a reasonably pure triglyceride low in sterol esters. The same distillation procedure was used for both soybean oil and Tower RSO. The soybean oil triglycerides (soybean oil MD7) were purified 5-fold, while Tower RSO triglycerides (Tower RSO MD7) were purified 14-fold by molecular distillation based on the sterol ester content (Table I).

Triglycerides from Tower RSO were further purified by passing Tower RSO MD7 through columns packed with silica gel H as described previously (10). The eluates were monitored by thin layer chromatography and the purified triglycerides were collected. Enough purified triglycerides were prepared by this method to make two fractions, designated Tower RSO adsorption chromatography (AC) A and B. Based on the sterol ester content (Table I), an additional 30- and 15-fold purification was achieved for fractions A and B, respectively.

The relative sterol composition of the free and esterified sterols within each oil were different (Table I). Changes in the relative proportion of sterols were evident during molecular distillation of both free and esterified sterols, while adsorption chromatography had no effect on the proportions. The relative concentration of brassicasterol in the sterol esters of Tower RSO was considerably less than that found in the free sterols of this oil; on molecular distillation, the concentration was further decreased.

The fatty acid composition of the test oils are shown in Table II. Relatively minor changes were observed in the fatty acid composition of the fractions obtained after molecular distillation and adsorption chromatography, and the oils retained their characteristic fatty acid composition.

TABLE III

Body Weight and Total Feed Consumed by Rats Fed the Diets for the Experimental Period[a]

Diet	Body weight[b] (g) Ottawa n=30	Saskatoon n=20	Mean	Feed consumed[c] (kg/rat) Ottawa n=30	Saskatoon n=20
Soybean oil	505	519	511	1.75	2.11
Soybean oil MD7	505	527	514	1.78	2.11
Tower RSO	508	515	511	1.88	2.02
Tower RSO MD7	531	534	532	2.00	2.11
Tower RSO AC A	481	492	485	1.75	1.95
Tower RSO AC B	498	505	500	1.82	2.06
SEM	9.6	11.7	7.4		

Analysis of variance for body weight[d]

Source of variation	d.f.	Sum of Squares	Mean Square
Location (L)	1	8,845	8,845
Diet (D)	5	59,572	11,914**
L x D	5	2,692	538
Error	288	793,790	2,756
Total	299	864,899	
Diet	5	59,572	11,914**
Among Soy	1	262	262
Soy vs. Tower	1	1,824	1,824
Tower vs. Tower AC B	1	2,746	2,746
Tower AC A vs. (Soy, Soy MD7, Tower, Tower AC B)	1	21,940	21,940***
Tower MD7 vs. (Soy, Soy MD7, Tower, Tower AC B)	1	21,418	21,418***

[a]Rats at Ottawa and Saskatoon were fed 108 and 114 days, respectively.

[b]Values are the mean of n rats per treatment.

[c]Values are derived by dividing the total feed consumed per group by the number of rats per diet. Statistical analysis was not possible.

[d]Analysis of variance was carried out only for body weight data. d.f., degrees of freedom; significantly different at the 1% (**) and 0.1% (***) level.

Growth of Rats

The mean body weight of rats fed the diets for the experimental period is shown in Table III. The rats fed at Ottawa weighed less and ate less feed than those at Saskatoon, owing in part to differences in duration of treatment periods, 108 days at Ottawa and 114 days at Saskatoon. The analysis of variance for body weights, however, showed no diet x location interaction, indicating that differences among diets were consistent at the two establishments. There was a significant diet effect; rats fed the diet which contained Tower RSO AC A gained the least weight and those fed Tower RSO MD7 the most weight. The total amount of feed consumed by each group at both locations is also shown in Table III. Because feed consumption was recorded by group, leaving only 12 measurements, an analysis of variance was not carried out. However, it may be noted that the group of rats fed Tower AC A ate less feed than the group fed Tower RSO MD7.

Heart and Liver Lipids

Lower heart weights were observed in rats fed the fractions of Tower RSO purified by adsorption chromatography (Table IV), a pattern similar to that of body weights (see Table III, Ottawa group). No differences in heart lipid levels were found between diets. The fatty acid composition of the heart lipids (Table IV) showed differences which reflect the composition of the dietary oils with regard to the levels of 18:1, 18:2, 20:1 and 22:1. No significant differences were observed in the heart lipids of rats fed the fully refined oil and their respective molecular distillates (MD7). Feeding Tower RSO fraction A obtained from absorption chromatography likewise resulted in a heart fatty acid pattern similar to that of Tower RSO. However, feeding Tower RSO AC fraction B resulted in significant changes in the levels of saturates (16:0 and 18:0), 20:4 and 22:6. These differences in heart fatty acids were observed despite the similarity of the fatty

TABLE IV

Weight, Lipid Content and Fatty Acid Composition of Heart and Liver Lipids of Rats Fed the Experimental Diets for 16 Weeks[a]

Diet		Weight (g)	Lipid (%)	Fatty acid (weight %)													
				14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:2	20:4	22:1	22:4 n-6	22:5 n-6	22:5 n-3	22:6 n-3
Heart																		
	Soybean	1.37	4.1	0.8	13.8	1.1	28.0	13.7	26.6	1.0	0.4	0.3	9.1	trace	0.5	0.3	0.6	2.4
	Soybean MD7	1.36	4.0	0.6	13.5	1.2	29.3	14.6	25.3	1.0	0.4	0.3	7.7	trace	0.6	0.2	0.4	1.8
	Tower RSO	1.45	4.1	0.6	13.8	0.9	29.1	22.4	17.6	0.9	0.7	0.1	8.4	0.2	0.7	0.3	0.5	2.3
	Tower RSO MD7	1.36	3.9	0.6	12.6	0.9	28.6	23.4	17.7	1.1	0.9	0.2	8.3	0.2	0.6	0.2	0.6	1.8
	Tower RSO AC A	1.26	4.1	0.7	11.5	1.0	30.4	23.2	16.6	0.6	0.8	0.2	9.0	0.2	0.8	0.2	0.5	2.2
	Tower RSO AC B	1.24	3.8	0.4	9.9	0.7	23.6	20.3	18.9	0.9	0.7	0.2	14.4	0.2	1.4	0.2	0.9	5.8
	LSD[b]	0.21	0.4	0.3	2.3	0.4	5.1	4.4	3.1	0.4	0.2	0.1	3.0	0.1	0.7	0.2	0.3	1.0
Liver																		
	Soybean	15.10	4.6	0.6	19.2	1.4	13.4	19.3	30.5	1.9	0.6	0.7	7.7	trace	0.6	0.3	0.2	1.1
	Soybean MD7	14.80	5.7	0.5	16.1	1.3	11.8	19.7	32.9	2.4	0.6	0.9	8.9	trace	0.4	0.3	0.3	1.8
	Tower RSO	18.27	2.6	0.7	20.7	2.4	13.6	33.5	14.7	1.8	0.8	0.3	7.6	0.2	0.2	0.3	0.4	1.6
	Tower RSO MD7	16.82	4.8	0.8	20.2	2.0	14.7	38.1	12.7	1.2	1.0	0.3	5.2	0.1	0.2	0.4	0.4	1.1
	Tower RSO AC A	16.88	4.1	0.8	19.0	1.6	18.0	37.3	11.0	0.7	0.9	0.3	6.3	0.1	0.2	0.4	0.3	1.2
	Tower RSO AC B	15.65	3.9	0.9	18.3	1.9	16.4	35.0	12.4	1.2	1.3	0.3	6.5	0.1	0.2	0.3	0.4	1.4
	LSD	4.21	1.6	0.4	4.4	0.5	4.8	5.5	5.5	1.1	0.8	0.3	2.6	0.1	0.4	0.2	0.2	0.9

[a]All values are obtained from 4 rats per treatment.

[b]LSD, least significant difference. Differences between means which are greater than the LSD are significant at the 1% level.

TABLE V

Incidence and Severity of Myocardial Lesions in Male Sprague-Dawley Rats Fed Experimental Diets for 16 Weeks at Two Establishments

Diet	Affected/examined			Severity[a] (combined)			
	Saskatoon	Ottawa	Combined	1-2	3-5	6-9	>10
Soybean oil	10/20	12/26	22/46	17	4	0	1
Soybean oil MD7	12/20	13/26	25/46	16	7	1	1
Tower RSO	13/20	21/26	34/46	22	8	2	2
Tower RSO MD7	15/20	18/26	33/46	15	11	2	5
Tower RSO AC A	8/20	11/26	19/46	12	7	0	0
Tower RSO AC B	9/20	20/26	29/46	16	12	1	0

χ^2 Analysis (of combined results)	Incidence[b] (d.f.)
All diet (D)	16.6** (5)
Two establishments (E)	0.8 (1)
D x E	6.4 (5)
Soybean oil vs. Tower RSO	6.7** (1)
Oils vs. MD7 fractions	0.1 (1)
Among Tower RSOs	12.9** (3)
Among Tower RSOs except Tower RSO AC A	1.4 (2)
Tower RSO AC A vs. All Tower RSOs	11.5*** (1)
Tower RSO AC A vs. AC B	4.4* (1)

[a]Severity = number of rats with lesion scores of 1-2, 3-5, 6-9 and >10 per three sections of heart examined. No statistical analysis was performed on the severity data because of insufficient numbers in many subgroups.

[b]A comparison of rats affected to number of rats examined was used in the analysis of variance. d.f. = degrees of freedom. Significant differences at the 5% (*), 1% (**) and 0.1% (***) levels.

acid composition of the two Tower RSO fractions A and B (Table II).

The fatty acid composition of the livers are shown in Table IV. As noted for heart lipids, clear differences were observed in the liver lipids between diets with regard to the level of 18:1, 18:2 and 22:1. No significant differences in the liver fatty acid composition, however, were observed between animals fed the fully refined oil and its corresponding fraction. It was evident that heart and liver lipids differed in which saturated fatty acids were predominant (liver, 16:0; heart, 18:0), and in the effects of dietary 18:1 and 18:2 (concentration of these fatty acids were greater in the liver of rats fed dietary oils rich in these acids).

Cardiopathology

The incidence and severity of cardiac lesions in male rats fed the same diets at the two establishments are given in Table V. Neither the difference between the two establishments nor the interaction between diet and establishment were significant. Therefore, the results were combined. Cardiac lesions were observed in all dietary groups. The incidence of cardiac lesions was significantly higher ($P<0.01$) in rats fed Tower RSO than in those fed soybean oil. It was evident that purifying the triglycerides of the two oils by molecular distillation had no effect on the incidence of cardiac lesions. A statistically significant difference was observed, however, among the Tower RSO and its 3 fractions. This difference was accounted for by the difference between Tower RSO AC A and the remaining Tower RSOs. Of interest was the significant difference between two virtually identical fractions obtained by a combination of molecular distillation and column chromatography on silica gel H.

The combined severity score from the two locations is also given in Table V. Most of the rats on each of the diets had only a few myocardial lesions per heart (the sum of three sections per heart). No statistical analysis was performed on severity data because the latter two levels of severity (6-9 and >10) had insufficient numbers of rats per diet to give a meaningful evaluation.

DISCUSSION

Numerous reports have appeared in the past decade assessing the cardiopathogenicity of diets rich in vegetable oils, fats or mixtures thereof in various animal species. These studies were prompted by an earlier report that rats fed rapeseed oil high in 22:1 developed necrotic

and fibrotic heart lesions (16). Subsequent reports have shown that the male Sprague-Dawley rat on a high fat diet is particularly predisposed to the development of heart lesions (2,8,17,18). This strain of rat readily develops heart lesions when fed a diet containing vegetable oil (2,8,18), animal fat (19) or a fat-oil mixture (4), but the incidence relative to other oils or fats is significantly higher when LEAR oils are fed (2), a result also seen in this study. It should be noted, however, that other strains of rat, namely Chester Beatty (17,20) and Wistar (18), showed only a low incidence and/or severity of heart lesions which was not increased with the intake of LEAR oils. Similar results were observed with mice (18), pigs (21-25), monkeys (26,27), ducks (28), and chicken (29) fed LEAR oils.

The etiology of degenerative heart lesions in the male Sprague-Dawley rat is not known. Furthermore, there is no conclusive evidence for the cause of the significantly higher incidence of heart lesions associated with the intake of LEAR oils. Cardiotoxic contaminants were considered as one possible cause (3). An extensive purification of a LEAR oil (Span RSO containing 4.8% 22:1) by either molecular distillation or by adsorption chromatography failed to remove cardiopathogenic agents from the oil (8,10). In this study, these two purification steps were used in combination in an attempt to provide a pure triglyceride from Tower RSO which would increase the likelihood of eliminating a possible cardiotoxin. The results again provide no evidence that molecular distillation removed a cardiotoxin from either Tower RSO or soybean oil.

When Tower RSO was purified by a combination of molecular distillation and adsorption chromatography, the resultant triglycerides still gave a high incidence of myocardial lesions in male rats. The fact that one of the two fractions gave a lower incidence of heart lesions relative to the other Tower RSO fractions, in spite of the similarity in chemical composition of the AC fractions, does not appear to be of physiological significance. Previous studies have shown a similar range of heart lesion incidence with LEAR oils (30). Furthermore, this particular group of rats (Tower RSO AC A) had decreased feed intake and weight gain which itself appears to be related to reduced lesion incidence (manuscript in preparation). Therefore, it seems more likely that the primary cardiopathogenic agent resides in the triglycerides of the oil, supporting the finding of a previous study (8).

Since myocardial lesions in male Sprague-Dawley rats do not appear to be due to cardiotoxic contaminants in the oils, we have suggested that the myocardial lesions in the fast growing male rat of this strain may be related to the dietary fatty acids in relation to the animal's nutritional requirements (2,7,8). Linolenic acid (18:3) has been implicated as a contributing factor in the incidence of heart lesions in male rats (9,18). The presence of 18:3 in the diet increases the level of polyunsaturates of the linolenic acid family (n-3) in cardiac total lipids (2,7,31) and particularly in phospholipids (32,33) which are membrane constituents. Substitution of these polyunsaturates into membranes appears to cause greater fragility of mitochondria (34) which may lead to changes in their function (35) and possibly myocardial lesions. The results of this study confirm that male Sprague-Dawley rats fed soybean oil also develop a relatively high incidence of heart lesions (46-60%). Both soybean oil and Tower RSO have a common level of 18:3 and similar cardiac levels of polyunsaturates of the n-3 family. However, this does not explain the difference in lesion incidence between the two oils. The results of a multiple regression analysis of aggregate data of heart lesions and dietary fats and oils indicate that in addition to 18:3, the low level of saturates and/or high level of 18:1 in LEAR oils may be contributing factors (36). Studies are currently in progress to investigate these relationships.

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Lipid Composition of Morris Hepatoma 5123c, and of Livers and Blood Plasma from Host and Normal Rats

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ABSTRACT

The lipid composition of Morris hepatoma 5123c was analyzed together with that of liver and blood plasma from both normal and tumor-bearing rats. The results showed that the liver of tumor-bearing rats contained higher amounts of glycerides, cholesteryl esters, free fatty acids and phospholipids than the liver of normal rats. In the blood plasma of tumor-bearing rats, there was an increase of free cholesterol and triglycerides; this latter difference, however, was not statistically significant. Acyl chain changes in the liver of tumor-bearing rats consisted of an increase of palmitic and oleic acids and a decrease of stearic and arachidonic acids in phosphatidylinositol. Morris hepatoma 5123c contained a lower amount of triglycerides than the livers (both host and normal) and showed a significant decrease of total phospholipids when compared to the host liver. The major acyl chain changes found in Morris hepatoma 5123c compared with both normal and host rat livers were: a) a higher percentage of arachidonic acid together with a lower proportion of palmitic acid in cholesteryl esters; b) an increase of stearic and arachidonic acids and a decrease of palmitic acid in triglycerides; and c) a higher level of palmitic and oleic acids associated with a lower percentage of stearic and C_{22} polyunsaturated acids in phosphatidylcholine.

INTRODUCTION

In previous investigations from this laboratory, lipids of two rat hepatomas – the fast growing Yoshida hepatoma AH130 and the slow growing Morris hepatoma 5123c – have been studied in comparison with the lipids of host livers both from whole homogenate (1,2) and subcellular fractions (3,4). Among the major changes observed in these studies, there was an increase of oleic acid together with a decrease of C_{20} and C_{22} polyunsaturated acids which affected phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol of Yoshida hepatoma AH130, and mainly phosphatidylcholine of Morris hepatoma 5123c; these changes were present almost to the same extent in the different subcellular fractions of the two hepatomas. Since differences noted in the lipid composition of tumors may represent effects of tumor-host relationships rather than molecular changes typical of neoplasia per se, studies are in progress in this laboratory to evaluate the abnormalities in tumor lipids together with possible changes induced by the presence of tumor in the lipids of liver and blood plasma of host animals.

Previous investigation (5) revealed that growth of Yoshida hepatoma AH130 induced a marked hypertriglyceridemia as well as limited changes in the fatty acid composition of the host liver. These variations in the lipids of the host, however, did not account for the peculiarities observed in the lipid composition of Yoshida hepatoma cells.

The present investigation deals with the lipids of Morris hepatoma 5123c as studied together with the lipids of liver and blood plasma from normal and tumor-bearing rats.

MATERIALS AND METHODS

Animals and Tumors

The Morris hepatoma 5123c, suspended in Hank's solution, was transplanted in the hind limbs of 150-200 g male rats (Buffalo strain) and used after 5-6 weeks. At this time, the tumor had reached an appreciable mass but showed only limited areas of necrosis. At the time of the experiment, two rats fasted overnight were exsanguinated under diethyl ether anaesthesia by heart puncture using heparinized syringes. Tumors from these two rats were quickly excised, freed from necrotic parts, weighed and then homogenized in the cold with 4 volumes of 0.04% $CaCl_2$ in a MSE homogenizer. Equal portions of the livers of these host animals were also pooled and homogenized in 4 volumes of the same solution, while blood was centrifuged at low speed to separate cells from plasma. Normal Buffalo rats of the same age as the host rats were used as controls. These rats were also starved overnight and exsanguinated, and blood and livers were processed as above.

Analytical Procedures

Total lipids of hepatoma, livers and blood plasma were extracted according to Folch et al. (6) and fractionated into neutral lipids and phospholipids by silicic acid column chromatography (3). Chromatographic procedures described previously (3) were used to resolve

TABLE I

Content of Neutral Lipid Classes in Normal and Host Rat Livers and Morris Hepatomas 5123c[a]

Lipid class	mg/g Lipid-free dry weight[b]		
	Normal liver (5)	Host liver (5)	Hepatoma 5123c (9)
Esterified cholesterol	1.1 ± 0.2	7.9 ± 1.6[e]	1.8 ± 0.2
Free cholesterol	5.6 ± 0.3	8.5 ± 0.7[e]	8.0 ± 0.6
Triglycerides[c]	52.5 ± 12.3	922.8 ± 246.4[e]	20.7 ± 4.6
Diglycerides[c]	2.0 ± 0.4	10.6 ± 2.9[f]	1.3 ± 0.2
Monoglycerides[c]	0.1 ± 0.0	1.0 ± 0.4[f]	0.1 ± 0.0
Free fatty acids[d]	3.0 ± 0.8	7.0 ± 1.3[f]	1.9 ± 0.2

[a]Values are the mean ± SE of the number of experiments listed in parentheses.
[b]Lipid-free dry weight was calculated as the difference between constant dry weight and total lipids determined gravimetrically. Water content represents 78 and 66% of wet weight of livers and hepatoma, respectively.
[c]818, 546 and 270 were the molecular weights used to calculate the amounts of tri-, di- and monoglycerides, respectively, in livers and hepatoma cells.
[d]Determined colorimetrically and using an average molecular weight of 270.
[e]Significantly different from normal rat liver at $P<0.01$.
[f]Significantly different from normal rat liver at $P<0.05$.

neutral lipids into cholesteryl esters (CE), triglycerides (TG), diglycerides (DG), monoglycerides (MG), free cholesterol (FC) and free fatty acids (FFA), and phospholipids into diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) (plus phosphatidylserine, PS), phosphatidylinositol (PI), phosphatidylcholine (PC), sphingomyelin (SP) and lysolecithin (LPC). TG, DG and MG were evaluated by glycerol determination (7) and by using molecular weights derived from the fatty acid composition of the individual glycerides in the various tissues examined. FC and esterified cholesterol (EC) were assayed according to Cramer and Isaksson (8), and FFA were determined following the method of Duncombe (9). Phospholipids were determined by multiplying x 25 the lipid-phosphorus assayed following the method of Martin and Doty (10) after digestion with sulfuric acid-perchloric acid (3:2, v/v). Fatty acid composition of individual lipid classes was determined by gas liquid chromatography as previously described (5).

Statistical Analyses

Statistical significance of the differences between normal and host rat livers, normal and host blood plasma, and Morris hepatoma 5123c and host or normal rat livers was assessed using the Student's *t* test. The minimum level of significance was considered the 5% level.

RESULTS AND DISCUSSION

As shown in Table I, in the livers of animals bearing Morris hepatoma 5123c, there was a remarkable increase of CE and glycerides; FC and FFA were also increased although to a lesser extent. No significant changes of liver neutral lipid classes were observed in rats bearing Yoshida hepatoma AH130 8-10 days after transplantation (5), while rats bearing 4-5 week old hepatoma 7288 CTC (11) as well as patients with malignant neoplastic diseases (12) showed reduced concentrations of liver TG in comparison to the controls. Sequential determination of liver lipids during tumor growth in mice bearing a glioma (13) or Ehrlich ascites carcinoma (14) showed an increase of liver TG a few days after transplantation, followed by a decrease at more advanced stages of tumor growth. A marked reduction of TG was also found in livers of advanced stage lymphoma-bearing mice (15).

Table I also shows that, in comparison to normal liver, hepatoma 5123c contained a lower amount of TG and a higher level of FC; the increase of EC and the decrease of FFA and DG were not statistically significant. The reduction of TG and FFA in hepatoma 5123c appeared still greater when the tumor was compared to the host liver, due to the accumulation of neutral lipids in the latter. A reduction of TG together with an increase of FC was also observed in hepatoma 7777 (16) and 7288 CTC (11) as compared with normal liver. In hepatoma 7794A, however, an increase of FC was found to be associated with an unchanged level of TG (16). The great accumulation of CE reported for hepatoma 7777 (16) was not observed in the present study.

Moreover, hepatoma 5123c did not contain any detectable amount of glyceryl ether diesters which were present in appreciable quan-

TABLE II

Content of Phospholipid Classes in Normal and Host Rat Livers and Morris Hepatoma 5123c[a]

Lipid class[b]	Normal liver n = 2	Host liver n = 6	Hepatoma 5123c n = 5
TPL	110.0 ± 7.3	138.0 ± 10.9[d]	99.9 ± 5.7
DPG	5.7 ± 0.3 (5.4)	6.1 ± 0.2 (4.4)	6.2 ± 0.5 (6.2)
PE (plus PS)[c]	33.8 ± 3.9 (30.4)	47.6 ± 2.6[d] (34.5)	33.2 ± 0.8 (33.3)
PI	8.6 ± 0.4 (7.8)	4.8 ± 1.3 (3.5)	4.5 ± 0.4 (4.5)
PC	54.7 ± 2.0 (49.4)	68.7 ± 2.5[d] (49.8)	50.1 ± 1.6 (50.3)
SP	6.1 ± 0.8 (5.5)	8.7 ± 2.3 (6.3)	4.5 ± 0.4 (4.5)
LPC	1.7 ± 0.4 (1.5)	2.1 ± 0.2 (1.5)	1.2 ± 0.1 (1.2)

[a]The values are means ± SE and are expressed as mg/g lipid-free dry weight. n = Number of experiments. Figures in parentheses represent percentages of total lipid phosphorus.

[b]TPL = Total phospholipids; DPG = diphosphatidylglycerol; PE (plus PS) = phosphatidylethanolamine (plus phosphatidylserine); PI = phosphatidylinositol; PC = phosphatidylcholine; SP = sphingomyelin; LPC = lysophosphatidylcholine.

[c]PE and PS were eluted together during phospholipid fractionation by silicic acid column chromatography (19).

[d]Significantly different from normal rat liver at $P<0.05$.

TABLE III

Lipid Concentrations in Blood Plasma from Normal Rats and Morris Hepatoma-Bearing Rats[a]

Lipid class	Normal rat (5)	Host rat (11)
Total phospholipids	95.0 ± 4.3	120.0 ± 13.8
Esterified cholesterol	31.0 ± 2.5	37.3 ± 3.8
Free cholesterol	14.4 ± 1.1	24.0 ± 2.4[d]
Triglycerides[b]	102.3 ± 9.1	238.2 ± 60.4
Free fatty acids[c]	16.8 ± 3.6	16.1 ± 2.4

[a]The values, expressed as mg/100 ml blood plasma, are the means ± SE of the number of experiments listed in parentheses.

[b]824 and 820 were the molecular weights used to calculate the amounts of triglycerides in normal and host rat blood plasma, respectively.

[c]Determined colorimetrically and using an average molecular weight of 270.

[d]Significantly different from normal rat blood plasma at $P<0.02$.

tities in Ehrlich carcinoma (17) as well as in hepatoma 7777 (16) and Yoshida hepatoma AH130 (5). Differences in the level of the ether-linked lipids among various tumors have been correlated with their growth rate (18).

Table II shows that, in comparison to the controls, animals bearing hepatoma 5123c had higher concentrations of total phospholipids (PL) due primarily to an increase of PC and PE (plus PS). An increase of total PL was also found in liver of rats bearing mammary carcinoma (19), in mice transplanted with human ovarian carcinoma (20), and in patients with malignant neoplastic diseases (12). On the other hand, rats bearing Walker carcinoma (19), hepatoma 7288 CTC (11) and Yoshida hepatoma AH130 (5) did not show significant differences in the liver PL in comparison with normal rats.

Table II also shows that hepatoma 5123c contained a lower amount of total PL than host and normal rat livers; the difference with the latter, however, was not statistically significant. It can be seen that the percent composition of phospholipids in the tumor was similar to that in the livers, indicating that all the major phospholipid classes were equally reduced in hepatoma 5123c. The decrease of PL observed in hepatoma 5123c as well as in other hepatomas (5,16,21-23) may be related to the reduced concentration of microsomal PL which was repeatedly reported in hepatomas as compared to liver (3,4,24-28).

As shown in Table III, rats bearing hepatoma 5123c had increased levels of plasma PL, EC and FC in comparison with normal rats; however, when submitted to the Student's *t* test, only the differences in the plasma FC concentration were statistically significant. Similar results were found for FC and EC in the plasma

TABLE IV

Fatty Acid Composition of Cholesteryl Esters, Triglycerides and Free Fatty Acids of Livers from Normal and Host Rats and Morris Hepatoma 5123c

Tissue	Percent (by wt) of total fatty acids[a]							
	16:0	16:1	18:0	18:1	18:2	18:3[b]	20:4	Others[c]
Cholesteryl esters								
Normal rat liver (5)	40.0 ± 3.4	8.4 ± 1.3	4.8 ± 1.0	25.4 ± 2.1	14.3 ± 1.6	1.2 ± 0.7	4.6 ± 0.6	1.3
Host rat liver (10)	31.0 ± 2.1[e]	6.3 ± 0.6	3.3 ± 0.4	39.7 ± 2.8	14.3 ± 1.2	0.8 ± 0.2	4.1 ± 0.9	0.5
Hepatoma 5123c[d] (7)	20.2 ± 0.5	3.3 ± 0.4	8.1 ± 0.7	26.1 ± 1.2	15.9 ± 1.0	0.5 ± 0.4	24.6 ± 1.9	1.3
Triglycerides								
Normal rat liver (5)	30.7 ± 0.8	7.4 ± 0.9	1.2 ± 0.1	38.4 ± 1.3	19.7 ± 2.5	1.4 ± 0.3	0.3 ± 0.1	0.9
Host rat liver (11)	29.3 ± 0.9	4.7 ± 0.5	1.4 ± 0.3	41.8 ± 1.4	19.0 ± 1.2	1.5 ± 0.3	1.3 ± 0.5	1.0
Hepatoma 5123c[d] (7)	24.6 ± 0.7	3.8 ± 0.4	5.4 ± 0.2	39.6 ± 1.3	19.0 ± 2.0	1.5 ± 0.4	4.1 ± 0.5	1.4
Free fatty acids								
Normal rat liver (5)	55.2 ± 3.8	3.6 ± 0.4	9.1 ± 1.2	18.4 ± 1.3	9.2 ± 1.6	0.6 ± 0.4	2.5 ± 0.8	1.4
Host rat liver (10)	53.1 ± 2.6	3.6 ± 0.5	7.6 ± 1.0	22.6 ± 2.0	9.8 ± 1.1	0.9 ± 0.4	0.7 ± 0.2	1.7
Hepatoma 5123c[d] (7)	33.9 ± 0.8	3.3 ± 0.6	23.0 ± 2.1	22.3 ± 2.1	10.8 ± 0.7	0.3 ± 0.1	4.3 ± 1.2	2.0

[a]Each values is the mean ± SE of the number of experiments listed in parentheses.
[b] Values of 18:3 also include small amounts of 20:1.
[c]Include 14:0 and 20:3.
[d]Significant differences between hepatoma 5123c and host or normal rat liver are reported in the text.
[e]Significantly different from normal rat liver at $P<0.05$.

of rats transplanted with Morris hepatoma 7777 (29). Moreover, as in animals bearing Yoshida hepatoma AH130 (5), Walker carcinoma (30) or Ehrlich ascites carcinoma (31-33), rats with hepatoma 5123c showed a higher level of plasma TG than normal rats, but the difference, although marked, was not statistically significant owing to the large variability of values in host rats.

From the examination of Table III it is also evident that, in agreement with previous findings from animals bearing hepatoma AH130 (5) and Ehrlich carcinoma (34), the presence of hepatoma 5123c did not significantly change the level of plasma FFA in host rats. It should be stressed, however, that all these animals were fasted overnight before blood sampling and that an increase of plasma FFA of tumor-bearing animals as compared to the controls has been observed only using fed animals (35).

As given in Table IV, the fatty acid profiles of EC, TG and FFA of the host liver were similar to those of the normal rat liver, except for a reduction of palmitic acid in CE. In comparison to host and normal rat livers, hepatoma 5123c showed an increase of arachidonic acid and a decrease of palmitic acid in CE, which were also noted in hepatoma 7777 (29) and hepatoma 7288 CTC (11). In TG, hepatoma 5123c had a lower level of palmitic acid and higher proportions of stearic and arachidonic acids in comparison to the livers, findings also reported in hepatoma 7288 CTC (11). A decrease of palmitic acid together with an increase of stearic acid was also observed in TG of Yoshida hepatoma AH130 when compared to the liver (5).

As shown in Table V, the only significant differences in acyl group composition of PL of host vs. normal rat liver were an increase of palmitic and oleic acids and a decrease of stearic and arachidonic acids in PI. It should be noted that oleic acid, although increased, was still a minor fatty acid in PI of the host liver. A significant increase of palmitic acid together with a decrease of arachidonic acid was found in the total liver PL of rats bearing Walker carcinoma (36) as well as in the liver PC and PI of rats bearing Yoshida hepatoma AH130 (5).

In PE (plus PS), hepatoma 5123c revealed a fatty acid profile similar to that of normal liver, but showed a slight decrease of palmitic acid and an increase of oleic acid when compared to host liver. Minor quantities of aldehydogenic chains of plasmalogens (not reported in the table) were also found in PE (plus PS) of hepatoma 5123c. In PC, hepatoma 5123c contained higher percentages of palmitic and oleic acids and lower proportions of stearic acid and of 22:5 and 22:6 fatty acids in comparison to both host and normal rat liver. In addition, hepatoma 5123c showed a significant reduction of arachidonic acid in PC when compared to host liver. Furthermore, an increase of palmitic, oleic and linoleic acids accompanied by a decrease of stearic acid was found in the PI of hepatoma compared with normal liver, while no significant variation in the tumor PI fatty acid profile was revealed with respect to the host liver. With the exception of minor differences, DPG fatty acid composition of hepatoma was rather similar to that of the livers. An increase of oleic acid coupled with a decrease of C_{20} and C_{22} polyunsaturated acids has been previously reported in various glycerophospholipids from different rat hepatomas (5,16,21, 22,24,37,38). It should be noted, however, that the magnitude of fatty acid changes in the slow-growing Morris hepatoma 5123c was smaller than that found in the poorly differentiated Yoshida hepatoma AH130 (5), hepatoma 7288 CTC (21) and hepatoma 7777 (24). An analogous difference may also be observed in the extent of fatty acid changes between the fast-growing 7777 and slow-growing 7794A hepatomas (16). Moreover, in the poorly differentiated hepatomas, the increase of oleic acid associated with the decrease of polyunsaturated acids appeared in different phospholipid classes while being mainly confined to PC in the case of hepatoma 5123c. Since octadecenoate isomers were not determined in the present study, it cannot be established whether the increase of "oleic acid" in hepatoma 5123c is in effect partly due to vaccenic acid. This latter was found to constitute an appreciable percentage of octadecenoic acid in several tumors (39,40).

As shown in Table VI, the fatty acid profile of sphingomyelin was similar in hepatoma and in livers with the exception of a lower proportion of oleic acid in host compared to normal rat livers.

Table VII shows that the fatty acid compositions of blood plasma CE, TG, FFA and PL in animals bearing hepatoma 5123c were similar to those in normal rats.

In conclusion, the present study has revealed that in comparison with the control group the liver of rats bearing a 5-6 week old Morris hepatoma 5123c shows an accumulation of CE and glycerides as well as slight changes in the PI fatty acid profile. From these results and those of other laboratories, it is difficult to ascertain common trends in the lipid changes induced by tumor growth in host tissues, since any comparison among different tumor-bearing animals is limited by such variables as dif-

TABLE V

Fatty Acid Composition of Phosphatidylethanolamine (plus Phosphatidylserine), Phosphatidylcholine, Phosphatidylinositol and Diphosphatidylglycerol of Normal and Host Rat Livers, and Morris Hepatoma 5123c

Tissue	Percent (by wt) of total fatty acids[a]										
	16:0	16:1	18:0	18:1	18:2	18:3[b]	20:3[c]	20:4	22:5	22:6	Others[d]
Phosphatidylethanolamine (plus Phosphatidylserine)											
Normal rat liver (2)	17.9 ± 1.5	0.6 ± 0.1	24.4 ± 3.1	6.2 ± 1.3	8.2 ± 2.0	---	1.1 ± 0.6	25.4 ± 4.2	1.4 ± 0.7	14.4 ± 0.8	0.4
Host rat liver (5)	22.1 ± 1.3	0.2 ± 0.1	25.8 ± 0.5	4.3 ± 0.7	6.0 ± 1.2	0.2 ± 0.1	1.2 ± 0.2	17.0 ± 1.6	2.0 ± 0.3	20.5 ± 3.7	0.7
Hepatoma 5123c[e] (8)	18.5 ± 1.0	0.5 ± 0.1	27.9 ± 0.9	8.4 ± 0.6	7.0 ± 0.3	0.1 ± 0.1	0.4 ± 0.1	20.0 ± 1.7	2.6 ± 0.4	13.3 ± 0.7	1.3
Phosphatidylcholine											
Normal rat liver (2)	23.4 ± 3.0	1.2 ± 0.2	22.0 ± 0.3	8.4 ± 0.9	22.5 ± 0.3	---	0.6 ± 0.2	13.9 ± 5.5	0.9 ± 0.2	6.0 ± 0.7	1.1
Host rat liver (5)	26.1 ± 2.0	0.6 ± 0.2	21.1 ± 1.6	9.9 ± 0.9	17.4 ± 1.8	0.1 ± 0.1	1.1 ± 0.2	12.4 ± 0.5	0.9 ± 0.4	9.6 ± 1.9	0.7
Hepatoma 5123c[e] (8)	32.3 ± 1.1	2.1 ± 0.3	13.9 ± 0.3	20.0 ± 0.9	17.8 ± 0.8	---	0.2 ± 0.1	9.3 ± 0.8	0.4 ± 0.2	2.7 ± 0.5	1.1
Phosphatidylinositol											
Normal rat liver (2)	5.6 ± 0.0	0.8 ± 0.2	49.8 ± 2.2	3.4 ± 1.3	2.3 ± 0.1	---	1.3 ± 0.7	29.9 ± 0.4	1.0 ± 0.4	5.4 ± 0.9	0.5
Host rat liver (5)	17.2 ± 1.4[f]	2.3 ± 2.0	35.4 ± 2.0[f]	6.2 ± 1.6[g]	5.9 ± 0.6	0.3 ± 0.2	2.5 ± 0.8	20.8 ± 1.9[g]	0.6 ± 0.3	7.3 ± 1.9	1.5
Hepatoma 5123c[e] (7)	15.1 ± 0.9	0.6 ± 0.2	39.3 ± 1.8	7.1 ± 0.6	5.9 ± 0.3	0.2 ± 0.2	1.8 ± 0.8	22.2 ± 1.1	0.9 ± 0.5	3.7 ± 0.8	3.2
Diphosphatidylglycerol											
Normal rat liver (2)	8.3 ± 1.0	7.6 ± 0.9	3.5 ± 0.8	26.6 ± 3.0	48.2 ± 2.7	---	---	2.9 ± 0.8	---	---	2.9
Host rat liver (5)	7.7 ± 1.8	6.3 ± 0.8	6.7 ± 1.6	16.4 ± 1.9	57.1 ± 3.1	0.6 ± 0.4	0.4 ± 0.2	2.7 ± 0.7	---	0.8 ± 0.8	1.3
Hepatoma 5123c[e] (7)	7.2 ± 0.5	4.6 ± 0.5	3.6 ± 0.6	21.7 ± 1.9	58.1 ± 2.5	0.2 ± 0.1	0.5 ± 0.3	2.5 ± 0.5	---	---	1.6

[a,b] As in Table IV.
[c] Values of 20:3 include either ω6 or ω9 isomer, this latter being present only in trace amounts.
[d] Include 14:0 and 20:5.
[e] Significant differences between hepatoma 5123c and host or normal rat liver are reported in the text.
[f] Significantly different from normal rat liver at $P<0.01$.
[g] Significantly different from normal rat liver at $P<0.05$.

TABLE VI

Fatty Acid Composition of Sphingomyelin of Normal and Host Rat Livers, and Morris Hepatoma 5123c

Tissue	Percent (by wt) of total fatty acids[a]											
	16:0	18:0	18:1	18:2	20:0	22:0	22:1	23:0	23:1	24:0	24:1	Others[b]
Normal rat liver (2)	26.3 ± 4.1	10.1 ± 0.7	15.2 ± 8.5	4.0 ± 1.6	2.0 ± 0.1	6.1 ± 1.4	1.6 ± 0.4	6.1 ± 1.6	1.2 ± 0.3	14.5 ± 3.3	10.7 ± 0.5	2.1
Host rat liver (5)	34.7 ± 2.2	12.8 ± 1.6	3.5 ± 0.4[d]	3.4 ± 0.7	1.7 ± 0.2	9.6 ± 2.0	1.2 ± 0.6	3.1 ± 0.9	3.1 ± 1.6	12.4 ± 1.9	11.6 ± 1.8	2.9
Hepatoma 5123c[c] (7)	33.1 ± 3.2	12.5 ± 1.6	7.3 ± 1.6	5.1 ± 1.0	1.7 ± 0.3	10.4 ± 1.2	1.6 ± 0.8	3.9 ± 1.8	0.6 ± 0.3	8.2 ± 1.7	14.2 ± 1.9	1.4

[a]As in Table IV.
[b]Include 14:0 and 16:1.
[c]Significant differences between hepatoma 5123c and host or normal rat liver are reported in the text.
[d]Significantly different from normal rat liver at $P<0.05$.

TABLE VII

Fatty Acid Composition of Blood Plasma Cholesteryl Esters, Triglycerides, Free Fatty Acids and Phospholipids of Normal Rats and Hepatoma 5123c Bearing Rats

Tissue	Percent (by wt) of total fatty acids[a]									
	16:0	16:1	18:0	18:1	18:2	18:3[b]	20:3[c]	20:4	22:6	Others[d]
Cholesteryl esters										
Normal rat blood plasma (6)	12.4 ± 0.1	4.8 ± 0.6	0.9 ± 0.1	14.3 ± 1.1	29.3 ± 1.8	0.1 ± 0.1	0.1 ± 0.1	34.6 ± 3.9	---	3.5
Host rat blood plasma (10)	14.3 ± 1.1	5.2 ± 0.5	2.0 ± 0.4	16.6 ± 1.5	26.9 ± 1.4	0.5 ± 0.1	0.5 ± 0.4	33.2 ± 3.1	---	0.8
Triglycerides										
Normal rat blood plasma (6)	26.0 ± 1.8	3.5 ± 0.4	2.3 ± 0.2	32.9 ± 1.1	26.6 ± 2.2	1.6 ± 0.4	---	1.8 ± 0.3	1.9 ± 0.9	3.2
Host rat blood plasma (11)	30.2 ± 1.2	3.4 ± 0.4	4.0 ± 0.6	33.3 ± 0.7	23.3 ± 0.7	2.0 ± 0.5	0.1 ± 0.1	3.1 ± 0.8	0.2 ± 0.1	0.4
Free fatty acids										
Normal rat blood plasma (5)	33.5 ± 1.0	7.6 ± 1.0	5.8 ± 0.4	28.7 ± 1.9	18.3 ± 1.5	1.9 ± 0.5	0.7 ± 0.3	1.1 ± 0.3	---	2.4
Host rat blood plasma (12)	33.8 ± 1.2	5.8 ± 0.4	8.5 ± 0.6	30.0 ± 0.9	16.4 ± 1.0	1.5 ± 0.4	---	1.7 ± 0.2	---	2.3
Phospholipids										
Normal rat blood plasma (4)	27.5 ± 1.4	0.7 ± 0.1	25.8 ± 1.6	9.9 ± 0.6	21.7 ± 0.4	0.5 ± 0.2	0.7 ± 0.2	10.2 ± 1.4	2.3 ± 0.7	0.7
Host rat blood plasma (8)	27.6 ± 1.5	0.5 ± 0.1	23.1 ± 1.8	9.4 ± 0.8	17.6 ± 1.1	0.4 ± 0.3	1.3 ± 0.2	13.6 ± 1.4	5.8 ± 0.5	0.7

[a,b]As in Table IV.
[c]As in Table V.
[d]Include 14:0, 20:5 and 22:5.

ferences in the biological characteristics of tumors, in the species and strains of host animals and in the timing of experimental observations.

This study further shows that changes in the acyl chain composition of neutral lipids and phospholipids of Morris hepatoma 5123c can be detected when comparing the tumor either with host liver or with normal rat liver; no such acyl chain changes are observed when comparing host vs. normal livers. These acyl chain variations, also found in other neoplastic systems, are indicative of tumor-specific anomalies with potential relevance for tumor cells behavior.

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Geometrical and Positional Isomer Content of the Monounsaturated Fatty Acids from Various Rat Tissues

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ABSTRACT

The percentage distribution of the geometrical and positional isomers in the hexadecenoates and octadecenoates isolated from triglycerides, phosphatidylcholines, and phosphatidylethanolamines of brain, heart, kidney, liver, lung, muscle, spleen, and adipose tissues from normal rats maintained on a laboratory diet has been determined. All of the octadecenoates and most of the hexadecenoates from the lipid classes of all the tissues consisted of more than 95% *cis* isomers. Generally, palmitoleic was the predominant hexadecenoate, but many of the tissue phospholipids contained relatively high percentages of the Δ6 and Δ7 isomers. Oleate and vaccenate were the predominant octadecenoates in all tissues. Except for brain and adipose tissues, the oleate to vaccenate ratios differed for each lipid class, as well as between most tissues. In contrast to the monoenes of the phospholipids, the triglyceride monoenes exhibited the same approximate: percentage composition; percentage of geometrical isomers; and percentage distribution of hexadecenoate and octadecenoate positional isomers. These data add to our basic information about the percentage distribution of geometrical and positional isomers of naturally occurring unsaturated fatty acids in the major lipid classes of various normal tissues. Some new concepts were advanced as possible explanations to some of the observed positional isomer distributions.

INTRODUCTION

Oleic (*cis* Δ9 octadecenoic) and vaccenic (*cis* Δ11 octadecenoic) acids are the predominant octadecenoates found in the major lipid classes of normal rat liver. The proportion of vaccenic acid is dependent upon the lipid class: phosphatidylethanolamines > phosphatidylcholines > cholesteryl esters > triglycerides (1). Oleic to vaccenic acid ratios in the lipid classes of normal liver are unaffected or marginally affected by extremes in diet (1). The importance of maintaining a particular ratio of these two fatty acids characteristic of each of the major lipid classes is not understood at this time, but it is known that all the major lipid classes of hepatomas and livers from animals fed a dietary co-carcinogen contain the same approximate proportions of these two monoene isomers (1-3).

The geometrical and positional isomer content of the major lipid classes of several normal tissues has been examined in the present study to determine: 1) the level of *trans* monoene isomers in the various normal tissues; 2) whether the oleic to vaccenic ratios unique to the individual lipid classes of liver exist in other tissues; 3) whether the octadecenoate isomers exhibit tissue specificity; and 4) to what extent isomeric hexadecenoates might exhibit tissue and lipid class specificity.

EXPERIMENTAL PROCEDURES

Male Buffalo strain rats (200–300 g) that had been maintained on a laboratory chow diet (Wayne Lab-Blox, Allied Mills, Inc., Chicago, IL) for at least four weeks were used. The animals were killed, the tissues excised, lyophilized and extracted twice by the Bligh and Dyer procedure (4). Neutral and polar lipids were separated by silicic acid chromatography (5), individual lipid classes isolated by thin layer chromatography (TLC) (6,7), methyl esters prepared by acid-catalyzed transesterification (8), and analyzed quantitatively by gas liquid chromatography (GLC) as described previously (8). Triglycerides were quantitated by high temperature GLC as the intact glycerides (8). Argentation TLC was used to separate *cis* and *trans* monoene fatty ester fractions from each lipid class. Hexadecenoates and octadecenoates were isolated from these fractions by preparative GLC and the double bond positions determined by GLC analysis of the ozonide cleavage products as described earlier (2). The ozonides were prepared by a modification (2) of the Beroza and Bierl procedure (9). The two geometrical isomer fractions were quantitated by GLC analysis of the samples with a known quantity of methyl docosanoate added.

The source and purity of lipid standards, solvents, chemicals and reagents were the same as given previously (2).

RESULTS

The chow diet contained 5.65% total lipid of which triglyceride was the major component. The fatty acid composition of the total lipids

TABLE I

Water and Lipid Concentrations in Various Rat Tissues[a]

Tissue	% Dry matter	% Total lipid of dry wt.	% Polar lipid	% Neutral lipid	% TG of neutral lipids
Brain	22.2 ± 1.2	41.1 ± 1.5	80.3 ± 0.7	19.7 ± 0.7	<1
Heart	22.9 ± 0.4	17.0 ± 2.5	80.0 ± 6.0	20.0 ± 6.0	87.0 ± 1.6
Kidney	24.1 ± 0.6	20.4 ± 0.7	74.9 ± 3.0	25.1 ± 3.0	40.0 ± 6.6
Liver	32.9 ± 1.0	15.2 ± 0.6	74.8 ± 1.8	25.2 ± 1.8	60.2 ± 4.0
Lung	22.1 ± 0.5	21.2 ± 2.5	58.8 ± 2.3	41.2 ± 2.3	62.0 ± 11.4
Muscle	26.7 ± 0.8	15.4 ± 3.9	13.8 ± 5.4	86.2 ± 5.4	97.6 ± 0.6
Spleen	23.3 ± 0.4	13.0 ± 0.8	61.4 ± 1.7	38.6 ± 1.7	41.0 ± 1.1
Adipose	>90[b]	84.9 ± 2.4	0.9 ± 0.5	99.1 ± 0.5	>99

[a]Percentages represent the mean ± standard deviation from analyses of four rats individually.
[b]The high percentage of lipid in the adipose tissue prevented an accurate determination of the water content.

TABLE II

Octadecenoate and Hexadecenoate Percentages Found in the Various Rat Tissues of Animals Maintained on a Chow Diet

	Percentages of the total fatty acids[a]					
	TG		PC		PE	
Tissue	18:1	16:1	18:1	16:1	18:1	16:1
Brain	—	—	27.9 ± 0.6	0.8 ± 0.1	24.4 ± 0.3	T[b]
Heart	38.8 ± 1.0	8.3 ± 2.9	11.1 ± 0.6	0.6 ± 0.2	8.4 ± 0.7	0.5 ± 0.1
Kidney	29.9 ± 0.5	6.4 ± 0.9	10.0 ± 0.2	0.9 ± 0.1	8.6 ± 0.3	T
Liver	31.2 ± 0.9	7.8 ± 1.6	12.1 ± 1.7	2.2 ± 0.9	9.9 ± 1.3	1.0 ± 0.4
Lung	37.0 ± 1.4	7.2 ± 1.1	9.2 ± 0.1	9.8 ± 0.5	19.0 ± 1.1	2.9 ± 0.6
Muscle	28.4 ± 0.6	6.2 ± 0.9	7.6 ± 0.2	1.3 ± 0.1	6.5 ± 1.2	T
Spleen	30.6 ± 1.2	8.2 ± 0.6	10.1 ± 0.2	1.4 ± 0.1	12.0 ± 0.8	T
Adipose	28.2 ± 0.5	6.2 ± 0.8	19.4 ± 1.2	1.2 ± 0.1	19.2 ± 0.4	0.9 ± 0.2

[a]Percentages represent the mean ± standard deviation from analyses of four rats individually.
[b]T denotes detectable quantities of less than 0.5%.

was 16:0, 14.3%; 16:1, 1.5%; 18:0, 4.2%; 18:1, 22.9%; 18:2, 46.5%; 18:3, 5.1% and >18:3, 3.1%. This composition is similar to that of soybean oil listed as an ingredient of the diet. There were no detectable quantities of *trans* monoenes on silver ion TLC. Palmitoleic acid (Δ9 isomer) represented ca. 95% of the hexadecenoate fraction with the Δ6, Δ7, and Δ11 isomers making up the remainder. The octadecenoate fraction consisted of 93% oleate and 6.8% vaccenate. The fish meal and animal liver listed as ingredients of the diet were probably the origin of the small amount of the Δ11 isomer. The 18:2 fraction consisted of >99% linoleic acid. These analyses are very similar to our previous (unpublished) analyses of Purina Rat Chow. The absence of unnatural *cis* and *trans* isomers is not unexpected since the ingredients used in these laboratory animal diets are usually unprocessed.

The water and lipid content of the various rat tissues is given in Table I. Except liver and adipose tissue, all the tissues contained the same approximate lyophilized dry matter content. Excluding brain and adipose tissue, the total lipid content represented ca. 15–20% of the tissue dry weights. The polar lipid fractions of brain, heart, kidney, and liver represented 75–80% of the total lipids. Lung and spleen polar lipids, primarily phospholipids, accounted for ca. 60% of the total lipids. Percentages of phosphatidylcholines and phosphatidylethanolamines, the major phospholipids of all the tissues, have been reported (7,10). Triglycerides were the major component of the neutral lipid fraction of all tissues, except kidney and spleen (Table I) which contained large amounts of cholesterol. The data in Table I along with the data that will be given in the following tables will permit the calculation of absolute amounts of octadecenoates and hexadecenoates in the lipid classes of various tissues on a wet weight or dry weight basis.

The hexadecenoate and octadecenoate percentages found in the major glyceride classes of the various tissues are given in Table II. The complete fatty acid composition of these tissue lipid classes will be published (11). Except for brain, all tissue triglycerides contained the highest percentages of octadecenoates and hexadecenoates, excluding lung. The octadecenoates in phosphatidylcholines from all tissues, excluding brain and adipose, represented 8–11% of the total fatty acids. The

percentage of octadecenoates in phosphatidylethanolamines showed the most variation: brain, lung, spleen, and adipose contained more than 10% and the other tissues contained less than 10% octadecenoate. Except for two or three values, the monoene percentages found in each of the lipid classes of the various tissues agreed well with values reported previously (10,12) for animals fed normal laboratory chow diets.

Table III gives the percentage of *cis* hexadecenoates in the total hexadecenoate fraction isolated from each of the major lipid classes of the various tissues. All the lipid class hexadecenoates of all the tissues were composed primarily of the *cis* isomer. The small percentage of the *trans* hexadecenoates, the difference between the *cis* percentages and 100, was the highest in the phosphatidylcholines. Generally, the tissue phosphatidylcholines that contained the highest percentages of *trans* hexadecenoates were the ones that contained the lowest percentages of total hexadecenoates (Table II).

Table IV gives the percentages of *cis* octadecenoates in the total octadecenoate fraction from each lipid class of each tissue. The *cis* isomers accounted for more than 95% of the total. Heart and liver phospholipids contained the highest percentage of the *trans* isomers, but these percentages were less than 5%.

The distributions of the positional isomers of the *cis* hexadecenoates from the major lipid classes of the various tissues are given in Table V. The Δ6, Δ7, and Δ9 were the predominant isomers, which is in agreement with that reported for hexadecenoates isolated from the total lipids of several tissues of rats fed chow and fat-free diets (13). Palmitoleic acid, Δ9 hexadecenoate, was the most abundant isomer; however, the sum of the other two isomers was equal to or greater than the percentage of palmitoleic in adipose and lung phosphatidylcholine. The phospholipids contained a higher percentage of the Δ6 and Δ7 isomers than the triglycerides. Liver was the only tissue that exhibited the same approximate hexadecenoate isomer distribution in all three lipid classes. The distribution of hexadecenoate positional isomers in brain phosphatidylcholines and liver triglycerides is similar to values reported previously (1,12). There was not sufficient sample to permit positional analysis of the *trans* hexadecenoates.

The percentage distribution of oleic and vaccenic acid, the predominant *cis* octadecenoates, in the three major classes of the various tissues is given in Table VI. Oleate represented more than 85% of the triglyceride octadecenoates in all tissues, except liver. Adipose was the tissue that exhibited the same approximate proportion of oleic and vaccenic acid in all three lipid classes. This suggests that the biosynthesis of these three lipid classes draws from the same source of monoene fatty acids, and perhaps other fatty acids as well, in the adipocyte. This observation also suggests that the phosphoglycerides of this tissue undergo little modification after synthesis. Heart phosphatidylcholines and phosphatidylethanolamines contained the same approximate proportions of oleic and vaccenic acids, and this tissue was similar to liver. The phospholipids of these two tissues contained higher percentages of vaccenate than oleate. The octadecenoates of total phospholipids from heart have been reported to consist of ca. 50% vaccenate (14). Liver vaccenate percentages are slightly higher in the two phospholipid classes than we had reported previously (1). Seasonal or age differences might have contributed to the slight difference between values. Brain contained a similar

TABLE III

Distribution of *Cis* and *Trans* Hexadecenoate Isomers in the Major Lipid Classes Obtained from Several Rat Tissues of Animals Maintained on a Chow Diet

Tissue	Percentages of geometrical isomers[a]		
	TG:*cis*	PC:*cis*	PE:*cis*
Brain	—	96.0	—
Heart	98.7 ± 0.5	92.0	94.5
Kidney	99.8 ± 0.3	93.1	99.1
Liver	98.6 ± 0.6	98.4	96.7
Lung	97.0 ± 3.0	98.8	94.2
Muscle	97.6	91.5	—
Spleen	99.7	93.4	—
Adipose	99.4	91.5	99+

[a]The percentage of the *trans* isomer is the difference between the *cis* values and 100. Percentages represent the mean ± standard deviation from analyses of four rats individually. Values without standard deviation represents the analysis of a pooled sample from four rats.

TABLE IV

Distribution of *Cis* and *Trans* Octadecenoate Isomers in the Major Lipid Classes Obtained from Several Rat Tissues of Animals Maintained on a Chow Diet

Tissue	Percentage of geometrical isomers[a]		
	TG:*cis*	PC:*cis*	PE:*cis*
Brain	—	99+	99+
Heart	98.5 ± 0.3	97.7 ± 0.3	94.6 ± 0.7
Kidney	99.7 ± 0.1	99.6 ± 0.3	99.4 ± 0.1
Liver	99.4 ± 0.1	97.3 ± 1.3	96.8 ± 3.2
Lung	98.0 ± 0.5	98.7 ± 0.1	98.9 ± 0.4
Muscle	99.6 ± 0.5	99.3 ± 0.1	98.6 ± 1.0
Spleen	99.9 ± 0.2	99.2 ± 0.3	98.7 ± 0.2
Adipose	99.4 ± 0.5	98.6 ± 0.9	99+

[a]The percentage of the *trans* isomer is the difference between the *cis* values and 100. Percentages represent the mean ± standard deviation from analyses of four rats individually.

TABLE V

Percentage Distribution of Positional Isomers of *Cis*-Hexadecenoate Fatty Acids Isolated from the Major Lipid Classes of Various Rat Tissues of Animals Maintained on a Chow Diet

	Percentages[a]								
	TG			PC			PE		
Tissue	Δ6	Δ7	Δ9	Δ6	Δ7	Δ9	Δ6	Δ7	Δ9
Brain	—	—	—	17.9	18.5	59.9	—	—	—
Heart	2.3 ± 0.8	5.5 ± 2.1	91.2 ± 2.3	5.8	16.1	74.6	13.0	11.8	70.7
Kidney	4.1 ± 2.4	7.2 ± 0.4	88.6 ± 2.6	19.4	19.4	61.3	8.5	17.2	74.3
Liver	1.3 ± 0.7	6.9 ± 1.8	90.4 ± 1.7	1.9	7.5	87.8	2.6	5.0	89.6
Lung	5.4	7.5	87.1	13.5	36.0	50.5	10.0	27.6	62.4
Muscle	8.7	4.3	87.0	15.0	24.1	58.5	—	—	—
Spleen	—	3.0	96.3	21.6	11.9	58.6	—	—	—
Adipose	2.6	7.4	88.2	30.6	22.3	45.1	—	—	—

[a]Percentages represent the mean ± standard deviation from analyses of four rats. Values without standard deviation represents the analysis of a pooled sample from four rats.

TABLE VI

Percentage Distribution of Positional Isomers of *Cis*-Octadecenoate Fatty Acids Isolated from the Major Lipid Classes of Various Rat Tissues of Animals Maintained on a Chow Diet

	Percentages[a]					
	TG		PC		PE	
Tissue	Δ9	Δ11	Δ9	Δ11	Δ9	Δ11
Brain	—	—	74.6 ± 0.2	25.4 ± 0.2	78.5 ± 1.2	21.5 ± 1.2
Heart	90.1 ± 1.8	9.9 ± 1.8	31.3 ± 1.5	68.7 ± 1.5	39.7 ± 2.1	60.3 ± 2.1
Kidney	89.0 ± 0.5	11.0 ± 0.5	68.8 ± 1.2	31.2 ± 1.2	90.5 ± 0.5	9.5 ± 0.5
Liver	80.9 ± 4.8	19.1 ± 4.8	36.1 ± 1.6	63.9 ± 1.6	31.7 ± 4.1	68.3 ± 4.1
Lung	90.5 ± 0.5	9.5 ± 0.5	70.0 ± 3.9	30.0 ± 3.9	82.5 ± 4.4	16.3 ± 4.4
Muscle	86.8 ± 2.1	13.2 ± 2.1	49.2 ± 2.8	50.8 ± 2.8	72.9 ± 6.8	27.1 ± 6.8
Spleen	88.8 ± 0.6	11.2 ± 0.6	50.5 ± 0.9	49.5 ± 0.9	67.1 ± 0.9	32.9 ± 0.9
Adipose	86.9 ± 0.3	13.1 ± 0.3	87.1 ± 1.2	12.9 ± 1.2	90.4 ± 0.7	9.6 ± 0.7

[a]Percentages represent the mean ± standard deviation from analyses of four rats individually.

proportion of oleic and vaccenic acids in both phospholipid classes. This is in good agreement with the observation made by Spence (13) earlier. Muscle and spleen phosphatidylcholines contained equal percentages of oleic and vaccenic acids, but phosphatidylethanolamines of the corresponding tissues contained higher percentages of oleic acid. The octadecenoates of kidney phosphatidylethanolamines contained a high percentage of oleate, similar to the triglycerides, whereas phosphatidylcholine octadecenoates consisted of approximately one-third vaccenate. Lung showed a similar distribution to kidney. Octadecenoates from the total phospholipids of kidney have been reported to contain ca. 25% vaccenate. Generally, the ratio of oleic to vaccenic acid was dependent on lipid class and the tissue origin.

DISCUSSION

Most of the data on the distribution of the geometrical and positional isomers of monounsaturated fatty acids in the various tissues is new, except for that on liver and brain. These data add to our basic information, and hopefully will contribute to our knowledge and understanding of the biological importance of these isomeric fatty acids.

These data demonstrate that various rat tissues from animals maintained for one month on a chow diet containing natural fats contain very low levels of *trans* hexadecenoates and octadecenoates. Large quantities of the octadecenoate fractions permitted an accurate determination of the *trans* isomer content despite the low percentages. The higher percentages of the *trans* octadecenoates in heart and liver phosphatidylcholines and phosphatidylethanolamines (Table IV) than in the other tissues may have resulted from the slow turnover of some isomers already present prior to being placed on the experiment. Since a large percentage of dietary fatty acids are removed and cleared through the liver (15) and large quantities are oxidized in the heart (16), any selectivity in metabolism might lead to accumulation of the *trans* isomers in these tissues. It is also possible that traces of *trans* fatty acids could be of dietary or bacterial origin. Fats from ruminants contain a substantial quantity of *trans* octadecenoates (17,18) that result in part from the biohydrogenation of dietary lipids by rumen microorganisms (19). The

possibility that the microflora of rodents or other simple stomach animals could also give rise to *trans* fatty acids does not appear to have been investigated.

Oleate and vaccenate were the predominant naturally occurring octadecenoate isomers in all the tissues examined, similar to what we had reported earlier for liver (1,20). The high percentage of vaccenate in liver phospholipids (1,20) was also common to most other tissue phospholipids, except adipose tissue and kidney phosphatidylethanolamine. Most of the tissues exhibited oleate to vaccenate ratios that differed for each of the major lipid classes, as was observed earlier for liver (1,20). In addition, many of the tissues contained oleate to vaccenate ratios in one or more classes unique to the tissue (Table VI). Some of these tissues may exhibit a further preferential distribution of the positional octadecenoate isomers at the 1- and 2-positions of glycerol in the phospholipid classes as was shown to occur in liver (21). These results clearly demonstrate that the distribution of octadecenoate positional isomers exhibits both lipid class and tissue specificity.

The importance of the preferential distribution of positonal isomers and the question of how presumably the same enzymes in different tissues create different oleate to vaccenate ratios are not understood. It is tempting to speculate that a higher percentage of either octadecenoate isomer reflects a tissue's need for a molecular species that contains the isomers. This may not be the case at all, but rather the class and tissue distributions of the octadecenoate isomers may be due to the origins of the isomers. We have shown that exogenous and endogenous oleate are not utilized equally for the biosynthesis of all lipid classes in cultured hepatoma cells (22). Preferential utilization of endogenous and exogenous octadecenoates in normal tissues could represent one way tissue lipid classes might contain different ratios of isomeric octadecenoates. It is possible that the origins of the octadecenoates used in the dihydroxyacetone phosphate (23,24) and α-glycerol phosphate (25,26) pathways might also differ, if one accepts the idea of multiple fatty acid pools or sources of which some are unavailable to certain acyl transferases, then the question of the preferential octadecenoate isomer distribution doesn't have to be explained on the basis of different enzyme specificity for the same substrate in different tissues. Multiple substrate pools and limited or restricted enzyme access to one or more sources of octadecenoates may only be partially responsible, if at all, but it would appear that such a concept might offer an alternative explanation to that of trying to interpret the observations totally on the basis of selectivity of multiple enzymes.

In contrast to the phospholipids, the monoenes from the triglycerides of the various tissues would appear to be of the same origin despite the large differences in concentrations (Table I). Generally, the percentage compositions of hexadecenoates and octadecenoates (Table II), the percentage of the geometrical isomers in the monoenes (Tables III and IV), and the percentages of the hexadecenoate and octadecenoate positional isomers (Table V and VI) were similar for all tissue triglycerides. Differences between tissue triglyceride compositions and the diet have indicated that the tissue triglycerides are not derived from a simple uptake of dietary fatty acid (11). How can the similarities in the hexadecenoate and octadecenoate positional isomer distribution in the various tissues be achieved when there are different routes of synthesis (27), different degrees of uptake (27), and an apparent lack of enzyme specificity in key enzymes (28)? One way might involve triglyceride biosynthesis in one or two tissues, possibly liver and adipose tissues, followed by transport to the various tissues. A central distribution of free fatty acids for triglyceride biosynthesis would also produce the same results. Transport of the triglycerides or fatty acids to the other tissues would need to be a recognizable complex to avoid mixing with plasma triglycerides and fatty acids that usually contain a higher percentage of polyunsaturated fatty acids (11,12). At the site of uptake in the tissues, any hydrolysis and resynthesis by one or multiple pathways should yield triglycerides with the isomeric octadecenoate composition if the process was compartmentalized and restricted to one or two sources of fatty acids or triglycerides. The described possible means by which all tissue triglycerides exhibit the same fatty acid and octadecenoate isomeric compositions may be incorrect, but hopefully it will initiate interest that will further our understanding of this biological process.

The biological function of oleate, vaccenate, or a specific ratio of these two octadecenoate isomers in any lipid class of any tissue is unknown; however, this does not mean they do not play an important role. Some indirect evidence that the lipid class specific ratios of oleate to vaccenate may be important is suggested by the fact these ratios are altered in the lipid classes from several tumors (1,2,29), plasma of host animals (30), and the liver of animals fed *Sterculia foetida* oil (3), a reported

carcinogen and co-carcinogen (31–33). The altered ratios of these two octadecenoate isomers associated with neoplasia may be secondary; however, it might represent our first evidence in the remarkably difficult task of demonstrating the essential role of a fatty acid that is synthesized de novo, in vivo.

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Fatty Alcohols in Capelin, Herring and Mackerel Oils and Muscle Lipids: I. Fatty Alcohol Details Linking Dietary Copepod Fat with Certain Fish Depot Fats

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ABSTRACT

It is shown that the shorter chain (C_{14}-C_{18}) minor fatty alcohols in copepods, fish body lipids, and commercial fish oils are all qualitatively present, and quantitatively similar in proportions to acids found in the depot fats of capelin and mackerel, and in some herring. Although these fatty acids can be formed de novo in fish, copepod alcohols offer an alternative dietary source. Monoethylenic fatty alcohol details, especially for the 22:1 isomers, are reviewed, and the latter are discussed as precursors of the 22:1 fatty acids of fish depot fats, specifically of the dominant 22:1ω11 isomer.

INTRODUCTION

The long chain (C_{20}-C_{22}) monoethylenic fatty acids of the depot fats of marine animals are plausibly derived from their diet (1), but may be modified slightly by species needs (1-3). At one time, the same fatty acids in fish such as herring *Clupea harengus* were thought to be biosynthesized on a basis of the needs peculiar to that species (4,5). A basic problem in accepting this hypothesis was that at that time the main 22:1 fatty acid isomer (6) was not clearly recognized as being 22:1ω11, whereas the precursor chain lengths have fatty acids of ω9 structure (e.g., 20:1ω9 can be formed by chain extension of 18:1ω9 and is the predominant 20:1 isomer in lipids of many fish, including certain freshwater species [7]). Hence, 22:1ω9 would have been expected to dominate herring oil fatty acids. Since that period, it has been determined that the longer chain fatty alcohols of the wax esters of copepods common in the North Atlantic include the same monoethylenic isomer types in each chain length as are found in herring oil (8,9). It was, therefore, considered desirable to compare the *unaltered* fatty alcohols found in the depot fats of fish feeding on copepods, specifically of commercially important species such as capelin *Mallotus villosus,* mackerel *Scomber scombrus* and herring, with those of the copepods. Both proportions among different alcohols and acids, and proportions among monoethylenic isomers, have been found to support the thesis that copepod fatty alcohols are converted directly to fatty acids on digestion by the fish. However, each species of fish may alter its overall depot fat composition to suit its particular needs.

EXPERIMENTAL

The commercial fish oils included a sample of capelin oil from fish taken off the south coast of Newfoundland in 1977, a Pacific herring oil (1973 production), an Atlantic mackerel oil and an Atlantic herring oil, both produced in the summer of 1973 at Shippegan, New Brunswick, from fish caught in the Gulf of St. Lawrence. The fish included capelin caught in the northern Gulf of St. Lawrence in the summer of 1975 (20 fish, av. length 20 cm, av. weight 13.4 g), mackerel caught off Halifax in the summer of 1978 (3 fish, av. length 36 cm, av. weight 762 g), and herring samples as follows: (a) Chedabucto Bay, January 1978; (b) southwest Nova Scotia, Mid-summer 1978 (2 fish, av. length 35.6 cm, av. weight 415 g); (c) southeast Nova Scotia, August 1978, (c1 = 2 male fish, av. length 21.5 cm, av. weight 405 g; c2 - 1 female fish, 29.8 cm., 245 g). The fish were measured, weighed, eviscerated and heads removed. Bodies (i.e., muscle and skin) were extracted by the method of Bligh and Dyer (10). The copepod lipids were the gift of the late Max Blumer and were recovered from unsorted northwest Atlantic copepods by centrifugation. The composition of these lipids was determined by the Iatroscan thin layer chromatography procedure (11). The commercial oils and the fish body lipids recovered from the chloroform layers were saponified by AOCS method Ca-6B-53. The recovered unsaponifiable materials were streaked on Prekote Adsorbosil 5 thin layer chromatographic plates (Applied Science Laboratories, State College, PA) and developed with hexane/diethyl ether/acetic acid (90:10:1). After spraying with

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2,7-dichlorofluorescein, the fatty alcohol bands were identified under UV light and extracted with hexane/$CHCl_3$ (1:1). The recovered alcohols were converted to acetates for gas liquid chromatographic analysis.

Wax esters and triglycerides were separated on and recovered from thin layer chromatography (TLC) on silicic acid plates. The fatty alcohols were analyzed by open-tubular gas liquid chromatography (GLC), with the liquid phase SILAR-5CP coated in stainless steel tubing 47 m in length and 0.25 mm I.D. (12). A portion of all esters was hydrogenated to verify quantitation (12) and to confirm identifications of components suggested by a standard alcohol mixture prepared by reduction of fish oil methyl esters to alcohols with $LiAlH_4$ (13). Data is presented to two decimal places for convenience in comparing small quantities, and accuracy is nominally within ± 10% for major components but decreases as percentages decrease. Monoethylenic acetates were concentrated by argentation TLC (14) and the alc 22:1 fraction collected from preparative GLC was monitored by open-tubular GLC. After oxidative ozonolysis in BF_3-MeOH and silylation (13), the products (monomethyl esters and half ester, half silyl ethers) were also determined by this technique. Fatty acids were recovered and examined by similar means (15).

RESULTS AND DISCUSSION

Unsaponifiables in Fish Body Lipids and Commercial Oils

The 1977 commercial capelin oil (Table I) contained 3.16% unsaponifiable material of which 15.5% was fatty alcohol. As far as is known, this oil came from nonfeeding fish in a prespawning condition and accordingly quite lean (16). Total lipid in the range 1 to 3% would be typical for these fish (17,18), and the 10.8% lipid of the Gulf of St. Lawrence capelin is indicative of the fat increase during the fall feeding period (18,19). The two results suggest that once deposited in depot fats of capelin during feeding, the fatty alcohols may not be especially affected by the mobilization of triglycerides. The concentration of alcohols in the total lipids, and hence in commercial oil, will be enriched accordingly. Lean capelin processed in Denmark (20) yielded ca. 1% oil with 3.54% unsaponifiable matter. The range in oils from different samples of fish was 1.34-3.56% unsaponifiables, with the proportion approximately inverse to oil recovery. Of the 3.54%, 52% was sterol but only 5-6% was fatty alcohol (roughly 30% 20:1 and 50% 22:1%). The data for fatty alcohols is thus remarkably

TABLE I

Sample Origin and Results of Examination of Lipids for Fatty Alcohols

Sample origin	%(w/w) Lipid in sample	%(w/w) Unsaponifiable material in lipid	%(w/w) Fatty alcohol in lipid	%(w/w) 16.0	Selected alcohol components Σ18:1	Σ20:1	Σ22:1
Capelin commercial oil (1977)	100	3.16	0.49	12.3	3.2	30.8	39.4
Capelin muscle and skin (1975)	10.8	1.96	0.19	6.8	1.6	18.7	68.4
Mackerel commercial oil (1977)	100	1.35	0.11	18.2	7.0	17.5	33.3
Mackerel muscle and skin (1978)	12.2	3.79	1.81	13.1	4.9	27.4	39.3
Pacific Herring commercial oil (1973)	100	1.08	0.05	18.4	35.3	5.0	5.6
Atlantic herring commercial oil (1973)	100	1.91	0.06	9.5	4.5	29.1	43.9
Atlantic herring muscle and skin (a)	3.3	1.62	<0.01	---	---	---	---
Atlantic herring muscle and skin (b)	17.8	0.59	0.03	10.7	7.6	21.5	22.7
Atlantic herring muscle and skin (c1)	22.8	0.74	0.01	---	---	---	---
Atlantic herring muscle and skin (c2)	17.0	0.58	<0.01	---	---	---	---
Atlantic herring, whole extract, Ref. (31)[a]	---			19.3	28.0	9.8	22.2
Atlantic herring, whole extract, Ref. (31)[a]	---	1.06	0.03	16.3	22.2	14.9	21.1
Icelandic herring oil, commercial Ref. (31)[a]	100	1.00	0.17	10.3	~5.0	~31	~52

[a]Additional material by private communication from A. Karleskind.

close to that given in Table I for a Newfoundland oil sample.

It is important to note that the physical storage of fat in mackerel and herring differs somewhat from that in capelin, where fat occurs mostly in the skin and bellyflap. In mackerel, the skin is still an important storage site, but muscle is equally important (21-23), while in herring the muscle is the chief depot fat site (22). This distribution factor may affect fat mobilization.

Mackerel are normally feeding when caught in the Gulf of St. Lawrence, and the 12.2% lipid in Nova Scotia coastal mackerel (Table I) also shows that feeding is in progress (23,24). The commercial mackerel oil contained 1.35% of unsaponifiable material, a value typical of Canadian marine oils from fish of this type (25) and also representative of values for lipid from muscle obtained earlier (24). The proportion of alcohols in this unsaponifiable material, ~10%, was similar to that in the capelin samples. However, the mackerel muscle sample unsaponifiables were exceptionally high (24). Of this material, about half was fatty alcohol. The remainder was apparently cholesterol (indicated by TLC). It seems that fatty alcohols are deposited in fat of mackerel much as suggested for capelin.

The commercial Pacific herring oil had a "normal" content of unsaponifiable matter but a very low level of total alcohols. This suggests that wax esters and related lipids were not common in the diet. The relatively low alc 22:1 (5.6%) and high alc 18:1 (35.5%) indicate that the food organisms were different in type of wax ester, but copepods from that region seem to include wax esters high in either 16:0 or 22:1 and not especially high in 18:1 (26-29). The commercial Atlantic herring oil also had a typical (25,30) total unsaponifiable content of (1.91%), but only a small proportion (3%) of alcohols in this lipid fraction. The alcohols did, however, show the high alc 22:1 (43.9%) typical of copepod lipids, but this could have originated in either or both of stomach contents or depot fat. The Atlantic herring muscle samples with very low (3.3%) lipid came from late winter fish, and initially it appeared reasonable to assume that in these herring, as distinct from the comparable lean capelin sample, mobilization of depot fat led to oxidation of alcohols to acids or even total catabolism of liberated fatty alcohols. This idea fitted the low level of fatty alcohol in the Pacific herring oil, but was offset by the Atlantic herring with 17.0% lipid and no fatty alcohol, and the samples with 17.8 and 22.8% lipid and only 0.02 and 0.01% fatty alcohol, respectively, in the body lipid. Literature data on alcohols from two herring oils (31) give total unsaponifiables of 1.06 (laboratory extract of whole herring, probably from the North Sea) and 1.0% (commercial Icelandic herring oil), with respective alcohol contents of 0.03 and 0.17% of oil or lipid (Table I). It will be noted that the fatty alcohols in the laboratory extract of commercial whole herring purchased in France resemble somewhat the Nova Scotia fish of lot 2 in having only ca. 20% of 22:1 alcohol. On the other hand, the commercial oil from Iceland has the four major alcohol components (Table I) in proportions very close to those of the local 1973 commercial oil. No definite conclusion can be drawn from these comparisons but the implication is that the Icelandic and Gulf of St. Lawrence fish were taken while feeding heavily on copepods and that the fatty alcohol is inclusive of stomach contents as well as fatty alcohols freshly deposited in the muscle. Small Danish herring (6.5% oil) had 0.02 – 0.04% alcohol in lipid, but large herring (12% oil) had alcohol $\leqslant$ 0.01% (20).

TABLE II

Class Composition (w/w%) of Copepod Lipid Recovered from a Mixed Northwest Atlantic Sample

Lipid class	w/w%
Polar lipid	5.6
Free fatty acid	0.8
Triglyceride	31.6
Wax ester	61.2
Sterol ester	0.8
Hydrocarbon	Trace

Copepod Lipids and Comparisons of Fatty Alcohols with Those in Fish Lipids

The copepod lipid sample (Table II) was dominated by the wax ester, a result in keeping with other research studies (8,32-35). The detailed study of the fatty alcohols from this lipid fraction provides, however, new information on the origin of fatty acids in the fish living on copepods. Table III shows that the important saturated alcohols 14:0, 16:0 and 18:0 are present in proportions similar to these of the fatty acids found in the depot of the fish studied (15). It had always been assumed that the minor fatty acids of fish such as iso 15:0, 17:0 etc. originated in the fish, in the course of fatty acid metabolism and turnover, or as dietary fatty acids, but the copepod fatty alcohols show that the complete spectrum of these materials can also be supplied in fatty alcohol form. These saturated alkyl chains are also available from the fatty acids of the wax esters and of the triglycerides of the copepod

TABLE III

Comparison of Saturated Copepod Fatty Alcohols with Those Recovered from Fish Bodies (Muscle with Skin on) or Commercial Fish Oils (w/w%)

	Saturated fatty alcohol acetates							
		Fish muscle and skin lipid			Fish commercial oils			
Fatty alcohol	Total copepod	Capelin	Mackerel	Atlantic herring	Capelin	Mackerel	Atlantic herring	Pacific herring
12:0	ND	0.12	ND	0.01	0.04	0.02	0.05	1.07
Iso 14:0	0.04	ND	0.01	0.19	ND	0.01	0.02	0.73
14:0	3.16	0.78	1.75	5.80	3.16	1.73	1.66	3.39
Iso 15:0	0.04	0.09	0.21	0.36	0.08	0.12	0.02	0.96
Anteiso 15:0	0.05	0.04	0.11	0.27	0.02	0.14	0.02	0.79
15:0	0.20	0.21	0.33	1.63	0.27	0.40	0.31	1.08
Iso 16:0	0.07	0.02	0.10	1.19	0.06	0.15	0.03	0.85
16:0	13.15	6.82	13.13	10.70	12.31	18.24	9.46	18.39
Iso 17:0	0.06	0.03	0.03	ND	0.05	0.15	0.01	0.13
Anteiso 17:0	0.02	0.02	0.01	ND	0.03	0.04	0.01	0.13
17:0	0.20	0.20	0.28	0.54	0.05	0.38	0.53	0.75
Iso 18:0	0.11	ND	0.11	ND	ND	0.12	ND	ND
18:0	0.47	0.71	0.39	2.95	0.98	1.49	3.27	12.41
19:0	0.07	0.03	0.09	ND	0.06	0.13	0.22	0.18
20:0	0.12	0.69	0.07	1.22	0.17	0.37	0.82	2.46
22:0	ND	ND	ND	ND	ND	0.11	0.31	0.43
24:0	0.01	0.08	ND	0.11	0.12	0.01	0.07	ND
7-methylhexadecanoate[a]	0.13	ND	0.04	0.29	ND	2.20	0.25	0.05
Total saturated	19.9	9.8	16.6	25.2	17.4	25.7	16.8	43.7

[a]Calculated from hydrogenated esters.

(15), and it appears that all originated in a common metabolic pool.

The saturated fatty alcohols recovered from the Atlantic fish body lipids and from the commercial oils all have compositions relating approximately to the corresponding copepod fatty alcohols. The different status of fatty alcohols in Atlantic herring bodies is supported by the enrichment of minor saturated alcohols such as iso and anteiso 15:0 and even 15:0. This effect may be peculiar to overall chain length since the percentage of 14:0 is also higher. The Pacific herring oil alcohols are very different in the emphasis on alc 18:0 and alc 20:0. There is, however, good reason to conclude that the residual saturated alcohols in the Atlantic fish body lipids originate in North Atlantic copepod wax ester alcohols. The saturated fatty alcohols in the commercial mackerel and Atlantic herring oils could be from the body lipids, or from stomach contents, or both sources, but the similarity serves to support the basic conclusion. The commercial capelin oil presumably came from lean, nonfeeding fish, so stomach contents should not be involved. It is remarkable that the composition of the alcohols from this oil sample matches the copepod composition very closely. Furnishing a fish with an excess of a minor fatty alcohol seems unlikely if copepods predominate in the diet, but in fish any accumulations of unusual shorter chain fatty acids are transitory, and the fatty acid composition returns to normal when input reverts to normal (36-38).

The totals for the monoethylenic fatty alcohols (Table IV) recovered from the fish bodies, or the commercial fish oils, are all similar in magnitude to the total for the copepods. In detail, this class of fatty alcohols is in all cases except one dominated by the C_{20} and especially C_{22} chain lengths. The Pacific herring oil is exceptional in this respect and clearly demonstrates the relation between 22:1 alcohols and acids (15,39). It is also noteworthy that the alc 20:1ω7 and alc 22:1ω7 isomers are rather more important, relative to the corresponding ω9 isomers, than one would expect on the basis of the proportions of alc 18:1ω9 and alc 18:1ω7. The only suggestion which can be made is that some mechanism, perhaps primed by an excess of 16:0, favors four-carbon elongation of alc 16:1ω7 to alc 20:1ω7, and that there is little selectivity in the conversion of alc 20:1ω7 and alc 20:1ω9 to the corresponding alc 22:1 isomers. The ratio of 18:1ω7 to 20:1ω7 is nominally 1:1 in all cases of Table IV. The four-carbon elongation has been discussed in fatty acid elongation in Brassica oils (14) where the ratio of ω7 and ω9 isomers was changed by suppression of elongation. One of the reasons for this study was to see if the peculiar concentration of alc 22:1ω11 could be explained. In this respect, these fatty alcohols from Pacific herring are normal in that the alc 22:1ω9 is about a tenth of alc 22:1ω11 (see also Table V). Whatever the exogenous food source or sources of alcohols for the Pacific herring, the proportions between the alc 22:1ω9 and alc 22:1ω7 isomers are unusual, but between alc 22:1ω11 and alc 22:1ω9 the usual proportions prevail. Thus, in this aspect of the marine food web, three mechanisms for chain elongation of monoethylenic fatty alcohols may have to be considered, respectively, affecting formation of alc 20:1ω11 and elongation to alc 22:1ω11 in one step, or a conventional progressive elongation in the ω9 series from alc 18:1ω9, or a special case, in some circumstances, of the ω7 series which must start with 16:1ω7.

The 22:1 alcohol and acid isomer comparisons are rearranged to proportions of 100% in Table V. There can be little doubt that the 22:1ω11 fatty alcohol is the origin of the unusual 22:1ω11 fatty acid. The three oxidative fission studies on fish fatty alcohols and acids suggest that 22:1ω13 acid usually exceeds alc 22:1ω13. This could be due to direct desaturation in the 9,10 position of 22:0 in either alcohol or acid form. Since 22:0 as a fatty acid is generally barely detectable (nominally ($\leqslant$ 0.1%) in marine oils (1,2,12,15), it may be that it is an unsuitable substrate for esterases or other processes and is disposed of by desaturation to the monoethylenic (22:1ω13) form. The absence (40) of 22:0 from sea water fatty acid containing 19.1% of 18:0 and 8.8% of 20:0 is another curious facet of the problem of the longer chain saturated acids in marine biochemistry. The 2.5% proportion of alc 22:1ω13 in the copepod lipids (Table V) may be compared with the figure of ca. 10% of the same alcohol isomer in a different sample of copepod wax ester (9). An alternative explanation therefore must be considered, that the dietary alcohol form is somehow altered in proportions of isomers in the course of the oxidation of all alc 22:1 isomers to fatty acids, reducing residual alc 22:1ω13.

In the polyethylenic alcohols (Table VI), with one exception, alc 18:2ω6 exceeds alc 18:3ω3, proportions familar in the fatty acids of most marine lipids (41,42). One of the surprising features of these alcohol analyses is that the C_{20} and C_{22} polyethylenic alcohols, except alc 22:6ω3, are at very low levels (including alc 20:5ω3). The unusual feature of

TABLE IV

Comparison of Copepod Monoethylenic Fatty Alcohols with Those Recovered from Fish Bodies (Muscle with Skin on) or Commercial Fish Oils (w/w%)

	Monoethylenic fatty alcohol acetates							
		Fish muscle and skin lipid			Fish commercial oils			
Fatty alcohol	Total copepod	Capelin	Mackerel	Atlantic herring	Capelin	Mackerel	Atlantic herring	Pacific herring
Σ14:1	ND	ND	ND	ND	ND	0.09	0.01	ND
Σ15:1	ND	ND	ND	ND	ND	ND	0.01	ND
16:1ω9	0.02	ND	0.03	0.25	ND	0.02	0.01	0.53
16:1ω7	3.72	0.53	3.00	2.84	7.067.06	2.14	0.90	2.20
16:1ω5	0.37	ND	0.26	ND	ND	0.54	0.10	0.58
Σ17:1	0.13	ND	ND	ND	ND	0.14	0.01	ND
18:1ω9	2.57	2.51	2.67	3.50	0.81	4.43	2.49	34.99
18:1ω7	1.62	1.06	1.91	3.21	1.75	2.18	1.74	1.65
18:1ω5	0.34	0.01	0.33	0.88	0.64	0.41	0.24	0.24
Σ19:1	0.03	ND	0.06	ND	ND	ND	ND	ND
20:1ω11	2.04	0.01	0.92	1.22	0.07	0.23	0.88	0.84
20:1ω9	28.86	16.93	24.69	17.37	26.05	15.93	25.48	2.58
20:1ω7	0.80	1.38	1.74	1.80	3.55	1.27	2.22	1.60
20:1ω5	0.01	0.01	0.15	0.24	0.66	0.01	0.50	ND
22:1ω11(+13)	33.73	57.70	32.85	20.06	35.02	31.23	39.80	4.61
22:1ω9ω	2.77	6.00	5.50	2.37	3.42	2.43	3.29	0.50
22:1ω7	0.68	1.48	0.93	0.62	0.70	0.25	0.71	0.48
Σ24:1	0.31	1.53	0.33	0.50	1.04	0.41	0.60	1.22
Total monoethylenic	78.9	89.2	75.3	54.9	80.7	61.1	79.0	52.0

TABLE V

Comparison of Proportions of Docosenoyl Isomers in Copepod Fatty Alcohols with Fatty Alcohols and Total Fatty Acid Docesenoic Acids of Fish Lipids

	Data from oxidative fission								
		Fish muscle and skin lipid				Commercial oil			
	Copepod alcohol	Capelin		Mackerel		Capelin			
		Alcohol	Acid	Alcohol	Acid	Alcohol	Acid		
22:1 Structure									
ω13	2.5	0.3	1.7	2.6	3.6	0.3	1.5		
ω11	87.6	88.2	87.1	81.0	88.8	89.7	87.5		
ω9	7.9	9.2	9.1	14.0	6.6	8.2	8.8		
ω7	2.0	2.3	2.1	2.4	0.9	1.8	2.2		
	Data from open-tubular GLC								
		Fish Muscle and skin lipid				Commercial oils			
	Copepod alcohol	Atlantic herring		Mackerel		Atlantic herring		Pacific herring	
		Alcohol	Acid	Alcohol	Acid	Alcohol	Acid	Alcohol	Acid
22:1 Structure									
ω11(+13).	89.8	86.8	78.4	91.9	91.5	90.9	92.5	82.5	79.8
ω9	8.2	10.5	19.0	7.3	7.2	7.5	6.0	8.9	17.7
ω7	2.0	2.7	2.3	0.7	1.4	1.6	1.5	5.6	2.4
ω5	---	0.0	0.4	---	---	---	---	---	---

TABLE VI

Comparison of Copepod Polyethylenic Fatty Alcohols with Those Recovered from Fish Bodies (Muscle with Skin on) or Commercial Fish Oils (w/w%)

	Polyethylenic fatty alcohol acetates							
	Fish muscle and skin lipid				Fish commercial oils			
Fatty alcohol[a]	Total copepod	Capelin	Mackerel	Atlantic herring	Capelin	Mackerel	Atlantic herring	Pacific herring
16:2ω6	ND	0.04	ND	ND	0.47	ND	ND	0.03
16:2ω4	0.02	0.01	0.12	ND	0.01	0.12	0.11	0.79
16:3ω3	0.14	ND	0.19	0.57	ND	0.29	0.12	0.61
16:4ω1	ND	ND	ND	ND	ND	ND	ND	0.13
18:2ω6	1.37	0.06	1.38	0.78	0.44	2.57	0.58	0.64
20:2ω6	0.17	ND	0.14	0.07	0.03	0.31	0.01	0.11
18:3ω6	0.01	ND	ND	3.11	ND	ND	0.01	0.05
18:3ω3	1.19	0.02	0.92	1.17	0.13	2.04	0.30	0.05
18:4ω3	0.06	ND	0.03	0.32	0.01	0.09	0.02	0.05
20:4ω6	0.04	ND	ND	0.56	0.01	0.16	ND	ND
20:4ω3	0.14	ND	0.09	0.01	0.01	0.36	0.07	0.01
20:5ω3	0.10	ND	0.13	1.30	ND	0.26	0.16	0.84
21:5ω3	0.14	ND	0.15	0.05	ND	0.25	0.07	ND
22:5ω6	0.01	ND	ND	0.01	ND	0.22	0.02	ND
22:5ω3	0.19	ND	0.29	0.01	ND	0.24	0.02	ND
22:6ω3	0.43	0.89	4.44	11.50	0.69	5.66	2.43	0.88
Total polyethylenic	4.0	1.0	7.9	20.0	1.8	12.6	3.9	4.2

[a]Other alcohols observed in trace amounts included 16:4ω3, 18:4ω1, 20:3ω3, and 22:4ω3.

this observation is that 20:5ω3 is the most likely fatty acid of this type to be found in the phytoplankter diet of copepods (43). Filter-feeding molluscs, for example, can readily assimilate such fatty acids (44). These results for the copepod sample are confirmed by an earlier study on a different sample (8) and the fatty acid-alcohol implications are considered elsewhere (15).

Experiments (45,46) with several diverse marine fish species show that the capability to oxidize fatty alcohols to acids is widespread. From the results of this study, three additional species can now be included, but there may be minor differences in natural feeds or in specific enzyme-fatty alcohol interactions requiring further study.

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Fatty Alcohols in Capelin, Herring and Mackerel Oils and Muscle Lipids: II. A Comparison of Fatty Acids from Wax Esters with Those of Triglycerides

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ABSTRACT

The fatty acids recovered from the triglycerides and wax esters of common northwest Atlantic copepods are compared with the fatty acids of wax esters recovered intact from certain fish skin and body lipid, and from commercial fish oils. The fish species, herring, capelin and mackerel, all feed on copepods, and many resemblances of the copepod lipid fatty acids to those of a previous analysis of similar copepods suggest that the basic dietary fat input for these fish may be quite constant. The two copepod fatty acid analyses differed quantitatively in triglyceride 20:1 and 22:1 and also in 20:5ω3 and 22:6ω3, confirming the primary role of the wax esters in copepods. Selectivity factors are discussed in comparing the copepod wax ester fatty acids with the fatty acids of the wax esters recovered intact from the fish lipids and oils. The basic role of copepods in supplying all types of fatty acids to fish depot fats is considered to be strongly supported by these findings.

INTRODUCTION

The small zooplanktonic crustacea classed as copepods often have lipid sacs containing depot fat reserves in the form of wax esters and triglycerides, usually with a higher proportion of the former (1,2). The long-held hypothesis (3) that the fatty alcohols of copepod wax esters were the basic origin of the longer chain monoethylenic fatty acids of capelin *Mallotus villosus*, mackerel *Scomber scombrus* and herring *Clupea harengus* was upheld by study of monoethylenic fatty alcohol details (4) conforming to general marine oil fatty acid patterns. However, it was noted that numerous minor fatty alcohols could be found which were qualitatively similar to those generally found in marine oils (4). In this section of the study, the total fatty acids of the copepod triglycerides and of all fish oils or body lipids are examined, and also the fatty acid moiety of the wax esters found in a copepod sample, in capelin lipids and commercial oils, and in a mackerel body oil.

EXPERIMENTAL

The details of origin and lipid recovery and fractionation for most samples are given elsewhere (4). The triglycerides of the copepod sample were isolated by preparative thin layer chromatography (TLC) and converted to methyl esters by reaction in 7% BF_3-MeOH for 60 min at 100 C in a screw cap centrifuge tube. All other samples used similar technology and represented whole oil or total lipid extracts from fish, or were acids recovered from analyses of wax esters for fatty alcohols. The methyl esters of fatty acids were examined by open-tubular gas liquid chromatography (GLC) as described for the acetates of fatty alcohols (4).

RESULTS AND DISCUSSION

In the saturated fatty acids of the copepod triglyceride, 14:0 at 19.8% (Table I) was three times higher than the 6.3% observed in a previous mixed copepod sample (5), but the 16:0 at 28.9% was close to the previous report of 25.5%. The percentages of three major saturated fatty acids of wax esters, 14:0, 16:0 and 18:0, were, however, all remarkably similar in proportions (respectively, 38.4, 11.2 and 0.3 vs. 32.2, 11.3 and 0.3) to those reported earlier. This supports the view that the wax esters are the basic lipid of copepods, the triglyceride being only an "auxillary" lipid reserve (6-9). The minor fatty acids of both copepod lipid samples were approximately parallel in proportions, and confirm previous details (5) such as the presence of more 20:0 and 22:0 in the triglyceride than in the wax ester acids.

The copepod monoethylenic fatty acids (Table II) show a very close similarity in the triglyceride and wax ester lipids except for the higher 16:1 in the wax ester fatty acids. This was also observed in the earlier copepod sample (5), the respective percentages for 16:1ω7 being 3.0 and 15.6. Thus, the obvious 16:1 difference from the former data is also in the triglyceride, the wax ester 16:1 being approxi-

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TABLE I

Saturated Fatty Acids (w/w%) of Copepod Triglycerides and Wax Esters, and of Total Lipid (Essentially Depot Fat Triglyceride), or of Wax Esters, Recovered from Fish Muscle and Skin Lipids

			Fish muscle and skin lipids					Fish commercial oils				
	Copepod		Capelin		Mackerel		Atlantic herring	Capelin		Mackerel	Atlantic herring	Pacific herring
Fatty acid	Triglyceride	Wax ester	Total acids	Wax esters	Total acids	Wax esters	Total acids	Total acids	Wax esters	Total acids	Total acids	Total acids
12:0	0.32	0.62	0.55	0.16	0.15	0.19	0.17	0.16	0.41	0.11	0.10	0.20
Iso 14:0	0.07	0.25	0.16	0.48	0.02	0.17	0.07	0.06	0.37	0.03	0.03	0.03
14:0	19.84	38.42	6.60	2.32	8.60	23.57	7.18	7.85	5.23	7.81	8.77	6.81
Iso 15:0	0.31	0.59	0.41	0.22	0.18	0.62	0.27	0.14	0.31	0.20	0.22	0.01
Anteiso 15:0	0.12	0.19	0.24	0.18	0.07	0.40	0.07	0.04	0.15	0.07	0.09	0.09
15:0	1.05	0.97	0.66	0.59	0.40	1.90	0.44	0.28	0.61	0.41	0.34	0.34
Iso 16:0	0.19	0.06	0.21	0.16	0.10	0.19	0.03	0.03	0.15	0.12	0.03	0.03
16:0	28.98	11.15	11.74	10.88	17.59	21.43	13.68	8.81	8.36	15.93	14.84	22.74
Iso 17:0	0.06	0.05	0.16	0.26	0.12	0.18	0.04	0.03	0.07	0.15	0.03	0.01
Anteiso 17:0	0.07	0.05	0.13	0.17	0.04	0.09	0.01	0.03	0.09	0.05	0.01	0.07
17:0	0.83	0.42	0.27	0.39	0.54	0.80	0.43	0.17	1.19	0.46	0.33	1.29
Iso 18:0	ND[a]	ND	ND	ND	ND	ND	0.01	ND	ND	0.07	0.04	ND
18:0	1.04	0.35	0.92	1.38	2.22	2.17	0.64	0.72	1.03	1.73	0.97	2.72
19:0	0.09	0.16	0.12	0.15	0.15	0.08	0.07	0.06	0.11	0.19	0.10	0.01
20:0	0.51	0.38	0.44	0.15	0.12	0.25	0.08	0.08	0.15	0.11	0.15	0.05
22:0	0.20	ND	ND	ND	ND	0.04	0.01	ND	ND	0.01	ND	ND
24:0	0.09	ND	ND	1.37	ND	0.11	0.07	0.17	0.37	0.03	0.01	ND
Total Saturated	53.8	53.7	22.6	18.9	30.3	52.2	23.3	18.6	18.6	27.5	26.1	34.4

[a]ND = not determined.

TABLE II

Monoethylenic Fatty Acids (w/w%) of Copepod Triglycerides and Wax Esters, and of Total Lipid (Essentially Depot Fat Triglyceride), or of Wax Esters, Recovered from Fish Muscle and Skin Lipids

			Fish muscle and skin lipids					Fish commercial oils				
	Copepod		Capelin		Mackerel		Atlantic herring	Capelin		Mackerel	Atlantic herring	Pacific herring
Fatty acid	Triglyceride	Wax esters	Total acids	Wax esters	Total acids	Wax esters	Total acids	Total acids	Wax esters	Total acids	Total acids	Total acids
Σ14:1	0.07	ND	0.32	ND	0.08	0.11	0.07	0.06	ND	0.11	0.07	0.22
Σ15:1	ND	ND	ND	ND	0.06	0.30	ND	ND	ND	ND	0.03	ND
16:1ω9	0.05	0.35	ND	0.96	0.42	1.80	0.09	0.03	1.58	0.29	ND	0.07
16:1ω7	8.89	12.33	12.89	7.32	9.57	8.65	15.74	15.42	18.16	8.20	7.22	7.53
16:1ω5	0.73	1.04	0.61	0.55	ND	0.28	0.32	0.73	0.12	0.54	0.52	0.45
Σ17:1	0.13	0.12	ND	0.24	0.16	0.06	ND	ND	0.11	0.15	0.04	ND
18:1ω9	3.14	3.46	6.15	6.90	9.47	7.23	4.36	4.40	5.95	8.61	12.27	29.72
18:1ω7	0.91	0.56	2.67	2.09	4.69	2.29	2.37	3.43	1.69	3.78	3.66	4.98
18:1ω5	0.41	0.15	0.35	0.78	0.59	0.50	0.53	0.62	0.65	0.54	0.64	0.41
Σ19:1	ND	ND	ND	ND	0.06	ND	ND	ND	ND	0.04	0.07	ND
20:1ω11	0.33	0.36	0.44	0.25	0.34	0.44	1.21	1.20	0.46	0.24	0.50	1.01
20:1ω9	4.12	4.37	14.83	3.36	7.41	5.75	12.03	14.53	9.34	10.59	14.37	4.40
20:1ω7	0.34	0.55	1.44	0.51	0.93	0.34	1.72	1.84	0.92	1.13	0.94	0.37
20:1ω5	0.02	0.01	0.06	0.07	0.03	0.05	0.18	0.23	0.10	0.09	0.19	ND
22:1ω11(+13)	5.16	4.59	18.41	2.92	9.40	9.85	15.40	17.45	5.90	12.74	20.92	3.92
22:1ω9	0.34	0.65	1.86	0.45	0.65	0.69	3.60	1.70	0.92	1.00	1.36	0.87
22:1ω7	0.11	0.11	0.42	0.08	0.09	0.12	0.43	0.42	0.19	0.19	0.33	0.12
Σ24:1	0.49	0.24	0.53	0.08	0.09	0.45	0.30	0.59	0.13	0.69	0.52	0.47
Total Monoethylenic	25.2	28.9	61.0	26.6	43.9	38.9	58.4	62.7	46.2	48.9	63.7	54.5

mately that observed earlier. Most importantly, both lipids differ in including 20:1 and 22:1 at ca. 5%, whereas, in the sample studied earlier, 20:1 was <1% and 22:1 present only in trace amounts.

Both fatty acid sources are quite low in 18:2ω6 and 18:3ω6 (Table III). As these are plentifully available from phytoplankters (10), it is likely that they are readily catabolized or converted to 20:5ω3 or 22:6ω3. In respect to 20:5ω3 both this copepod sample and that previously studied show triglyceride > wax ester for 20:5ω3 and confirm that the triglyceride can contain appreciable amounts of 22:6ω3 (27% in the earlier study), whereas both wax esters have very little 22:6ω3. The greater accumulation of 18:4ω3 (also readily available from phytoplankters) in wax ester than in triglyceride is also confirmed, and the proportions suggest that this is temporarily stored as an inert component, although in the copepod sample studied earlier, the low proportion (0.8%) in triglyceride relative to 20:5ω3 (14.1%) and 22:6ω3 (27.0%) suggests that 18:4ω3 can be converted to the higher polyethylenic acids if this is necessary.

The balance of the copepod polyunsaturated fatty acids are all present in minor proportions, but it is of considerable interest to find that fatty acids such as the unusual 16:2ω4, which are definitely of exogenous origin (10), were not converted to fatty alcohols in any significant proportion of the total (4), but instead are either catabolized or stored as acids in the wax esters rather than in the triglyceride (Table III, see also [5]). This preference for C_{16} fatty acids may simply reflect a general tendency for wax esters in marine animals to total 34 or 36 carbons (5). Since the copepod fatty alcohols include 31% C_{20} and 34% C_{22} in the monoethylenic alcohols alone, considerable C_{14} and C_{16} fatty acid is required to achieve this optimum, hence the high proportions of 14:0 and 16:0 (Table I). A total biosynthesis specificity may be lacking and unsaturated exogenous fatty acids of the correct chain length may be simply incorporated into wax esters, along with the saturated fatty acids which may be either synthesized de novo or be partly of exogenous origin (10). The previous copepod sample included 2.9% of 16:4 in the wax esters, probably 16:4ω1 (5), and in the current sample the 16:4 figure is 1.4%, but of the different isomer 16:4ω3. Both can be of phytoplankter origin (10).

Intact wax esters were recovered from capelin and mackerel body and muscle lipid, and from commercial capelin oil. The fatty acids of the wax esters can then be compared with the actual fatty acids of the corresponding depot fats of the fish, termed total fatty acids in Table I-III. The contribution of the fatty acids from the polar lipids can be ignored in total lipids as being too low to be important in these particular fish species (11) when fat contents were high (4).

The deposition of the fatty acids from copepods depends on the availability of lipases active against wax ester and triglycerides (12-15), and on the subsequent catabolism or conversion process in the fish body. The recovered capelin body muscle wax esters, and the commercial capelin oil wax esters, contained quite low levels of saturated acids (19% respectively, Table I) compared to the totals in the copepod wax ester (54%). On the other hand, the mackerel body and muscle wax ester had a total of 52% saturated esters, containing two-thirds the 14:0 of the copepod wax ester, and twice the 16:0 plus 18:0.

The capelin wax esters appeared to contain approximately the same monoethylenic acids as the copepod wax esters with differences in each chain length which are difficult to explain. The proportions among the monoethylenic isomers in each chain length are, however, relatively consistent except for the higher proportion of 18:1ω7 to 18:1ω9. The mackerel wax ester fatty acids include the same ranges of monoethylenic fatty acids except for 22:1, which is somewhat higher. The mackerel and capelin 18:1ω7/18:1ω9 proportions are consistent, but the different (lower) proportions of 18:1ω7 in the copepod wax ester fatty acids is confirmed by the earlier study (5), the weight percentages being, respectively, 3.28 and 0.64 for 18:1ω7 and 18:1ω9.

The minor polyunsaturated wax acids from the two capelin samples (Table III) are close in detail to those of the copepod wax esters. However, a remarkable enrichment appears in the 20:5ω3 of the capelin wax esters ~40% for capelin body, 22% for commercial capelin oil, vs. 5.8 in the copepod wax ester and 8.6% in the copepod wax ester sample examined earlier (5). This is extended to the 22:6ω3, 6.3 and 5.3%, respectively, as against 0.6 and 0.5 in the copepod wax ester fatty acids. The 18:4ω3, notable in the copepod wax esters, has dropped to about 2% or approximately to the higher end of the range of values typical of fish oil fatty acids (Table III). In the mackerel wax ester, the total polyunsaturated acids are quite low at 8.9%, and compared to the "normal" percentage of, for example, 18:2ω6, it can be concluded that 18:4ω3, 20:5ω3 and 22:6ω3 are all partially excluded instead of being enriched as in the capelin. Despite this, both mackerel

TABLE III

Polyethylenic Fatty Acids (w/w%) of Copepod Triglycerides and Wax Esters, and of Total Lipid (Essentially Depot Fat Triglyceride), or of Wax Esters, Recovered from Fish Muscle and Skin Lipids

			Fish muscle and skin lipids					Fish commercial oils				
	Copepod		Capelin		Mackerel		Atlantic herring	Capelin		Mackerel	Atlantic herring	Pacific herring
Fatty acid	Triglyceride	Wax esters	Total acids	Wax esters	Total acids	Wax esters	Total acids	Total acids	Wax esters	Total acids	Total acids	Total acids
16:2ω6	ND	0.20	0.76	ND	0.04	ND	0.03	0.84	0.04	ND	ND	0.75
16:2ω4	0.09	0.52	0.13	0.03	0.48	0.09	0.25	0.06	0.15	0.45	0.23	0.27
16:3ω3	0.26	0.01	0.96	0.09	0.48	0.17	0.88	0.82	0.11	0.52	0.39	0.44
16:4ω3	0.32	1.35	ND	ND	0.49	ND	1.30	ND	0.01	0.44	0.39	0.01
16:4ω1	ND	ND	1.52	0.82	ND	0.08	ND	1.47	2.10	ND	0.03	0.42
18:2ω6	0.97	0.83	0.92	1.03	1.04	1.21	1.11	0.78	0.86	1.28	0.78	0.67
20:2ω6	0.04	0.04	0.17	0.08	0.13	0.09	0.03	0.05	0.06	0.24	0.09	0.14
18:3ω6	0.16	0.14	0.09	0.12	0.10	0.06	0.02	0.01	0.13	0.07	0.04	0.07
18:3ω3	1.08	1.00	0.87	0.74	0.74	0.62	0.45	0.20	0.36	0.99	0.39	0.18
18:4ω3	3.23	5.63	1.57	1.80	1.96	1.30	1.60	1.36	1.87	2.47	0.93	0.67
20:4ω6	0.29	0.19	0.29	0.60	0.27	0.17	0.22	0.14	0.52	0.36	0.24	ND
20:4ω3	0.38	0.62	0.21	0.52	0.55	0.31	0.34	0.28	0.27	0.50	0.22	0.29
20:5ω3	8.38	5.81	6.11	40.19	9.39	2.74	7.29	9.35	22.38	7.64	2.85	5.31
21:5ω3	0.21	0.24	0.36	0.47	0.21	0.11	0.10	ND	0.24	0.41	0.13	0.09
22:5ω6	0.09	0.01	0.07	ND	0.02	0.05	0.20	0.03	ND	0.01	0.03	ND
22:5ω3	0.60	0.23	0.52	0.64	1.17	0.15	0.75	0.60	0.74	0.57	0.37	0.22
22:6ω3	4.90	0.64	1.87	6.30	8.73	1.73	3.72	2.70	5.32	7.66	2.70	1.53
Total Polyethylenic	21.0	17.5	16.4	54.5	25.8	8.9	18.3	18.7	35.2	23.6	10.2	11.1

and capelin total acids, when compared to recovered wax ester fatty acids, show an enrichment of nearly two-fold in 22:6ω3 relative to 20:5ω3. Whatever transfer process is taking place must be basically similar in both species. The overall impression of this particular lot of wax esters recovered from the mackerel body and skin is that it includes a high proportion of unaltered wax esters. The recovery of fatty alcohol from the total lipid was 1.8%, the highest of all samples examined (4). This also suggests an unselective assimilation process. It seems unlikely that this is peculiar to this one sample (3 fish were pooled), especially as mackerel are notorious heavy feeders and rapidly increase their depot fat during a few months in the Gulf of St. Lawrence (16). The distribution of fat among the various body organs of mackerel and capelin is different with species (17,18) but this is consistent with season and not apt to affect the digestive process. Hydrolysis of wax esters in the gut of several marine fish species is slower than that of triglycerides (13), and absorption of unhydrolyzed wax ester is established (12) for the gourami (*Trichogaster cosby*).

Pancreatic lipase is known not to be very active against 20:5ω3 in triacyl glycerol form (15), but with methyl esters of these acids as substrate several fish intestinal fluids did not discriminate (13) in hydrolyzing 20:4ω6 or 20:5ω3. The remarkable level of 20:5ω3 (Table III) in the fatty acids of the wax esters recovered from the capelin body and muscle lipid, or the capelin commercial oil, could be due to an adverse selectivity in capelin, leaving unhydrolyzed wax ester rich in 20:5ω3 (and 22:6ω3) to cross the intestinal wall intact as reported in the gourami (12). Alternatively, 20:5ω3 could cross the intestinal wall as the acid or monoglyceride and for unknown reasons be preferentially re-esterified into wax esters in the blood. Preparations of hepatopancreas and digestive organ of the myctophid *Diaphus gladulifer* can execute this type of lipid class formation with labeled 16:0 and 18:1, both being recovered in wax ester from (19). However, it should be observed that myctophids generally are expected to include wax esters as a necessary lipid (2), whereas in capelin no biochemical role for wax esters is known despite consistent reports of high proportions of fatty alcohols in the total lipid (1,20).

Unpublished observations (2) indicate that the formation of new glycerol in the gut mucosa may be a limiting factor in the formation of fish body triglycerides. If there were a deficiency in glycerol, then the freshly absorbed monoglyceride derived from copepod triglyceride, which could retain 20:5 (and 22:6ω3) in the 2-positions (21), might be a preferred source of glycerol if the fatty acid were simply transesterified to the fatty alcohol. The latter would be accumulating in cells, in the absence of glycerol, to esterify the fatty acids newly formed by oxidation of the fatty alcohols. Any discussion of the 20:5ω3 and 22:6ω3 has to consider if these are incorporated into the phospholipids of fish muscle and organs as preformed C_{20} and C_{22} fatty acids, or are elongated in situ from C_{18} precursors 18:3ω3 or 18:4ω3. There is a modest reduction in 18:3ω3 in going from copepod to fish, and a major reduction in 18:4ω3. There is no reason to suspect that copepod 18:4ω3 is converted into the high proportions of 20:5ω3 observed in the wax ester recovered from capelin (Table III), but in the myctophid homogenate (19) labeled acetate plus unlabeled substrate (i.e., fatty alcohol) did lead to 12% inclusion of label in wax esters, 34.9% in free fatty acids, and 36.8% in phospholipids.

The total fatty acids of the various fish depot fats (Table I-III) are all quite normal relative to similar published analyses for eastern Atlantic samples (11,22), provided that the total of 16:1 and 18:1 is taken and these two acids are regarded as interchangeable (23,24). This effect can also be observed in the two local Atlantic herring samples of Table II (23.4% and 24.3%, respectively, for the body plus skin and for the commercial oil). The local capelin lipids (Table III) do show the percentage of 20:5ω3 to be about four times that of 22:6ω3, whereas in a recent publication (3) the Norwegian capelin have 20:5ω3 and 22:6ω3 nominally equal and totaling two to three times the percentages in the western Atlantic oils given in Table III. The Pacific herring oil, as already pointed out (4), differs from Atlantic herring oil in various respects possibly linked to dietary factors. The absence of 20:4ω6 in this oil sample is especially remarkable.

This investigation of the deposition of wax esters and fatty alcohols in fish bodies confirms the major importance of copepods as sources of preformed alkyl chain of all types, ranging from the iso acids always found in marine lipids at low (<1%) levels, to the polyunsaturated fatty acids which nominally distinguish the depot fats of marine fish and mammals from those of other higher life forms. The unusual 22:1ω11 monoethylenic isomer is already preformed as the alkyl chain of the fatty alcohol in the copepod and previous speculation on the role of 22:1 synthesized do novo in herring biochemistry (24) must be reconsidered since the

proportions of 22:1ω9 which can be biosynthesized by herring is always very minor relative to 22:1ω11. The herring appears to deposit relatively little wax ester or alcohol, whereas the mackerel and capelin do show this capability, although in different ways, and therefore warrant further investigation.

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The Role of Gastric Lipolysis on Fat Absorption and Bile Acid Metabolism in the Rat

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ABSTRACT

In vivo studies were carried out in young Sprague-Dawley rats to examine the role of gastric lipolysis on fat absorption and bile acid metabolism. When fed by gastric perfusion 5 times (corn oil, 4 g/day) their usual dietary intake of fat, rats deprived of lingual lipase by the creation of an esophageal fistula had a significant degree of fat and bile acid malabsorption as well as a shortened bile acid half-life when compared to animals with a gastrostomy. The % fat absorption, bile acid loss and bile acid pool were normal in 2 groups of esophageal fistula rats fed the same quantity of corn oil or twice (8 g/day) that amount as a fine emulsion. In view of a negligible gastric lipase activity in animals with an esophageal fistula and of decreased hydrolysis of a triglyceride test meal, these data suggest that gastric lipolysis is of physiological importance in situations where lipolytic mechanisms are stressed by a large fat intake. Its principal role is to potentiate intestinal lipolysis by facilitating the emulsification of dietary lipids through its formed products and, therefore, the contact of pancreatic lipase with its substrates.

INTRODUCTION

It was shown more than 20 years ago that dietary triglycerides undergo a certain degree of lipolysis in the stomach (1). Lipolytic activity other than the one dependent on pancreatic enzymes was located in the stomach and found to be particularly active on milk triglycerides and medium chain triglycerides in both the suckling rat (2) and in man (3). Gastric lipolysis is due to a pregastric lipase originating in von Ebner's glands (Fig. 1) located beneath the circumvallate papillae on the posterior third of the dorsal surface of the tongue in both the rat (4) and in man (5).

During early postnatal life, the demands on the mechanisms for lipid absorption are high. Not only is food intake large in relation to body weight, but the lipid content accounts for more than 50% of total calories (6). In contrast to this high demand on the mechanisms for lipid digestion and absorption, the intestinal lipolytic mechanism has a low activity (7) and leads to a significant degree of fat malabsorption (8) particularly in preterm infants (9,10). The problem is further compounded by an excessive fecal loss of bile acids which varies with the source of fat and with the extent of fat malabsorption (10), a small pool size (11) and impaired micellar solubilization of lipolytic products (12). Only the normal full term neonate on breast milk can achieve a normal coefficient of fat absorption. It is likely that the bile-salt stimulated lipase present in human milk (13) and the high lingual lipase activity found in gastric aspirates of newborns and even of prematures (14,15) increase the efficiency of fat absorption.

In healthy adult humans, preduodenal lipolysis is considered to be a minor component of the overall lipolytic system. The importance of gastric lipolysis is perhaps limited to the infantile period (3). The physiological function

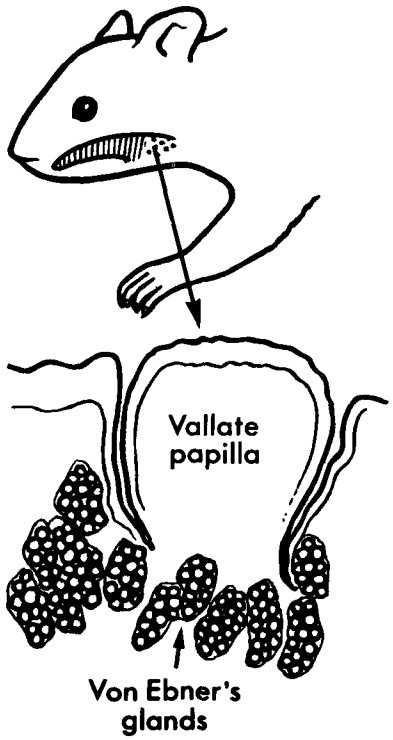

FIG. 1. Von Ebner's glands are a group of branching tubuloalveolar serous glands. The glands are embedded in the underlying muscular tissue of the tongue. Their ducts open into the trough at the base of the circumvallate papillae on the posterior dorsal 1/3 of the rat tongue.

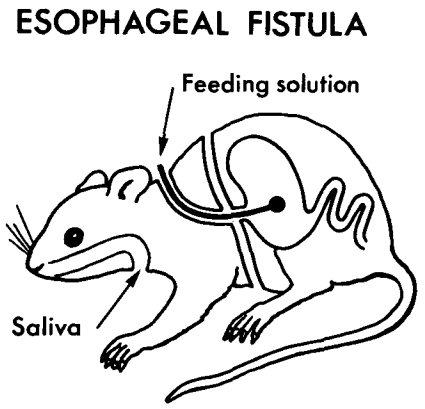

FIG. 2. The esophagus is transected in the neck and its proximal portion is brought to the skin. The distal portion is tied around a feeding tube.

and significance of lingual lipase and of gastric lipolysis remains unsettled. The present study was undertaken to determine the role of gastric lipolysis on fat absorption and bile acid metabolism in young rats.

PROCEDURES

Lipase and Lipolysis in the Stomach of Rats.

In a first step, we studied lipase activity in the stomach of young male Sprague-Dawley rats weighing 180 to 200 g. After sectionning the cervical portion of the esophagus, the pylorus was tied around a silastic tube to prevent contamination of gastric contents with pancreatic lipase. Controls had the pyloric part of the surgical procedure only. Exhaustive washing out of the stomach with saline was carried out. Two hr later, gastric contents were collected for a period of 3 hr. After centrifugation, lipase activity was immediately measured using a tributyrin (Sigma, St. Louis, MO) substrate (16).

In a second step, we studied gastric lipolysis of a fatty test meal. By cervical esophagostomy, a feeding tube was placed in the stomach and the cervical end of the esophagus was tied. A thread with a slip-knot was passed around the pylorus. Controls had the pyloric procedure and underwent a gastrostomy. They were then fed a liquid diet for 20 hr. Six hr after discontinuation of the alimentation, the pylorus was tied and one ml of a test meal was pushed in the stomach. The test meal was composed of corn oil 5% and ^{3}H-glyceryl-triolein 0.5 μCi added to casein hydrolyzate 5% at pH 5.5. It was blended. Exactly 1 hr later, exhaustive washing out of the stomach with a chloroform-methanol mixture was carried out. Glyceryl products were separated by thin layer chromatography (17), counted and expressed as the % of triglycerides hydrolyzed per hour.

Esophageal Fistula and Gastrostomy Animals Fed Corn Oil

An esophageal fistula was created in male Sprague-Dawley rats weighing 170 to 180 g. Through a midline incision in the neck, the esophagus was freed from the trachea and sectioned. The cephalic end was brought to the skin to allow drainage of saliva (Fig. 2). A 5F feeding tube was positioned in the stomach through the caudal esophagus. Control animals underwent a gastrostomy through which a feeding tube was positioned in the fundus. Both groups of animals were fed continuously at a rate of 3.3 ml/hr the following mixture: casein hydrolyzate 2%, sodium caseinate 3%, dextrimalose 9% and KCl 25 meq/l (7.5 meq/l for the control group). Twenty four hr after the operation, 4 g/day of corn oil was added to the carbohydrate-protein liquid meal via a separate syringe. Every 6 hr, 1 g of corn oil was given over a 30 min period. This feeding program provided the animals with 82 kcal per day; its lipid content was 5 times the amount contained in rat chow. Forty eight hr later, ^{14}C Na cholate (New England Nuclear, Chicago) 1.4 μCi was administered I.V. Stools were collected in separate aliquots over the following 4 to 6 days and analyzed for total fat (18), nitrogen (19) and bile acids (20). The decline of bile acid specific activity (DPM/mg bile acid) was plotted to estimate the bile acid 1/2 life (21).

Esophageal Fistula Animals Fed a Corn Oil Emulsion

In a second set of experiments carried out in rats with an esophageal fistula, the corn oil (4 to 8 g/24 hr) was mixed with the carbohydrate-protein liquid meal. The mixture was sonicated and the emulsion stabilized with gum acacia 1%. The emulsion remained stable for periods of 8 hr and was fed at a rate of 3.5 ml/hr. After 24 hr, a 3 day stool collection was made for fat and bile acid excretion. On the 4th day, a biliary fistula was created for the estimation of pool size from a 6 hr collection of bile using Eriksson's washout technique (22).

RESULTS

Lipase activity ($\bar{X} \pm SE$) expressed as μmole/min/ml in 6 rats deprived of lingual lipase was 0.02 ± 0.02 as compared to 3.82 ± 1.50 in 10 controls.

Lipolytic products (%) after a test meal were determined in 6 rats with transection of their

esophagus and in 6 controls. Monoglycerides were lower ($p<0.05$) in experimental animals (0.10 ± 0.10) than in controls (2.08 ± 0.70). Free fatty acids were also lower ($p<0.05$). Values of 4.75 ± 1.76 were obtained vs 10.05 ± 3.55 in controls. There was no significant difference for the diglycerides.

Rats with an esophageal fistula who were fed corn oil by push had softer and more frequent stools than gastrostomy controls. The former lost weight at an average rate of 4 g daily. However, their daily electrolyte patterns were normal and they showed no signs of dehydration. In contrast, a mean daily weight gain of 2.5 g was noted in the gastrostomy animals. As seen in Figure 3, rats deprived of lingual lipase lost more fat in their stools than gastrostomy controls. The calculated % of fat absorption was 59.7 ± 7.0 in the fistula group and 88.5 ± 7.5 in controls. There was little difference in nitrogen losses. The % absorption was 90.2 ± 15 and 92.0 ± 15 for the experimental animals and the controls, respectively. Figure 4 shows that the daily loss of bile acids was nearly doubled in the esophageal fistula group. Figure 5 is a representative plot of the decline of bile acid specific activity. It illustrates the significant difference in bile acid 1/2 life between the two groups of rats (Fig. 4).

The groups of esophageal fistula rats fed 4 g and 8 g of emulsified corn oil had a normal % fat absorption (Fig. 6). There was no difference between the 2 groups of animals in terms of daily bile acid loss and bile acid pool (Fig. 6).

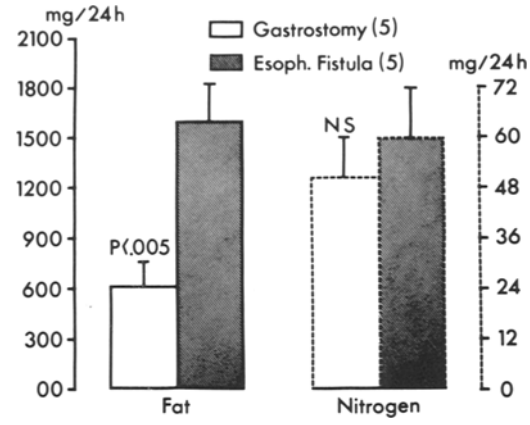

FIG. 3. The daily fecal fat and nitrogen loss ($\bar{X}$ ± SE) in rats with an esophageal fistula or a gastrostomy. They were fed 4 g/daily of corn oil by gastric perfusion. The number of animals are in parentheses. NS = non significant.

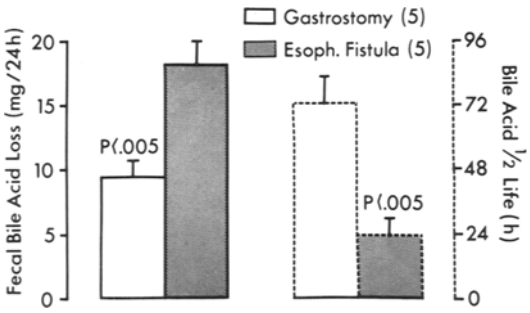

FIG. 4. Daily bile acid loss and bile acid half-life in the enterohepatic circulation calculated by the ratio of fecal radioactivity per mg bile acid over a 4 to 6 day period. Results are expressed as $\bar{X}$ ± SE. Number of animals are in parentheses.

DISCUSSION

These data show that rats in whom salivary flow was diverted through an esophageal fistula and given by gastric perfusion 5 times their usual intake of lipids lost weight, had diarrhea and fat malabsorption. The daily loss of fecal bile acids which is the only accurate method of measuring daily hepatic synthesis in conditions where there is a significant interruption of the enterohepatic circulation of bile acids (23) was increased. The rate of disappearance of a labeled bile acid from the enterohepatic circulation can be monitored by measurement of the fecal radioactivity in relation to the excretion of a nonabsorbable marker (24). In this study, it was obtained by the more accurate technique of calculation of the decay constant of fecal bile acid specific activity (21) and showed a shortened half-life for cholic acid and its metabolites.

When the same quantity of corn oil (4 g) or even twice that amount was given as a fine emulsion to 2 groups of animals deprived of saliva and therefore of lingual lipase, both fat and bile acid absorption were normal. The somewhat increased bile acid pool in the rats receiving 8 g daily of the corn oil emulsion could have been secondary to the effect of polyunsaturated fats on the bile acid pool (25).

The role of unabsorbed lipids on bile acid absorption has not been extensively studied. Experimental studies show that, in the rat, fatty acids inhibit the absorption of bile acids in the jejunum (26) and in the ileum (27). In both cystic fibrosis children (28) and in prematures (12), a close relationship was found between fat and bile acid malabsorption.

Lingual lipase is a polypeptide with a molecular weight between 40,000 and 50,000. In contrast to pancreatic lipase, it has a pH optimum of 5.4. It is resistant to acid, is not inhibited by bile acids and yields partial gly-

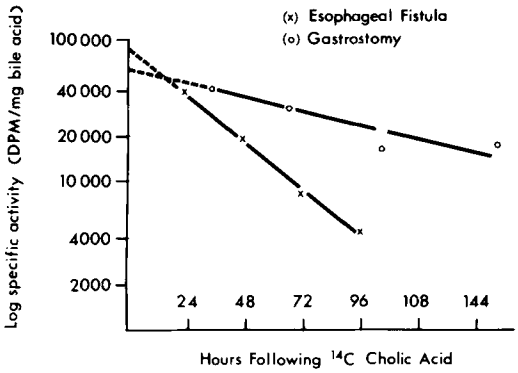

FIG. 5. The rate of disappearance of cholic acid and of its metabolites from the enterohepatic circulation was plotted over a 4 to 6 day period by the determination of the decay constant (k) of bile acid specific activity. The half-life (0.69/k) shown in this figure was 24 hr for the esophageal fistula animal and 73 hr in the gastrostomy rat.

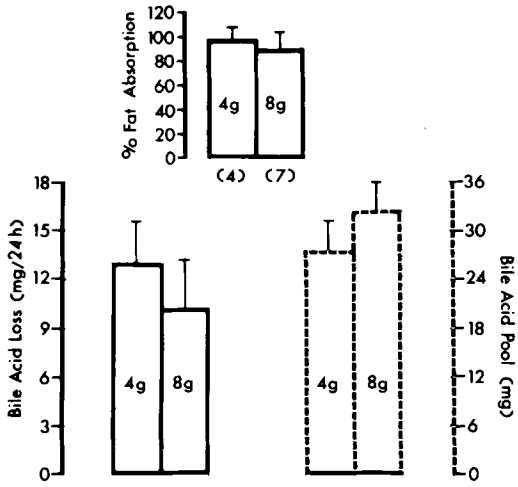

FIG. 6. The % fat absorption, bile acid loss and bile acid pool in (4) and (7) esophageal fistula rats, respectively, fed 4 and 8 g of corn oil as a fine emulsion. Results are expressed as $\bar{X} \pm SE$.

cerides and free fatty acids in both the rat (4) and in man (29,30). The rates of gastric lipolysis assessed in 3 infants with pyloric stenosis was thought to account for a third of the total lipolysis (31). More recently, gastric lipase activity was found to be considerably increased during test meals in the neonatal period (32).

In the present study, lipase activity was negligible in the stomach of rats deprived of lingual lipase and in which reflux of pancreatic lipase was prevented by ligature of the pylorus. Lipolytic activity against triglycerides was significantly lower in experimental rats as compared to controls.

Although this is the first study investigating the physiological importance of lingual lipase on the overall process of fat digestion and absorption, it is tempting to surmise that the present results provide a partial explanation for the observation that, in low birth weight newborns fed via a nasoduodenal tube, 50% more fat was lost in the stools when compared to those fed via a nasogastric tube (33). The results showing that animals with diversion of salivary flow have a good tolerance for large amounts of fat given as a fine emulsion suggests that in situations where normal digestive mechanisms are stressed by a high lipid intake or by defective lipolysis, lingual lipase may be essential. It not only catalyzes the first step in the digestion of dietary fat, but perhaps more importantly it facilitates through its formed products the dispersion and emulsification of dietary lipids in order to increase the effectiveness of pancreatic lipase.

ACKNOWLEDGMENT

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METHODS

Synthesis of Saturated, Unsaturated, Spin-Labeled, and Fluorescent Cholesteryl Esters: Acylation of Cholesterol Using Fatty Acid Anhydride and 4-Pyrrolidinopyridine

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ABSTRACT

A rapid, high yield method for the preparation of cholesteryl esters is described. The method is a modification of the catalytic procedure previously applied to the acylation of sn-glycero-3-phosphorylcholine (Patel, K.M., J.D. Morrisett, and J.T. Sparrow, J. Lipid Res., 20:676 (1979). Cholesteryl esters are formed in excellent yield by acylating cholesterol with fatty acid anhydride or fatty acid and dicyclohexylcarbodiimide in methylene chloride containing 4-pyrrolidinopyridine. The versatility of the method is demonstrated by the preparation of the cholesteryl esters of saturated, unsaturated, spin-labeled, and labile fluorescent fatty acids.

INTRODUCTION

The value of fluorescent probe molecules for studying structure and organization of membranes and serum lipoproteins has been well established. Unfortunately, many such probe molecules are structurally different from the lipid matrix into which they have been introduced; hence, a rigorous correlation between their fluorescence properties and environmental structure may not always be valid. These problems have been reduced by the development of a new class of naturally occurring, conjugated, linear polyene fatty acids, parinaric acid and eleostearic acid (1,2). However, a considerable obstacle to their use in any form other than free fatty acid has been due to their polymerization at elevated temperatures, double bond migration under acidic conditions, and their tendency to isomerize or oxidize under all but the mildest conditions. Our need for fluorescent cholesteryl esters for the study of nonpolar regions of serum lipoproteins served as an impetus to define nondestructive reaction conditions suitable for esterification of conjugated polyene fatty acids.

Cholesteryl esters have been synthesized (3) in the past by heating cholesterol and the appropriate fatty acid at 200 C for 3-4 hr under CO_2. Cataline et al. (4) used benzene as a solvent and acid catalysis to obtain cholesteryl esters, but their yields were poor. These methods have been limited to saturated cholesteryl esters. Acid halides (3,5-7) can also be used, but they afford poor yields and are often difficult to prepare in pure form. Mahadevan and Lundberg (8) utilized transesterification of a fatty acid methyl ester with cholesteryl acetate and sodium ethylate. Fatty acid anhydrides also react with cholesterol, but their reactivity decreases with increasing molecular weight (9), the anhydrides of palmitic and higher acids being inert to acylation. Morrisett (10) used the spin-labeled fatty acid anhydride with pyridine but found that longer reaction times were required, and the yield of cholesteryl esters was poor. These procedures have proven unsuited to the preparation of cholesteryl parinarate.

Recently we reported the use of 4-pyrrolidinopyridine (11) as a catalyst for the acylation of *sn*-glycero-3-phosphocholine with fatty acid anhydrides. The rapidity of the reaction and the stability of parinaric acid to the reaction conditions led us to use this same base for the acylation of cholesterol. We have developed two procedures: one involves the acylation of cholesterol with a slight excess of fatty acid anhydride and 4-pyrrolidinopyridine in benzene or methylene chloride at ambient temperatures to obtain high yields of cholesteryl esters; the other involves reacting equimolar quantities of cholesterol, fatty acid, and dicyclohexylcarbodiimide with a catalytic amount of 4-pyrrolidinopyridine in methylene chloride. The purification of the product was accomplished by chromatography on a silica gel column with a step gradient of hexane/ethyl acetate. The cholesteryl ester was collected and the solvent evaporated to give a crystalline compound.

The effectiveness of the procedure has been verified by the preparation and characterization of not only cholesteryl *cis*- and *trans*-parinarate,

TABLE I

Cholesteryl ester	% Yield		Melting point	
	Method A	Method B	Obs.	Lit.
Butyrate	94	---	100	100
Myristate	88	75	69-70	70
Palmitate	90	---	77	77
Elaidate	95	---	62	---
Linoleate	92	---	41	42
cis-Parinarate	59	60	67	---
trans-Parinarate	27	---	102-104	---
8-doxyl palmitic acid	---	75	---	---

but long chain saturated and unsaturated, short chain, and spin-labeled cholesteryl esters, all of which can be synthesized in a pure form in a relatively short time.

EXPERIMENTAL

Materials

Cholesterol and butyric, palmitic, myristic, oleic, and linoleic acids were purchased from Nu-Chek Prep. (Elysian, MN). All *trans*-9,11, 13,15-octadecatetraenoic acid (*trans*-parinaric acid) and *cis, trans, trans, cis*-9,11,13,15-octadicatetraenoic acid (*cis*-parinaric acid) were isolated as described by Sklar et al. (2) and stored frozen in benzene until required for use. The acid anhydrides were prepared with dicyclohexylcarbodiimide (Schwarz-Mann, Orangeburg, NY) by the method of Lapidot and Selinger (12). 4-Pyrrolidinopyridine was synthesized according to Patel and Sparrow (13). 8-(4′,4′-Dimethyl-oxazolidinyl-N-oxyl)-palmitic acid (8-doxyl palmitic acid) was prepared by a modification of the Hubbell and McConnell (14) procedure. The progress of the reaction was monitored by thin layer chromatography (TLC) on silica gel plates (Brinkmann, Westbury, NY) eluting with hexane/ethyl acetate (8:2). Melting points were determined by differential scanning calorimetry on a Perkin Elmer DSC-2 or with a Fisher melting point unit.

Synthesis of Cholesteryl Esters

Method A. Cholesteryl Myristate. In a typical run, cholesterol (100 mg, 0.258 mmole) was mixed with myristic anhydride, (170 mg, 0.387 mmole), and 4-pyrrolidinopyridine) 38.2 mg, 0.258 mmole) and 4 ml dry benzene or methylene chloride were added; the reaction mixture was stirred at room temperature under a nitrogen atmosphere for 10-12 hr. After 12 hr, the reaction mixture was loaded on a silica gel column (2.5 x 25 cm), pre-equilibrated with hexane. The column was eluted with a step gradient of 100 ml each of hexane, hexane/ethyl acetate (9:1), hexane/ethyl acetate (8:2), hexane/ethyl acetate (7:3). Thirty fractions of 10 ml each were collected. Fractions 12 to 20, containing cholesteryl ester as indicated by TLC, were combined and the solvent evaporated to give 135 mg (88% yield) of cholesteryl myristate, m.p. 69-70 C, lit (6) 70 C.

Method B. Cholesteryl cis-parinarate. Cholesterol (140 mg, 0.37 mmole) was added to 5 ml of methylene chloride containing *cis*-parinaric acid (100 mg, 0.36 mmole) and 4-pyrrolidinopyridine (5.5 mg, 0.036 mmole). After adding dicyclohexylcarbodiimide (75 mg, 0.36 mmole), the reaction mixture was stirred under argon at room temperature for 5 hr and purified as in Method A except the column was poured and eluted under argon to give cholesteryl *cis*-parinarate, 140 mg (60%), m.p. 67 C.

RESULTS AND DISCUSSION

Short chain, long chain unsaturated, and spin-labeled cholesteryl esters have been synthesized; the yields and melting points are shown in Table I. In addition to melting points determined in a capillary, differential scanning calorimetry was used to confirm the liquid crystalline behavior of several of the products. We have, for example, verified that cholesteryl myristate prepared by either procedure exhibits identical melting behavior. Crystalline cholesteryl myristate melts at 71 C, the smectic mesophase is converted to the cholesteric mesophase at 79 C, which is converted to the isotropic phase at 85 C. Physical and chemical properties of the spin-labeled cholesteryl ester were similar to those reported by Morrisett (10).

Because the cholesteryl parinarate isomers have not been previously reported, additional characterization was undertaken. The ester exhibits the characteristic IR C=O stretch at 1730 cm^{-1} (in carbon tetrachloride). The isomeric integrity of cholesteryl *cis*-parinarate and cholesteryl *trans*-parinarate was confirmed by comparing the UV absorption spectra of

the fatty acid starting material and ester product. Two spectral features, the wavelength of the absorption peak maxima and the spectral resolution ("sharpness"), are characteristic of the *cis*- and *trans*-isomers (15). These spectral features were unchanged by esterification under the reaction conditions and purification procedures. Both cholesteryl parinarate isomers have relatively high melting points. Considering that the melting points of the starting fatty acids, *cis*-parinaric acid (84 C) and *trans*-parinaric acid (95 C) (2) are also high, it is reasonable that their cholesteryl esters would also have high melting points.

The lower yields of the cholesteryl parinarate derivatives result neither from incomplete esterification, nor from destruction of the chromophore; UV analysis of the reaction mixture reveals that both isomers of parinaraic acid are stable under the reaction conditions. TLC shows the reaction to be essentially complete after 5 hr. The low yield of cholesteryl *trans*-parinarate resulted from degradation of the product during silicic acid chromatography which can be substantially reduced when columns are poured and eluted rapidly in an atmosphere of argon.

The synthetic procedure presented here has broad utility for the preparation of esters; phospholipids (11), methyl esters and triglycerides can also be synthesized by the procedures described herein (unpublished experiments). Thus, this procedure represents a general methodology for the acylation of hydroxyl groups which is especially useful in lipid synthesis.

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Synthesis and Characterization of [1-^{13}C] - and d_8-Arachidonic Acid

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ABSTRACT

Methyl d_8- and [1-^{13}C] 5,8,11,14-eicosatetraenoate (arachidonate) were prepared from a common synthetic precursor, 4,7,10,13-nonadecatetrayn-1-ol. The purified products were characterized by gas chromatography-mass spectrometry. Mass spectra of *t*-butyldimethylsilyl esters of d_8- and [1-^{13}C]-arachidonic acid showed a most intense $[M-57]^+$ peak at high mass. The isotopic purity of methyl [1-^{13}C] arachidonate was 99% and that of methyl d_8-arachidonate was 56%. When d_8-arachidonic acid was prepared by direct deuteration of 5,8,11,14-eicosatetraynoic acid, the isotopic purity of the sample was 86%.

INTRODUCTION

Fatty acids labeled with stable isotopes have been very useful in investigations of lipid metabolism (1,2) and lipid membrane structure (3,4). To study the metabolism of arachidonic acid and prostaglandins, it is often necessary to have available the acid in a stable isotopic form. We report here the preparation of [1-^{13}C] - and d_8-arachidonic acid from a common intermediate. These acids have been characterized by gas chromatography (GC), argentation thin layer chromatography (TLC) and mass spectrometry (MS).

EXPERIMENTAL PROCEDURES

Authentic fatty acids and their methyl esters were obtained from Applied Science Division, Milton Roy Company (State College, PA). *t*-Butyldimethylsilyl (*t*-BDMS) esters of fatty acids were prepared by the method of Corey and Venkateswarlu (5). Methyl esters of fatty acids were analyzed by a Perkin-Elmer 3920B gas chromatography equipped with a flame ionization detector. The glass capillary column, 25 m x 0.25 mm inner diameter (ID), was coated with SILAR 10C. The oven was maintained at 180 C and the helium flow rate was 2 ml/min. GC-MS was carried out at George Washington University with a Hewlett Packard 5992A gas chromatograph-mass spectrometer equipped with a jet separator. All mass spectra were determined at 70 eV. The gas chromatographic column, 1 m x 2.0 mm ID, was packed with 3% SE 30 on 100/120 mesh GAS-CHROM Q. The column temperature was 220 C and the helium flow rate was 20 ml/min. For identification purposes full scan spectra (60 to 440 amu) were obtained at a scan speed of 330 amu per second. For accurate isotopic abundance measurements narrow scan spectra (359 to 379 amu for the $[M-57]^+$ ion of d_8-arachidonic acid) were obtained at a scan speed of 100 amu per second. Isotopic abundances were calculated according to Biemann (6). Determination of trace amounts ($< 1\%$) of undeuterated arachidonic acid in the deuterated sample was carried out by selected ion monitoring using Hewlett-Packard software. Nonadeca-4,7,10,13-tetrayn-1-ol (I): This compound was prepared by the method of Sprecher (7), mp 46-48 C.

Methyl d_8 Arachidonate

Method A. 6.12 g (2.28 m mol) of compound I in 100 ml of deuterated ethanol (99.5%, Aldrich Chem. Co., Metuchen, NJ) and 0.2 g of quinoline were stirred with 2 g of Lindlar catalyst (8) at atmospheric pressure of deuterium gas (99.5%, Matheson Gas Products, E. Rutherford, NJ). The reduced product, d_8-nonadeca-4,7,10,13-tetraen-1-ol (II), was purified by HI-FLOSIL silica gel column chromatography by eluting with petroleum ether/ether 80:20 (v/v). The eluants were monitored by GC. The purified II was converted to the corresponding mesylate by the procedure of Baumann and Mangold (9). Next, the d_8-mesylate was converted to the nitrile by heating with NaCN in dimethyl sulfoxide for 3 hr at 85 C (10). Finally, the d_8-nitrile was converted to d_8-methyl arachidonate by treating with 25% anhydrous HCl in methanol at room temperature for 2 hr (10). The methyl ester was purified by preparative TLC using petroleum ether/ether/acetic acid 80:20:1 (v/v) as developing solvent. Preparative argentation TLC was used to remove the *trans*-isomers and over reduced products (11).

Method B. Two g of 5,8,11,14-eicosate-

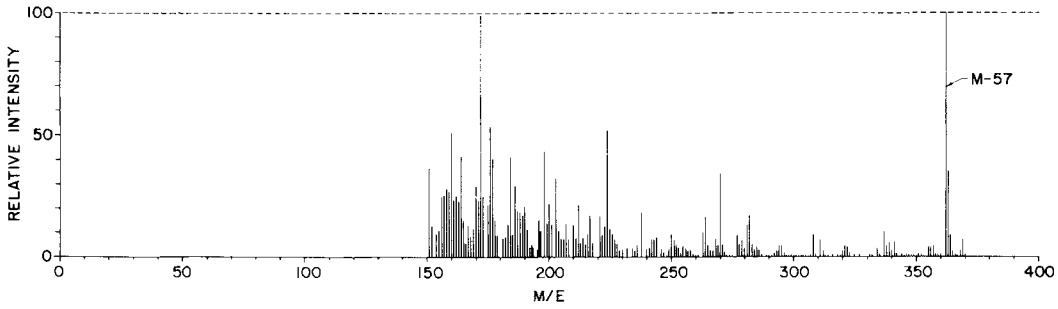

FIG. 1. Partial MS spectrum of the *t*-BDMS ester of [1-^{13}C] arachidonic acid.

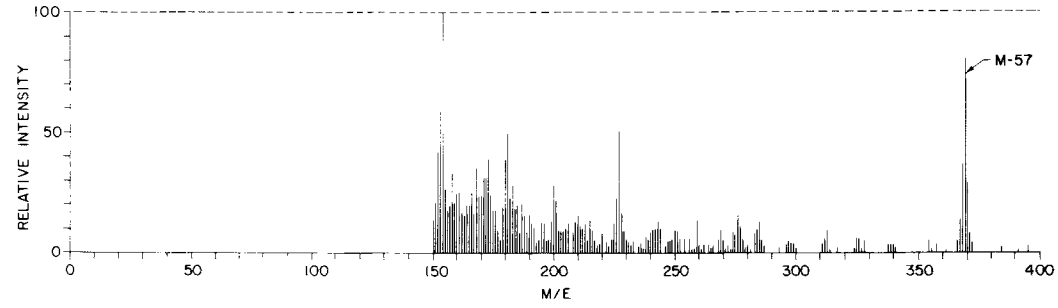

FIG. 2. Partial MS spectrum of the *t*-BDMS ester of d_8-arachidonic acid prepared by Method A.

traynoic acid (a gift of Dr. W.E. Scott from Hoffmann-La Roche Lab., Nutley, NJ) in 50 ml of ethyl acetate was reduced as described above. The reduced product was converted to the methyl ester by use of diazomethane in ether. The crude ester was further purified as described in Method A.

Methyl [1-^{13}C]-Arachidonate. 2.83 g (1.05 m mol) of compound I was reduced with 1 g of Lindlar catalyst in 60 ml of absolute ethanol and 0.1 g of quinoline at atmospheric pressure of hydrogen gas. The reduced product was purified by HI-FLOSIL column chromatography as described above and converted to the corresponding mesylate in the usual way. The isolated mesylate was heated with K^{13}CN (99%, Koch isotopes, Cambridge, MA) in dimethylsulfoxide for 3 hr at 80 C. The dried ^{13}C-nitrile was hydrolyzed to give methyl ^{13}C-arachidonate which was purified by preparative TLC and argentation TLC as described above.

RESULTS AND DISCUSSION

The purified methyl d_8- and ^{13}C-arachidonate migrated with the authentic methyl arachidonate (Rf = 0.18) on 20% $AgNO_3$-impregnated Silica Gel G plate developed in chloroform/ ethanol 97:3 (v/v). The methyl arachidonate was completely separated from methyl linoleate (Rf = 0.68) and methyl linolenate (Rf = 0.35) on this plate. When a portion of methyl d_8-or ^{13}C-arachidonate was mixed with authentic methyl arachidonate and analyzed by GC, a single peak was obtained on 25 m x 0.25 mm ID SILAR 10C capillary column.

A portion of methyl d_8- or ^{13}C-arachidonate was completely reduced with platinum oxide. When the reduced d_8- or ^{13}C-product was mixed with authentic methyl arachidate (20:0), a single peak was obtained on the SILAR 10C column. The results suggested that the purified d_8- and ^{13}C-product had four double bonds. The unequivocal identification of the two compounds was carried out by GC-MS.

Since the mass spectrum of methyl arachidonate at 70 ev showed the most intense ions in the low mass region, the accurate measurement of the parent molecular ions was difficult. Therefore, we employed *t*-BDMS derivatives of ^{13}C- and d_8-arachidonic acid (11) to increase the relative intensity of the high mass ions of these fatty acids. Although the *t*-BDMS ester of arachidonic acid did not give $[M-57]^+$ ion as the base peak, it displayed a pronounced $[M-57]^+$ ion, m/e 361, at high mass. The results

were consistent with data reported by Phillipou et al. (12). As shown in Figures 1 and 2, the t-BDMS ester of ^{13}C- and d_8-arachidonic acid also displayed the most intense $[M-57]^+$ peaks at high mass, m/e 362 and m/e 369, respectively. From the $[M-57]^+$ peaks the nominal molecular weights of ^{13}C- and d_8-arachidonic acid were calculated to be 305 and 312, respectively. The isotopic purity of [1-^{13}C]-arachidonic acid was 99%. To measure accurate isotopic abundance ratios, narrow mass range spectra were obtained at slow scan speeds as described in Experimental Procedure.

When the deuterium distribution for d_8-arachidonic acid prepared by Method A was calculated as described, 56% of the sample contained eight deuterium atoms per molecule, 30% contained seven deuterium atoms, 11% contained six deuterium atoms, and 4% contained five deuterium atoms. When the deuterium distribution for d_8-arachidonic acid prepared by Method B was calculated, the material consisted of a mixture of 86% octadeutero-, 10% heptadeutero-, 3% hexadeutero- and 0.5% pentadeuteroarachidonic acid. Selected ion monitoring GC-MS showed that 0.5% undeuterated arachidonic acid was present in the d_8-arachidonic acid prepared by Method B.

The values obtained by Method B agreed well with the values recently reported in the literature (13).

Results from both Method A and B suggested that the only deuterons in the molecule were those on the olefinic carbons, and these deuterons were the only ones which could be replaced. The extent of deuterium-proton exchange was significantly higher by the Method A which required three steps after reduction to convert the deuterated alcohol to the methyl d_8-arachidonate. Therefore, a single-step deuteration is recommended to prepare isotopically pure d_8-arachidonic acid.

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COMMUNICATIONS

Prenatal Protein Depletion and Δ9, Δ6 and Δ5 Desaturases in the Rat

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ABSTRACT

Pregnant rats were kept throughout gestation on a control diet (i.e., 25% protein), on a low protein diet (i.e., 5% protein) or on a fat-free diet. At 20-21 days of gestation, the rate of 9-, 6-, and 5-desaturation was measured, using microsomes from maternal and fetal livers and placenta microsomes. The effect of protein malnutrition was more evident upon Δ6-desaturase activity from maternal liver, while a less severe reduction in the activities of Δ9- and Δ5-desaturases was observed. No measurable activities of Δ5- and Δ6-desaturases were observed in fetal liver and placenta, while a low activity of Δ9-desaturase was detected in both tissues from the three groups under study. We concluded that Δ6-desaturation is greatly affected by maternal protein deprivation, and this fact could affect the normal supply of polyunsaturated fatty acids for the normal fetus growth and tissue development.

INTRODUCTION

As early as 1966, Williams and Hurlebaus (1) showed that the enzyme systems involved in the biosynthesis of arachidonate from linoleate appear to be impaired by protein deficiency. The linoleic acid chain is elongated and desaturated to form C_{20}-C_{22} polyunsaturated fatty acids with four and five double bonds. Apparently, these sequences of metabolic events involved in the synthesis of arachidonic acid are limited by the in vivo activity of Δ6-desaturase, since the desaturation of linoleic to γlinolenic acid is a rate-limiting step in the conversion of linoleic to arachidonic acid (2). Following the ingestion of [1-^{14}C]linoleic acid by pregnant rats, Pascaud et al. (3,4) demonstrated that: 1) fetal arachidonic acid is originated essentially in the maternal liver, and 2) this acid is selectively transferred from mother to fetus through the placenta.

The present study forms part of a continuous investigation on the effect of maternal protein deficiency and essential fatty acid deprivation on the activities of fatty acid desaturase enzymes from maternal and fetal livers and placentas.

MATERIALS AND METHODS

[1-^{14}C]Palmitic acid (54.0 mC/mmole, 99% radiochemically pure), [1-^{14}C]linoleic acid (52.0 mC/mmole, 99% radiochemically pure), and [1-^{14}C]eicosatrienoic acid (61.0 mC/mmole, 99% radiochemically pure and 98% pure *cis*-isomer) were purchased from Radiochemical Centre (Amersham, England).

Animals and Diets

Female Wistar rats (weighing 150-170 g) were divided after mating into three groups and were fed the following diets ad libitum throughout the pregnancy: *Control diet:* casein 25%, dextrine 66%, maize oil 5%, salt mixture 4% (5) and vitamins (6); *Fat-free diet:* casein 25%, dextrine 71%, salt mixture 4% and vitamins; *Low protein diet:* casein 5%, dextrine 88%, maize oil 5%, salt mixture 4% and vitamins.

On the 20th or 21st day of pregnancy, the mothers were decapitated and the maternal livers, fetal livers and placentas removed. Fetal livers from the litters and placentas were pooled.

Assay for in vitro Desaturation

Microsomes isolation. Maternal livers, placentas and fetal livers were homogenized and the microsomes isolated by differential centrifugation as previously described (7). The microsomal protein was estimated by the biuret method (8).

Incubation Procedure

[1-^{14}C]Palmitic acid, [1-^{14}C]linoleic acid and [1-^{14}C]8,11,14-eicosatrienoic acids were diluted to a specific activity of ca. 1.7 to 2.0 μC/μmole with the corresponding unlabeled

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pure fatty acid.

The assay conditions were as follows: 5 mg of microsomal protein were incubated in an open test tube with 100 nmoles of the diluted labeled fatty acid in a Dubnoff shaker at 37 C for 15 min in a total volume of 1.5 ml of 0.15 M KCl, 0.25 M sucrose containing μmoles ATP, 2; CoA, 0.1; NADH, 1.2; $MgCl_2$, 7.5; glutathione, 2.2; NaF, 62; nicotinamide, 0.5 and phosphate buffer (pH 7.0), 62.

After incubation, the mixture was saponified and the extracted free fatty acids esterified. The conversion of [1-^{14}C] palmitic acid to [1-^{14}C] palmitoleic acid was measured by thin layer chromatography (TLC) of the fatty acid methyl esters on $AgNO_3$-impregnated silica gel plates (9). The areas containing methyl esters were scraped off and counted directly in vials in a Packard scintillation spectrometer. The values were corrected for quenching by comparison with external standard. The conversion of [1-^{14}C] linoleic acid to γ[1-^{14}C] linolenic acid and [1-^{14}C] eicosatrienoic acid to [1-^{14}C] eicosatetraenoic acid were measured by radio gas liquid chromatography (GLC) in a Packard gas chromatograph. A glass column packed with 10% DEGS in Chromosorb WAW was used.

RESULTS AND DISCUSSION

Evidently, the fat-free diet fed during gestation produces in the rat an increase in the activity of Δ9-desaturase from maternal liver as is shown in Figure 1. These data agree with those reported in the literature (10,11). More recently, Jeffcoat and James (12) demonstrated the controlling effect of dietary linoleic acid in stearoyl CoA desaturase activity since the activity of the enzyme is diminished by ca. 60% in the first 18 hr of feeding a polyunsaturated fatty acid diet. In consequence, this control is not operative when rats are fed a fat-free diet. On the other hand, the effect of feeding a high carbohydrate, low protein diet, throughout gestation, resulted in a significant decrease in the microsomal capacity of maternal liver to desaturate palmitic acid when compared with normal diet, despite the stimulatory effect of the high carbohydrate content of the protein deficient diet on the activity of Δ9-desaturase as was described by Oshino and Sato (10) and Inkpen et al. (11).

At the same time, a very low capacity to desaturate palmitic acid was observed in the microsomal fraction from fetal livers and placentas of mothers fed the different diets under study. Apparently, neither protein deficiency nor fat deprivation during gestation

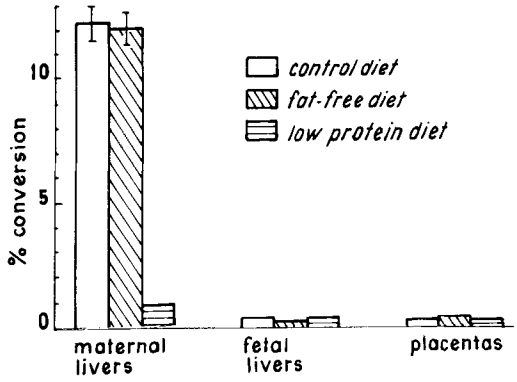

FIG. 1. Microsomal Δ9-desaturase activity from maternal and fetal livers and placentas from rats fed on different diets throughout the gestation. Each bar is the mean of five sample determinations ± standard deviation.

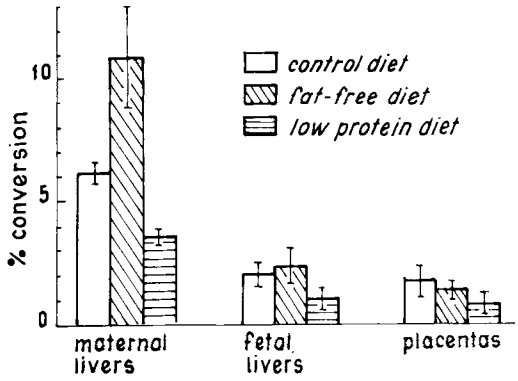

FIG. 2. Microsomal Δ6-desaturase activity from maternal and fetal livers and placentas from rats fed on different diets throughout the gestation. Each bar is the mean of five sample determinations ± standard deviation.

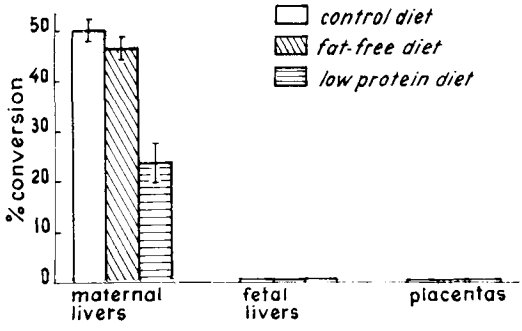

FIG. 3. Microsomal Δ5-desaturase activity from maternal and fetal livers and placentas from rats fed on different diets throughout the gestation. Each bar is the mean of five sample determinations ± standard deviation.

are able to modify the rather low activity of Δ9-desaturase enzyme in these two tissues.

Figure 2 shows the Δ6-desaturase activity from maternal and fetal livers and placenta of rats fed normal diets, restricted in protein and fat-free throughout gestation. A drastic decrease in the capacity of maternal liver microsomes to desaturate linoleic acid was promoted by feeding dams a protein deficient diet. On the other hand, no significant change in the activity of Δ6-desaturase was observed in liver from rats maintained on a fat-free diet during gestation.

Apparently, malnourished rats exhibited, during gestation, a failure in the activity of liver Δ6-desaturase, which is not observed when adult rats were fed a protein-free diet for 7 weeks. These results were obtained despite the arachidonic acid (20:4) impoverishment and the consequent accumulation of linoleic acid which is the substrate of Δ6-desaturase and precursor of 20:4 in the liver lipids of these protein deficient animals (13).

Apparently, the protein depletion, provoked in the rats by the protein deficient diet together with the progressive demand of nutrients according to its metabolic needs by the fetuses throughout the gestation, resulted in a decrease in the specific activity of Δ6-desaturase, probably due to a failure in the synthesis of the enzyme. The developmental changes in microsomal enzymes are probably associated with the quantitative increase of the endoplasmic reticulum (14), and these changes are really influenced by nutritional state. According to Jansen and Chase (15) and Kwong and Barnes (16), there were not any differences in food consumption of pregnant rats freely fed a low or a high casein diet; then the observed changes in enzyme activities could be related to the protein restriction during gestation.

No activity of Δ6-desaturase was detected in our experimental conditions in the microsomal fraction of fetal rat livers and placentas of mothers fed the different diets under study. Satomi and Matsuda (17) have reported a similar rate of conversion of linoleate to γ-linolenate by liver microsomes of pregnant rats and fetuses using in the assay 5 mg of microsomal protein and 12.5 nmoles of potassium linoleate 1-^{14}C.

Apparently, the discrepancy with these results could be due to our different assay conditions of the enzymatic activities, since we have assumed that in order to be able to compare these enzyme-catalyzed reactions between the three classes of tissues under study, it is necessary to saturate the enzyme with its substrate, since only under these conditions is the enzyme concentration the rate limiting factor of the reaction (18). Using the same assay conditions described in Methods for Δ6-desaturase, we found rather similar enzymatic activities in liver microsomes of 30-day-old rats fed a control diet and those observed in adult rats fed the same diet (unpublished results).

Besides the already discussed Δ6-desaturation of mother and fetal liver and placenta, the activity of Δ5-desaturase has also been demonstrated (Fig. 3). Maternal livers convert eicosa-8,11,14-trienoic acid to arachidonic acid at a higher rate than Δ6-desaturation is accomplished, and this conversion is not affected by mothers fed a fat-free diet.

Although Δ5-desaturase is less responsive to dietary and hormonal manipulations (18-20), a reduction of about 50% in its activity from control animals was observed in animals fed a low protein diet.

Similarly, as was observed for Δ6-desaturase, no detectable activity of Δ5-desaturase was evident in our experimental conditions shown by fetal liver and placentas.

Considering that the activities of the enzymes under study were measured in the liver microsomal fractions from the same animals, we concluded that dams fed on diets markedly restricted in proteins throughout the gestation show a drastic decrease in the activity of hepatic Δ6-desaturase, while a less severe reduction in the activities of Δ9 and Δ5-desaturases was observed.

These findings are consistent with those that demonstrated that protein diet increased Δ6-desaturation while Δ9- and Δ5-desaturases were less responsive to those dietary manipulations (21).

It must be emphasized that, even under normal dietary conditions, no measurable in vitro activity of Δ5- and Δ6desaturases was observed in the microsomal fraction of fetal liver and placenta.

According to the results of the present experiment, we can conclude that Δ6-desaturation, the key controlling step in the biosynthesis of polyunsaturated fatty acids, is profoundly affected by the maternal protein deprivation, and this fact could affect the normal supply of polyunsaturated fatty acids for normal fetal growth and tissue development.

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Correlations between Surface Area and the Rate of Enzymatic Desaturation with Methyl Branched 8,11,14-Eicosatrienoic Acid

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ABSTRACT

Methyl-branched derivatives of methyl 8,11,14-eicosatrienoate form stable liquid-expanded monolayers. Surface areas are expanded by the methyl branch. The expansion effect is a function of surface pressure. At high surface pressure, the greatest expansion occurs with a mid-point methyl branch. At low surface pressure, surface area increases continuously as the methyl group is moved along the carbon chain from carbon 19 to carbon 5. Desaturase activity varies inversely with surface area, and a linear correlation exists between surface area at low surface pressure and the desaturation rate. These data support the concept that lipid structure and its effect on short range forces between molecules is an important factor in desaturase activity.

INTRODUCTION

Monolayers in lipids with branched hydrocarbon chains, particularly lipids isolated from bacterial waxes and lipids with bactericidal activity, have been studied extensively (1-12). These studies show that methyl-substituted saturated fatty acids have expanded surface areas and that a methyl group attached at the fifth to the twelfth carbon atom along the main aliphatic chain exerts a maximum expansion effect on the first detectable surface area of the film (12). It is interesting that methyl-substituted stearic acids with the methyl branch in the middle of the main chain (large surface area derivatives) are poor substrates for a Δ9-desaturase (13). Furthermore, methyl-branched derivatives improve as substrates when the position of the methyl branch approaches either end of the fatty acid (13) and surface areas approach the surface area of the parent unbranched compound (10,12). These correlations between surface area and desaturase activity suggest that surface area may affect the geometry of the enzyme-substrate complex.

A recent study (14) of enzymatic desaturation with methyl-branched 8,11,14-eicosatrienoic acids shows Δ5-desaturase activity decreases as the methyl branch is moved along the main aliphatic chain from carbon 19 to carbon 5. This methyl branch effect which does not appear to correlate with the known surface properties of methyl-branched saturated acids (1-12) prompted us to examine the surface properties of methyl branched eicosatrienoic acids.

MATERIALS AND METHODS

Six methyl-branched isomers of methyl 8,11,14-eicosatrienoate with methyl branches located on carbons 5,10,13,17,18 and 19 were synthesized and purified as previously described (15). The methyl esters were dissolved in purified n-hexane (16) and applied to a Teflon Langmuir trough (50 x 10 x 1 cm) containing 0.1 M sodium chloride as the subphase. Surface pressure (Π) was measured by the Wilhelmy plate technique utilizing a Cahn R. G. recording balance. A platinum foil 1 cm wide was used as the dipping plate. The movable bar was milled from Teflon and was propelled by a high-torque variable speed motor. The compression rate was 20 Å^2/molecule/min. The temperature was 24-26 C.

RESULTS AND DISCUSSION

Methyl-branched derivatives of methyl 8,11,14-eicosatrienoate behaved as stable liquid-expanded films when they were spread on 0.1 N sodium chloride alone (neutral pH). Monolayers of these methyl esters all collapsed at pressures between 13 and 14.5 dynes/cm and generated a plateau that was maintained throughout the post-collapse region of the Π-A isotherm (apparent area/molecule decreased to 10 Å^2). In contrast to the methyl esters of methyl-branched unsaturated acids, collapse pressures of methyl-branched saturated acids varied as much as 14 dynes/cm (9-11). Furthermore, many of these saturated branched acids showed an immediate post-collapse decrease in surface pressure (9-11), which is characteristic of collapse from a metastable film (16,17).

Surface areas of the methyl-branched derivatives of methyl 8,11,14-eicosatrienoate are highly interesting. As the surface pressure approaches collapse, surface area shows the anticipated expansion of a mid-point methyl branch (see data at 12.5 dynes/cm in Fig. 1). The effect of the mid-point methyl branch is lost with a decrease in surface pressure, and at

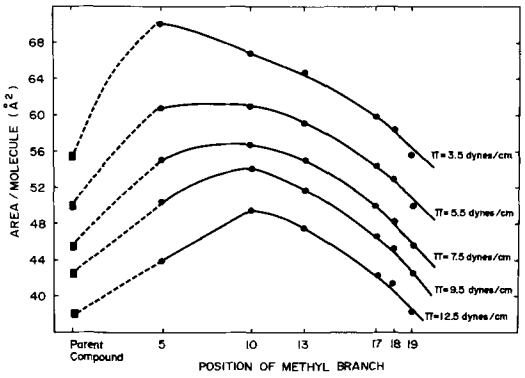

FIG. 1. Surface areas of methyl-branched methyl 8,11,14-eicosatrienoates at various surface pressures. The surface area at the specified surface pressure was obtained from the II-A isotherm at 24-26 C.

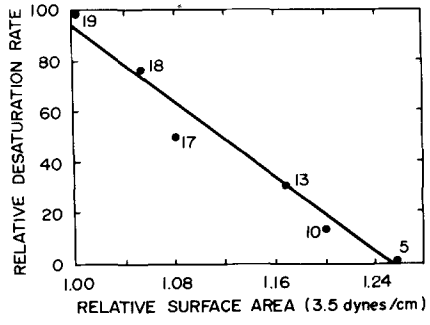

FIG. 2. Correlation between relative desaturation rates (14) and relative surface areas (surface area derivative/surface area parent compound) for methyl-branched methyl 8,11,14-eicosatrienoates. The correlation coefficient was 0.98 for the linear regression equation. Numbers in the figure designate the point of the methyl branch.

low surface pressure the 5-methyl derivative is more expanded than the 10-methyl derivative (see data at 3.5 dynes/cm in Fig. 1). Thus, maximum expansion with a mid-point methyl branch occurs only in the most tightly packed film.

The methyl branch has a very different effect on the surface properties of saturated fatty acids (9-11). These branched acids are characterized by a mid-point expansion which is maximal at low surface pressures (less than 5 dynes/cm). Significant film expansion begins with the 5-methyl and 15-methyl derivatives and increases to a maximum with the 8-methyl derivative. In contrast, surface areas of methyl-branched unsaturated fatty acids at 3.5 dynes/cm increase continuously as the methyl branch is moved along the carbon chain from the methyl terminal toward the carboxyl terminal (Fig. 1).

Desaturase activities with both methyl-branched saturated acids and methyl-branched unsaturated acids are closely correlated with the surface area at low surface pressure. The desaturation of methyl-branched stearic acids to the corresponding 9-monoenoic acids ceases with the 5-methyl through 15-methyl derivatives and increases to a maximum as the methyl branch is moved either from the 15-methyl position toward the methyl terminal or from the 5-methyl position toward the carboxyl terminal (13). Thus, desaturase activity varies inversely with the surface areas of methyl-branched saturated acids. The desaturation rate with methyl-branched derivatives of 8,11,14-eicosatrienoic acid decreases continuously as the methyl branch is moved along the carbon chain from the methyl terminal toward the carboxyl terminal (14). Indeed, a linear correlation exists between the relative desaturation rates and the relative surface areas of methyl-branched unsaturated acids (Fig. 2).

Several investigators (13,18) have discussed the importance of lipid structure in the enzyme-substrate interactions required for highly specific desaturase activities. Surface area data show that the position of a methyl branch will have a significant effect on short range forces between hydrocarbon molecules. The methyl branch effect is different with saturated and with unsaturated fatty acids, yet surface areas are correlated with enzyme activities in both series. These data support the concept that lipid structure and its effect on short range forces between molecules is an important factor in desaturase activity.

ACKNOWLEDGMENT

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Metabolism of Erucic Acid in Perfused Rat Liver: Increased Chain Shortening after Feeding Partially Hydrogenated Marine Oil and Rapeseed Oil

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ABSTRACT

The metabolism of [14-^{14}C] erucic acid was studied in perfused livers from rats fed on diets containing partially hydrogenated marine oil or rapeseed oil for three days or three weeks. Control rats were given groundnut oil. Chain-shortening of erucic acid, mainly to 18:1, was found in all dietary groups. In the marine oil and rapeseed oil groups, the percentage of chain-shortened fatty acids in very low density lipoproteins-triacylglycerols (VLDL-TG) exported from the liver increased after prolonged feeding. A similar increase was found in liver TG only with partially hydrogenated marine oil. This oil, rich in *trans* fatty acids, thus seemed to be more effective in promoting chain-shortening. The fatty acid composition of the secreted and stored TG differed both with respect to total fatty acids and radioactively labeled fatty acids, indicating that at least 2 different pools of TG exist in the liver. The lack of lipidosis in livers from rats fed dietary oils rich in 22:1 fatty acids is discussed in relation to these findings. In conclusion, a discussion is presented expressing the view that the reversal of the acute lipidosis in the hearts of rats fed rapeseed oil or partially hydrogenated marine oils is, to a large extent, derived from the increased chain-shortening capacity of erucic acid in liver.

INTRODUCTION

Diets rich in oils containing very long chain monounsaturated fatty acids, as rapeseed oil and partially hydrogenated marine oils, lead to the development of an acute accumulation of triacylglycerols (TG) in the heart and skeletal muscles of several animals (1). In the young rat, the fat content reaches a peak after 3-7 days of feeding, after which an adaptation takes place and the fat content decreases almost to a normal value (1,2). The mechanism of this adaptation is not quite clear. It may take place in the heart itself (3), or it may arise from a change in the supply of fatty acids reaching the heart from the blood.

We have previously demonstrated a significant decrease in the percentage of 22:1 fatty acids in rat serum very low density lipoprotein-triacylglycerols (VLDL-TG) after 3 weeks, compared to 3 days, on a diet containing rapeseed oil (4). We also observed a considerable decrease in the relative amount of 22:1 fatty acids in VLDL-TG secreted from perfused livers from rats fed rapeseed oil or marine oil for 3 weeks. The decreasing TG-content in the heart after prolonged feeding may thus, at least in part, be explained by an adaptation of the fatty acid metabolism in the liver.

The decrease in the 22:1 content was accompanied by an increase in 18:1. This led us to suggest that an increased chain-shortening might have taken place in the liver. This has been the subject of a closer investigation, and in this communication we report the results from a study of the metabolism of ^{14}C-erucic acid in perfused livers from rats fed diets containing partially hydrogenated marine oil, rapeseed oil or groundnut oil. Special attention was paid to the composition of fatty acids in VLDL-TG secreted from the livers. A preliminary report has been presented (5).

MATERIALS AND METHODS

Animals and Diets

Male weanling rats (60 g) of the Wistar strain were purchased from Møllegaard Laboratory, Denmark. After 3 to 5 days on a standard pellet diet, the animals were fed semisynthetic diets as described by Thomassen et al. (4) for 3 days or 3 weeks. Thirty percent of the calories were supplied as dietary oils, either partially hydrogenated marine oil, rapeseed oil or groundnut oil. The oils, including analytical data, were obtained from DeNoFa and Lilleborg Co., Norway (Table I).

After 3 days of feeding, the rats weighed ca. 100 g and after 3 weeks 200 g. Before the experiments, the animals were fasted for ca. 3 hr (from 0800 to 1100).

Liver Perfusion

The rats were killed after being anesthetized with ether, and the livers were perfused essentially as described by Seglen (6) for an

TABLE I

Fatty Acid Composition of Dietary Oils (% of Methylated Total Fatty Acids[a])

Fatty acid	Rapeseed oil	Partially hydrogenated[b] marine oil	Groundnut oil
14:0	0.7	6.5	0.7
16:0	2.8	16.1	17.6
16:1	0	6.9	0
16:2	0	1.2	0
18:0	1.0	7.7	2.8
18:1	16.3	12.8	36.2
18:2	17.9	2.1	40.8
18:3,20:0	9.2	4.9	0.7
20:1	0.5	11.5	1.1
20:2		3.7	
20:3		1.4	
22:0		5.0	
22:1	42.7	11.5	
22:2		3.7	
22:3		2.2	

[a]% Calculated from triangulation of peaks on the chromatograms.
[b]54.3% of the total fatty acid double bonds were *trans* isomers.

experimental period of 3 hr. Three hours of perfusion was necessary to ensure that the radioactivity recovered in the VLDL-TG fraction was high enough for the samples to be analyzed by radio gas chromatography. The perfusion fluid consisted of 50 ml Krebs-Ringer bicarbonate buffer, pH 7.45, 0.1% glucose and 0.028% $CaCl_2$, and was continuously gassed with 95% O_2-5% CO_2. After a 10 min flow-through perfusion with this medium, the system was closed to make a recirculating perfusion. Erucic acid suspended in a bovine serum albumin solution was added to final concentrations of 1 mM and 15 mg/ml, respectively, including 0.08 μCi/ml [14-^{14}C]erucic acid.

The perfusate was circulated through the liver at a flow rate of 3 ml/min/g liver. The weights of the livers after 3 days of feeding were ca. 5 g, and after 3 weeks, ca. 10 g. Samples (1-2 ml) were taken at half-hour intervals during the perfusion for analysis of the distribution of label into water-soluble fractions and various lipid fractions.

Isolation of VLDL and TG

The VLDL fraction from liver perfusate was separated by centrifugation of 5 ml samples for 18 hr at 4 C using 115,000 g. The layer of VLDL (d.- 1.006)floated on top of a separation layer of saline (d.= 1.006). Lipids were extracted with chloroform/methanol (2:1) after the method of Folch et al. (7).

The liver was chilled immediately after perfusion, and ca. 1 g was used for lipid extraction. The lipids were extracted essentially as above after the modified method described by Christie (8).

Lipid classes were separated by thin layer chromatography using 0.4 mm Silica Gel H. The solvent system was petroleum ether (60-70 C)/diethylether/acetic acid (113:20:1). Zones corresponding to free fatty acid (FFA) and TG fractions were scraped into glass-stoppered tubes and prepared for gas chromatography.

Chemicals

[14-^{14}C]Erucic acid (99% radiopurity) was from CEA (Commissariat a l'Energie Atomique), Gif-sur-Yvette, France, and erucic acid (99% pure) from Sigma Chemical Co., St. Louis, MO, (code no. E 7250). Bovine serum albumin was essentially fatty acid free (Sigma A-6003). Other chemicals were commercial products of high purity.

Gas Chromatographic Analysis

Methylation of the TG samples was performed as described by Thomassen et al. (4), and analyzed by radio gas chromatography using a Pye 104 gas chromatograph connected to an ESI Nuclear (Surrey, England) radioactivity detector with a 1:1 outlet splitter. Fatty acid methyl esters were separated at 185 C using 10% SP 2340 on Supelcoport 100/120 (Supelco, Inc., Bellefonte, PA.) The peaks were identified on the basis of the retention time compared with known standards (Supelco). The distribution of the radioactivity among the peaks was calculated from counting data

recorded on a Printing Autoscaler 5680 (ESI Nuclear). The percentage of each methylated fatty acid was determined by triangulation.

Analytical Methods

Protein was determined by the method of Lowry et al. (9). Lactic dehydrogenase activity, to test the viability of the perfused liver, was measured with a Sigma diagnostic kit no. 500. Triacylglycerol-glycerol was determined according to the method of Wieland (10) and measured fluorimetrically.

Radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer model no. 3385.

RESULTS

Erucic Acid Uptake in Perfused Liver

The time course of the uptake of ^{14}C-erucic acid from the perfusion medium and the incorporation of radioactivity into TG and oxidation products were studied in all dietary groups. However, only minor differences were detected. Thus, in Figure 1, only the results from an experiment using liver from a rat fed marine oil for 3 weeks are shown as an example. The albumin-bound ^{14}C-erucic acid was taken up almost completely by the livers during the 3 hr perfusion, and a gradual increase in radioactivity could be detected in TG and in water-soluble products in the medium.

The gross similarity between the 3 dietary groups with respect to erucic acid uptake and secretion of oxidation products and TG can be seen from the data presented in Table II. Here, the distribution of radioactivity in the liver perfusates after a perfusion of 3 hr is shown. A statistically significant decrease in radioactivity in the lipid fraction was seen after 3 weeks of feeding in all 3 dietary groups. This was most likely due to a more efficient removal of free erucic acid from the medium, since no significant decrease could be detected in the secretion of radioactively labeled TG. The amount of radioactivity recovered as secreted TG was very low, less than 5% of that added as ^{14}C-erucic acid even after 3 hr perfusion. Fifteen to 25% was recovered as water-soluble products. Here a significant increase was seen in the marine oil group after 3 weeks of feeding, while no significant differences were found in the rapeseed oil group or in the control (groundnut oil) group. This may be related to the extensive increase in chain-shortening activity found in the marine oil fed animals after 3 weeks, as discussed later.

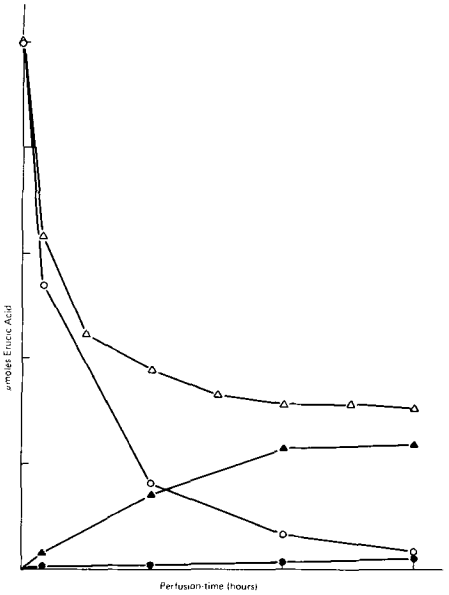

FIG. 1. Time-course of free ([14-^{14}C]erucic acid disappearance, and formation of water-soluble products and triacylglycerols in liver perfusate. The liver was taken from a rat fed marine oil for 3 weeks (as an example). The values are expressed as μmoles erucic acid. Fifty μmoles free ^{14}C-erucic acid were added to the perfusate at zero time. △—△ total label in the perfusate; ○—○ label in free fatty acids; ▲—▲ label in water-soluble products; ●—● label in TG.

Chain-Shortened Products in VLDL-TG

The distribution of radioactivity from [14-^{14}C] erucic acid into acyl groups in VLDL-TG was investigated (Table III). The appearance of a substantial amount of radioactively labeled fatty acids shorter than 22:1 showed that erucic acid was chain-shortened in perfused livers from rats in all three dietary groups. The identified products of the chain-shortening process were mainly 18:1, but also 20:1 and 16:1 fatty acids.

In the control groups, no significant differences between 3 days and 3 weeks could be detected, either in the pattern of chain-shortened fatty acids, or in the total percentage of chain-shortened radioactively labeled fatty acids. In the 2 other groups, however, a significant increase was seen in the percentage of chain-shortened fatty acids after 3 weeks. In the marine oil group, this was due to an increased percentage of 18:1, while in the rapeseed oil group an increase was also found in 16:1 (Table IV). This strongly implies that the decrease in the percentage of *total* 22:1 in VLDL-TG, also seen in the animals fed marine oil or rapeseed oil for three weeks (Table IV),

TABLE II

Distribution of Radioactivity from [14-^{14}C]Erucic Acid in Liver Perfusate after 3 hr Recycled Perfusion. Content in Some Lipid Classes and Water-Soluble Oxidation Products[a]

	Rapeseed oil		Partially hydrogenated marine oil		Groundnut oil	
	3 days (6)[b]	3 weeks (4)	3 days (4)	3 weeks (5)	3 days (6)	3 weeks (5)
Total perfusate	19.4 ± 5.3[c]	14.2 ± 3.6	15.6 ± 1.9	15.3 ± 2.1	16.0 ± 2.1	15.9 ± 5.5
Water sol. fraction	8.5 ± 2.8	7.8 ± 2.1	9.3 ± 1.4	12.0 ± 1.5^{xx}[d]	7.7 ± 1.2	10.4 ± 3.5
Lipid fraction	10.9 ± 2.8	6.4 ± 2.8^{xx}	6.3 ± 0.9	3.3 ± 0.9^{xxxx}	8.3 ± 2.1	5.5 ± 2.0^{xx}
FFA	8.3 ± 2.3	4.0 ± 1.7^{xxx}	4.4 ± 1.3	1.6 ± 1.0^{xxxx}	5.1 ± 2.6	3.1 ± 1.7
TG	1.5 ± 0.7	1.6 ± 0.9	1.4 ± 0.6	1.2 ± 0.4	2.7 ± 1.0	1.8 ± 0.7

[a]All the results are expressed as μmoles erucic acid from 50 μmoles added to the perfusion buffer. The fractions were separated as described in Materials and Methods.

[b]Figures in parentheses are numbers of animals per group.

[c]Mean ± standard deviation.

[d]Significantly different from 3 days group: x = 0.05>p>0.025, xx = 0.025>p>0.01, xxx = 0.01>p>0.005, xxxx = 0.005>p (t-distribution).

TABLE III

Distribution of Radioactivity from (14-^{14}C) Erucic Acid into Fatty Acids in Liver Perfusate VLDL-TG[a]

	Rapeseed oil		Partially hydrogenated marine oil		Groundnut oil	
Fatty acid	3 days (5)	3 weeks (4)	3 days (4)	3 weeks (5)	3 days (5)	3 weeks (5)
16:1	8.0 ± 3.0	12.5 ± 1.4^{xx}	4.9 ± 4.1	6.5 ± 2.1	1.3 ± 1.5	1.3 ± 1.8
18:1	40.6 ± 13.9	62.3 ± 5.9^{xx}	34.8 ± 4.8	75.6 ± 9.0^{xxxx}	33.9 ± 7.6	34.9 ± 6.3
20:1	5.2 ± 4.0	3.8 ± 3.0	7.4 ± 2.7	3.7 ± 2.0^{xx}	5.0 ± 1.8	3.2 ± 3.1
22:1	46.2 ± 16.7	21.5 ± 3.8^{xx}	52.9 ± 5.6	14.2 ± 11.0^{xxxx}	59.8 ± 8.8	60.6 ± 7.0
Chain-shortened fatty acids[b]	53.8 ± 16.8	78.5 ± 3.8^{xx}	47.1 ± 5.6	85.8 ± 11.1^{xxx}	40.2 ± 8.8	39.4 ± 15.4

[a]All the results are presented as % of total radioactivity (cpm) in VLDL-TG. For further information see legends to Table II.

[b]Sum of % of total radioactivity in 16:1, 18:1 and 20:1.

TABLE IV

Fatty Acid Composition of Liver Perfusate VLDL-TG[a]

Fatty acid	Rapeseed oil		Partially hydrogenated marine oil		Groundnut oil	
	3 days (6)	3 weeks (4)	3 days (4)	3 weeks (5)	3 days (5)	3 weeks (5)
16:0	14.1 ± 3.8	24.8 ± 1.3^{xxxx}	18.8 ± 4.4	27.6 ± 7.8^{x}	17.1 ± 3.2	14.0 ± 3.7
16:1	3.4 ± 1.4	6.0 ± 1.5^{xx}	9.5 ± 3.4	10.8 ± 2.8	2.0 ± 1.1	1.8 ± 0.5
18:0	3.6 ± 2.2	2.5 ± 1.8	2.1 ± 1.0	2.0 ± 0.5	3.1 ± 0.8	2.9 ± 2.0
18:1	33.5 ± 9.5	39.4 ± 1.6	32.2 ± 3.7	44.1 ± 5.8^{xxx}	28.6 ± 4.1	25.9 ± 6.1
18:2	12.9 ± 6.7	12.7 ± 3.5	9.0 ± 4.9	4.2 ± 3.7	16.9 ± 1.5	19.8 ± 5.9
20:1	3.5 ± 0.9	3.2 ± 1.2	4.5 ± 1.8	1.9 ± 0.5^{xxx}	1.9 ± 0.4	2.3 ± 0.8
22:1	24.7 ± 14.4	8.7 ± 1.7^{x}	22.2 ± 10.3	5.3 ± 3.9^{xxx}	28.8 ± 3.6	30.8 ± 7.8

[a]All the results are presented as % of total methylated fatty acids (represented by area on readout from gas chromatogram). For further information see legend to Table II.

was due to an increased chain-shortening capacity in the liver.

Chain-Shortened Products in Liver-TG

In the TG remaining in the liver after the perfusion, radioactively labeled acyl groups shorter than 22:1 could also be detected. The pattern, as well as the total percentage of chain-shortened radioactively labeled fatty acids, differed from that of the secreted VLDL-TG (Table V). This implies that at least 2 different pools of TG exist in the liver. In liver-TG, as in VLDL-TG, a significant increase in the percent of chain-shortened fatty acid was found after 3 weeks in the marine oil group. This was, however, not so in the rapeseed oil group. The marine oil thus seems to be more effective than rapeseed oil in promoting the chain-shortening ability.

Only small differences were observed in the *total* fatty acid composition of liver-TG between 3 days and 3 weeks of feeding (Table VI). The decrease in 22:1 seen in VLDL-TG after 3 weeks was apparent also in the liver-TG in the rapeseed oil group. Also there was a tendency to a lower percentage of 22:1, which was, however, not statistically significant.

DISCUSSION

Livers from fat-fed rats rapidly took up and metabolized erucic acid when perfused in vitro. The acid was partly oxidized to water-soluble products, partly chain-shortened, and some was incorporated unchanged into liver lipids. The feeding period and nature of the dietary oil influenced to some extent the fate of the erucic acid. Thus, the feeding of rapeseed oil, and more especially of marine oil, for 3 weeks led to a marked increase in the chain-shortening activity in the liver as measured by the incorporation of radioactively labeled fatty acids shorter than 22:1 into TG secreted from the liver in VLDL. An increase in the percentage of chain-shortened acyl groups was also seen in liver-TG with marine oil-fed animals but not with animals fed on the rapeseed oil diet. This may be due to a dilution effect, since the rapeseed oil-fed animals received more 22:1 acids in the diet and consequently had a higher content of this acid in the liver lipids. It may, however, also indicate that the partially hydrogenated marine oil, which is rich in *trans* fatty acids, is more effective than rapeseed oil in inducing the chain-shortening ability. This difference has also been observed in a study with hepatocytes isolated from rats fed rapeseed oil or partially hydrogenated marine oil (11). An increased

TABLE V

Distribution of Radioactivity from (14-^{14}C) Erucic Acid into Fatty Acids in Liver Triacylglycerols[a]

Fatty acid	Rapeseed oil		Partially hydrogenated marine oil		Groundnut oil	
	3 days (4)	3 weeks (4)	3 days (3)	3 weeks (4)	3 days (4)	3 weeks (3)
16:1	2.8 ± 2.2	3.6 ± 1.1	3.0 ± 1.7	4.4 ± 3.6	2.6 ± 3.0	2.3 ± 2.0
18:1	26.6 ± 10.9	32.9 ± 8.4	17.2 ± 2.2	59.8 ± 21.8^{xx}	19.0 ± 12.9	16.7 ± 2.0
20:1	5.3 ± 6.1	3.2 ± 2.4	5.1 ± 3.4	5.1 ± 4.1	2.9 ± 3.8	5.3 ± 3.6
22:1	68.7 ± 14.9	60.2 ± 8.2	74.7 ± 3.5	31.0 ± 21.3^{xxx}	75.5 ± 15.3	75.7 ± 4.2
Chain-shortened fatty acids[b]	34.7 ± 13.5	39.8 ± 8.2	25.3 ± 3.8	69.0 ± 21.4^{xx}	24.5 ± 15.3	24.2 ± 4.1

[a]All the results are presented as % of total radioactivity (cpm) in liver-TG. For further information see legends to Table II and III.

[b]Sum of % of total radioactivity in 16:1, 18:1 and 20:1.

TABLE VI

Fatty Acid Composition of Liver Triacylglycerols[a]

Fatty acid	Rapeseed oil		Partially hydrogenated marine oil		Groundnut oil	
	3 days (4)	3 weeks (4)	3 days (4)	3 weeks (3)	3 days (5)	3 weeks (5)
16:0	17.1 ± 3.2	20.0 ± 2.8	25.5 ± 3.7	32.0 ± 5.2	19.5 ± 4.9	17.0 ± 3.8
16:1	3.1 ± 0.6	2.9 ± 0.5	7.7 ± 5.3	11.5 ± 3.2	2.3 ± 1.3	2.0 ± 0.7
18:0	4.8 ± 2.6	2.2 ± 0.7	5.3 ± 1.8	3.9 ± 1.4	3.9 ± 1.1	3.1 ± 0.9
18:1	35.9 ± 2.4	43.4 ± 8.2	37.2 ± 6.5	36.6 ± 2.0	33.0 ± 5.0	32.5 ± 7.2
18:2	12.0 ± 3.9	12.0 ± 3.6	7.9 ± 2.8	5.9 ± 1.3	22.7 ± 5.8	29.8 ± 3.6
20:1	4.8 ± 2.6	6.6 ± 2.6	2.2 ± 2.4	3.9 ± 2.2	0.7 ± 0.8	1.4 ± 0.5
22:1	23.8 ± 10.4	11.3 ± 4.2^{x}	13.2 ± 12.3	4.2 ± 0.8	16.6 ± 8.2	12.8 ± 6.1

[a]All the results are presented as % of total methylated fatty acids (represented by area on readout from gas chromatogram). For further information see legend to Table II.

chain-shortening of 22:1 fatty acids has also been noted with the hypolipidemic drug, clofibrate, added to the diet (12,13). It is suggested from those studies that the location of the chain-shortening process is extramitochondrial, most likely peroxisomal. Preliminary results (14) show a 50-100% increase in peroxisomal fatty-acyl-CoA oxidizing activity in livers from animals fed partially hydrogenated marine oil compared to groundnut oil-fed animals. This may indicate that the increased chain-shortening capacity observed in the present study was also due to a proliferation of peroxisomes in the liver. The pattern, as well as the total percentage of chain-shortened radioactively labeled fatty acids, was very different in secreted VLDL-TG and liver-TG. This indicates that at least 2 different pools of TG exist in the liver, which has also been suggested by others (Kondrup, J.F., F. Lundquist and S.E. Damsgaard, personal communication, and 15-18). A better understanding of the mechanisms involved in the selection of TG for export vs. storage may be of great importance in the total understanding of the metabolism of 22:1 fatty acids. A preferential secretion of 22:1 fatty acids, as indicated by the results from a previous study (4), may explain why there is no, or very little, increase in the amount of TG in livers after feeding rapeseed oil (19,20).

The dietary fatty acids are introduced to the blood as chylomicrons. It has been reported that, of radioactively labeled chylomicrons, ca. 30% are taken up by the liver, 30% by adipose tissue, and the remainder by other organs, including the heart (21). In addition, the fatty acids are shuttled between the liver and other organs as plasma free fatty acids (FFA) and VLDL-TG. Of the plasma FFA, about one-third is taken up by the heart and one-third by the liver (22). These data indicate that, of the dietary fat, a considerable fraction of the fatty acids (which may be estimated to be at least 50%) will pass through the liver and be exposed to the chain-shortening process, while a smaller fraction will reach the heart more directly. Chain-shortening of erucic acid has also been observed in perfused heart (3). The capacity was, however, calculated to be at least 130 times higher in the liver than in the heart (23). Very little, if any, chain-shortening of erucic acid is found in isolated adipocytes (Christophersen, B.O., and J. Norseth, personal communication). This illustrates the importance of the chain-shortening process in the liver for the total conversion of 22:1 fatty acids in the body. An increase in the chain-shortening activity in the liver, as demonstrated in this study, may explain the decrease in the percentage of 22:1 fatty acids observed in TG secreted from perfused livers after prolonged feeding with rapeseed oil and partially hydrogenated marine oil (4). Consequently, it may also explain why the lipidosis in hearts of animals on an erucic acid diet is only temporary.

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Fatty Acid Composition of Heart Cells Exposed to Thermally Oxidized Fats

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ABSTRACT

Corn oil and olive oil were thermally oxidized, and the free fatty acids from the fresh fats, and from the distillable non-urea-adductable (DNUA) fractions of the thermally oxidized fats were prepared. These were added as emulsions to the medium of primary cultures of heart endothelial and muscle cells from neonatal rats. After exposure for 24 hr, the fatty acid composition of the triacylglycerol (TC) and phospholipid (PL) fractions of the cells was determined. Reflecting the nature of the fat used, the corn oil treatment produced relatively higher concentrations of linoleic acid in the TG and PL fractions compared to the olive oil treatment, in which case the oleic acid level was influenced. Treatment of the cultured cells with components derived from oxidized corn oil or oxidized olive oil resulted in lower concentrations of linoleic and arachidonic acids in the PL moieties compared to the fresh fat controls. However, there were marked increases in arachidonic acid in the TG fractions of both the endothelial and muscle cells. These changes due to the DNUA from thermally oxidized fats indicate a distinct metabolic response to the derivatives formed during thermal oxidation of the fats.

INTRODUCTION

The importance of fat in the Canadian and American diets is evidenced by the amount consumed. Much of this is from fried foods. Fats used in commercial food-frying operations or in home cooking often undergo physical and chemical changes. These altered fats may be organoleptically acceptable in human diets, but have exerted growth-depressing effects when fed to animals (1). Several workers have shown that thermally oxidized fats contain potentially harmful derivatives of fatty acids (2-4). The oxidation products of fatty acids can be concentrated in a distillable non-urea-adductable (DNUA) fraction. The monomeric and dimeric materials present are absorbed by experimental animals, and may exert toxic effects (5-8).

The influence of heated fat derivatives on the metabolic activity of tissues can be studied with whole animals, perfusion techniques, tissue slices, or cell culture techniques. The latter techniques have been employed by several workers in the fields of medicine, nutrition, and biochemistry for metabolic studies. Beating heart cells in culture were utilized for the first time by Harary et al. (9) as a model system for tests on intact myocardium. The main purpose of their study was to utilize this system to investigate the fatty acid metabolism of heart cells and its relationship to myocardial function.

In our laboratory, investigations have been carried out regarding the nutritional effects of thermally oxidized fats on experimental animals (10,11). In addition, we are using heart cell culture as a tool to study biological reactions involved in the toxicity of thermally oxidized fat components at the cellular level. In this study, the effects of the DNUA fraction on the fatty acid composition of heart endothelial (E) and muscle (M) cells were investigated.

MATERIALS AND METHODS

Preparation of Fat Samples

The two fats used in this study were corn oil (Mazola corn oil, Best Food Division) and olive oil (Pompeian Brand, Baltimore, MD). Thermally oxidized fats were prepared by heating the two oils continuously in stainless steel beakers at 180 C for 72 hr with 8 hr of continuous aeration every day by means of a mechnical stirrer. The DNUA material was prepared by the method of Crampton (12) with modification (13). The free fatty acids from fresh fat (FFA) and from the DNUA of oxidized fat (OFA) were obtained by a saponification procedure described earlier (14).

Primary Cultures of Heart Cells

Primary cultures of rat heart endothelial (E) and muscle (M) cells were obtained from neonatal rat hearts. Approximately 12 rat pups (2-5 days old) were used each time a heart cell culture was prepared. The pups were killed by an overdose of ether in a beaker. Their hearts were excised aseptically, and transferred to a petri dish containing phosphate buffered saline (PBS, GIBCO, Grand Island, NY) and chopped finely. The triturated tissue was separated into single cells by trypsinization using a 0.25% trypsin solution (GIBCO, Grand Island, NY). Heart E cells were separated from the M cells as described previously (14,15).

The cells were grown in a culture medium HB597 (Connaught Laboratories, Ltd., Willow-

dale, Ontario), supplied with 5% fetal calf serum (GIBCO, Grand Island, NY). These were in the form of monolayered coverslip cultures, enclosed in stoppered leighton tubes (Bell Co., Vineland, NJ). After four days' growth, they were exposed to the different treatments for 24 hr.

Administration of Lipid Fractions to Culture Medium

Lipid fractions (FFA from fresh fats, and FFA from DNUA of thermally oxidized fats) were administered to the culture medium (100 μg/ml) in the form of an emulsion with bovine serum albumin (Fraction V, poor in unesterified fatty acids) dissolved in phosphate buffered saline. The required concentration of each free fatty acid fraction, dissolved in hexane, was transferred to a 100 ml sterilized bottle, and the solvent was evaporated completely. The solution of bovine serum albumin (40 mg/ml of PBS) was added to the lipid fractions. A ratio of free fatty acid fraction to bovine serum albumin of 1:60 (w/w) was maintained. The bottle was screw capped and incubated at 40 C with occasional shaking for 2 hr. This incubation period was sufficient to obtain an emulsion of free fatty acids ready for administration into the culture medium. Further details have been described elsewhere (14).

Iodine Values and Carbonyl Values

The iodine values of fresh and thermally oxidized fats were determined by the method of the Association of Official Analytical Chemists (16). Carbonyl values for the thermally oxidized fats were determined by the method of Bhalerao et al. (17).

Fatty Acid Analyses of Fats

Methyl esters of the fatty acids (from fresh fats or DNUA fractions) were prepared by incubating them with 6% sulfuric acid in methanol at 80 C for 15 hr in screw-capped vials. The esters were extracted with petroleum ether which was evaporated under nitrogen, and the dried sample was stored at -20 C until analyzed by gas liquid chromatography (GLC). The esters were dissolved in carbon disulfide (10 μg/ml), and a 2 μl aliquot was injected into the gas chromatograph (Varian, Aerograph Series 500). The stainless steel balanced columns (100 cm x 2 mm I.D.) were packed with 3% EGSP-Z on acid-washed Gaschrom Q (Applied Science Laboratories, State College, PA). A flow rate of 50 ml/minute was used for the nitrogen carrier gas. The columns were run isothermally at 180 C, and detection of component fatty acids was by flame ionization. Identification of the peaks was accomplished by comparing the relative retention times with those of standards (NuChek Prep., Elysian, MN). Quantitation of peak area was carried out with an electronic digital integrator equipped with a teletype and paper tape punch (Technical Marketing Associates, Ltd., Toronto, Ontario). The fatty acid concentrations are expressed as percentages of the total fatty acids by weight.

Isolation, Separation, and Analyses of Cellular Lipids

Cellular lipid was extracted by means of the Folch procedure (18) and fractionated by thin layer chromatography (TLC). Glass plates coated with Silica Gel G of 0.5 mm thickness, and a solvent system consisting of heptane/isopropyl ether/acetic acid (60:40:3) were used (19). Preparation of the methyl esters of the fatty acids and their analysis by GLC were similar to above.

RESULTS

Analyses of Fats

Corn oil and olive oil are vegetable oils high in linoleic and oleic acids respectively, with other common fatty acids in much smaller amounts. The fatty acid compositions of the two fresh fats are shown in Table 1.

Unsaturated fatty acids are known to be more resistant to urea adduction than the saturated fatty acids. Compositions of the DNUA fractions obtained from thermally oxidized corn oil (OCO) and thermally oxidized olive oil (OOO) are shown in Table II. The two major fatty acids present in the DNUA samples, identifiable by GLC, were oleic and linoleic acids. Several other peaks mainly representing oxidation products probably ranging in chain length from 7-20 carbons as estimated by retention times, constituted 22% and 32% of the OCO and OOO.

Iodine and carbonyl values of the fat samples are in Table III, as indicators of oxidative deterioration. Heating and oxidation of the fats resulted in greatly reduced iodine values. For the thermally oxidized fats, olive oil had a higher carbonyl value than corn oil revealing more oxygen uptake.

Fatty Acid Composition of Heart Cells

The concentrations of specific fatty acids in the fresh fats were reflected in the fatty acid composition of the heart cells. Therefore, cells exposed to CO (Tables IV and V) had higher

TABLE I

Fatty Acids of Fresh Fats[a]

Fatty acids	Corn oil	Olive oil
14:0	tr	---
15:0	tr	---
16:0	9.0	12.0
16:1	0.5	0.9
17:0	tr	tr
18:0	2.0	2.5
18:1	23.0	75.0
18:2	62.0	8.0
18:3	---	1.0
20:0	1.2	0.5
20:1	1.3	0.5

[a]Expressed as percentage of total fatty acids by weight and trace (tr) indicates less than 0.5%. Each value represents the mean of three determinations.

TABLE II

Concentration of Oleic and Linoleic Acids in the DNUA Fractions of Thermally Oxidized Fats[a]

Fatty acids	OCO	OOO
18:1	35.3	30.0
18:2	42.5	37.9
Others[b]	22.2	32.1

[a]Expressed as percentage of total fatty acids by weight: OCO = distillable non-urea-adductable fraction (DNUA) from thermally oxidized corn oil, and OOO = DNUA from thermally oxidized olive oil. Each value represents the mean of three determinations.

[b]Includes oxidized fat components not adducted by urea. Their chain lengths ranged from 7-20 carbons.

levels of linoleic acid in the PL and TG fractions than for the OO treatment (Tables VI and VII). Likewise, cells treated with OO showed very high levels of oleic acid in their PL and TG fractions. OCO (Tables IV and V) produced low levels of linoleic acid and arachidonic acid in the PL fractions compared to CO controls. These cells also had a higher proportion of saturated fatty acids. For both E and M cells there were marked increases in the arachidonic acid content of the TG fractions of the heart cells treated with OCO concomitant with reductions in levels of linoleic acid. This effect was more pronounced in the M cells.

The cells treated with OOO also exhibited a reduction in levels of linoleic and arachidonic acids in their PL fractions (Table VI and VII) even though the OOO contained more 18:2 (38% in Table II) than did the OO (8% in Table I). The OOO treated cells showed lower levels of oleic acid, increases in the levels of linoleic acid, and at least two-fold increases in the relative proportions of arachidonic acid in the TG fractions compared to the OO treated cells. These increased levels of linoleic acid for the TG fractions were not seen in the case of the OCO treatment. Again, the cells exposed to OOO exhibited relatively more saturated fatty acids in the PL fractions.

DISCUSSION

High levels of 18:1 and 18:2 in the DNUA fractions of OCO and OOO confirmed that these unsaturated fatty acids are more resistant to urea adduction than the saturated fatty acids. Also, linoleic acid was more resistant than oleic acid as revealed by the results for OOO in Table II. The total level of the minor components was considerably higher in the OOO material.

Cultured cells are known to be very sensitive to physical and biochemical stimuli present in their environment. Therefore, it is quite conceivable that the metabolic effect of certain compounds would be more extensive and rapid in cultured cells than would be expected in the intact organ of the live animals. Heart cells appeared to respond rapidly to fatty acids administered to the culture medium. Major changes associated with fatty acid composition of the heart cells treated with heated fat components were: a) lower levels of linoleic and arachidonic acids and higher levels of

TABLE III

Carbonyl Values and Iodine Values of Thermally Oxidized Fats[a]

	Thermally oxidized corn oil	Thermally oxidized olive oil
Carbonyl value	165.3 ± 2.7	192.5 ± 1.5
Iodine value[b]	100.0 ± 4.8 (133.1 ± 1.5)	60.5 ± 2.4 (86.0 ± 0.5)

[a]Each value represents the mean of three determinations ± SEM.

[b]Values in parentheses are for fresh fats.

TABLE IV

Fatty Acid Composition of Phospholipid and Triacylglycerol Fractions of Heart Endothelial Cells Treated with Free Fatty Acids from Fresh or Oxidized Corn Oil[a]

	Treatments			
Fatty acids[b]	CO PL	OCO PL	CO TG	OCO TG
14:0	tr	---	1.0	---
16:0	27.3	35.6	17.3	18.6
16:1	2.0	---	2.5	7.8
18:0	12.7	20.5	20.5	13.4
18:1	26.0	23.2	23.3	17.6
18:2	12.0	8.0	22.0	7.6
20:2	3.0	---	---	---
20:3	tr	---	---	---
20:4	5.8	2.1	12.9	30.9
22:6	1.2	---	---	---
Others	9.5	10.6	0.5	4.1

[a]Expressed as % of total fatty acids by weight: tr=trace, CO=fresh corn oil, and OCO=DNUA fraction of thermally oxidized corn oil.

[b]Pooled samples from 4 or more cell cultures, and means from at least 3 determinations by GLC.

TABLE V

Fatty Acid Composition of Phospholipid and Triacylglycerol Fractions of Heart Muscle Cells Treated with Free Fatty Acids from Fresh or Oxidized Corn Oil[a]

	Treatments			
Fatty acids[b]	CO PL	OCO PL	CO TG	OCO TG
14:0	---	---	tr	tr
16:0	26.6	33.3	26.9	23.0
16:1	tr	---	tr	---
18:0	10.4	18.5	15.3	10.5
18:1	33.4	27.8	23.2	12.7
18:2	13.3	6.3	24.2	2.9
20:0	---	---	---	---
20:3	2.5	1.6	---	---
20:4	4.9	1.7	10.0	43.4
Others	8.5	10.8	---	7.3

[a]Expressed as % of total fatty acids by weight: tr=trace, CO=fresh corn oil, and OCO=DNUA fraction of thermally oxidized corn oil.

[b]Pooled samples from 4 or more cell cultures, and means from at least 3 determinations by GLC.

saturated fatty acids (16:0 and 18:0) in the PL fractions, and b) a dramatic increase in the arachidonic acid concentration of TG fractions, compared to that for control cells. Lower levels of linoleic and arachidonic acids in the PL fractions of cells exposed to OFA would indicate that heated fat components are producing an intracellular environment deficient in essential fatty acids for PL synthesis; but this could not have been the case because the DNUA fractions contained ca. 40% linoleic acid, and the culture medium also contained fetal calf serum as a source of EFA.

It was reported previously (14) that heated fat-treated heart cells have a tendency to take up an exogenous fatty acid very rapidly and to incorporate it preferentially into the TG fraction. Therefore, a higher concentration of unsaturated fatty acids in the TG fractions of such cells should be expected, due to increased

TABLE VI

Fatty Acid Composition of Phospholipid and Triacylglycerol Fractions of Heart Endothelial Cells Treated with Free Fatty Acids from Fresh or Oxidized Olive Oil[a]

	Treatments			
Fatty acids[b]	OO PL	OOO PL	OO TG	OOO TG
14:0	---	0.7	2.0	tr
16:0	23.7	34.9	16.0	19.8
16:1	0.5	1.0	---	---
18:0	11.7	18.4	5.4	8.8
18:1	40.8	23.4	52.4	16.8
18:2	8.9	5.8	11.4	23.4
20:0	---	1.0	---	---
20:1	1.0	---	---	---
20:2	2.0	tr	---	---
20:3	1.1	tr	---	---
20:4	6.4	2.5	11.0	22.3
22:6	0.9	tr	---	---
Others	3.0	12.0	2.8	8.5

[a]Expressed as % of total fatty acids by weight: tr=trace, OO=fresh olive oil, and OOO= DNUA fraction of thermally oxidized olive oil.

[b]Pooled samples from 4 or more cell cultures, and means from at least 3 determinations by GLC.

TABLE VII

Fatty Acid Composition of Phospholipid and Triacylglycerol Fractions of Heart Muscle Cells Treated with Free Fatty Acids from Fresh or Oxidized Olive Oil[a]

	Treatments			
Fatty acids[b]	OO PL	OOO PL	OO TG	OOO TG
14:0	---	2.1	tr	tr
16:0	28.9	35.9	15.7	18.0
16:1	0.7	0.5	---	---
18:0	11.7	19.9	4.0	8.9
18:1	38.9	22.8	51.3	13.4
18:2	5.2	2.3	7.7	16.9
20:2	1.5	---	tr	tr
20:3	1.9	---	tr	tr
20:4	3.5	2.6	11.7	28.0
22:5	---	---	---	---
22:6	---	---	---	---
Others	7.7	14.9	9.0	14.0

[a]Expressed as % of total fatty acids by weight: tr=trace, OO=fresh olive oil, and OOO= DNUA fraction of thermally oxidized olive oil.

[b]Pooled samples from 4 or more cell cultures, and means from at least 3 determinations by GLC.

TG synthesis. TG accumulation in the cells in the form of lipid droplets actually was observed under an inverted phase contrast microscope. This would tend to produce an intracellular condition with a deficiency of EFA for PL synthesis. Oxidized fat components also may exert inhibitory effects on PL synthesis.

Another reason for lower levels of EFA in the PL fraction of cells exposed to OFA could be deterioration of these unsaturated acids in the membranes as a result of the treatments. which could be oxidative in nature. Similar observations have been made by Kajimoto et al. (20,21) and Gabriel et al. (10). These authors

observed lower levels of linoleic and arachidonic acids in the PL fractions of several tissues of rats fed thermally oxidized fats in their diet.

A higher level of arachidonic acid, accompanied by a lower level of linoleic acid, also was found in the TG fractions for cells from the OCO treatment. This may have indicated an enhanced conversion of linoleic acid into arachidonic acid, a condition much less evident for the OOO group. Therefore, it appears that oxidized fat components had some effect on the cellular enzymes involved in the desaturation and chain elongation of fatty acids. In vivo studies by Rao and coworkers (22) showed a rapid rate of elongation and desaturation of fatty acid chains in the livers of rats fed heated fats. Cortesi and Privett (23) studied the acute toxicity of hydroperoxides of methyl linoleate. These investigators reported a very high level of arachidonic acid in the serum and lung tissue lipids of their experimental animals.

The very high levels of arachidonic acid seen in the TG of cultured cells is not found in the heart tissue of animals fed oxidized fat. However, we must consider the environmental conditions to which the cultured heart cells were exposed. The OCO and OOO supplied mainly 18:1 and 18:2 of the normal fatty acids found in the controls. Consequently, these two fatty acids would be taken up in substantial quantities by the cells for metabolism. Also, the data for the TG fractions indicate that the 18:2 was taken up by the cells in preference to 18:1, and much of the 18:2 was converted to 20:4.

Cultured heart cells can serve as a useful model system to carry out toxicological studies related to lipid metabolism, in that the experimental environment can be altered and controlled to observe the effects of specific treatments.

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Correlation between Skeletal Muscle Free Fatty Acid Extraction and Vascular Decompensation during Hemorrhagic Hypotension[1]

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ABSTRACT

The objective of this study was to determine whether or not a relationship exists between free fatty acid (FFA) extraction by skeletal muscle and onset of irreversible shock. Hind limb skeletal muscle vasculature of anesthetized dogs was surgically isolated from cutaneous tissue and subjected to a modified Wigger's hemorrhage shock protocol which was divided into five stages (I-V). Since the first signs of irreversibility began in Stage II, this stage of hypovolemic hypotension was subdivided into IIa, IIb and IIc. Arterial and venous blood samples were taken during each stage for subsequent blood gas and FFA analysis. The data indicated that the onset of severe tissue ischemia and metabolic acidosis occurs concurrently with increased uptake of FFA and skeletal muscle vasodilation (decompensation). A possible physiological explanation for these observations could be related to an increased synthesis and release of PGE_1. This agent has been shown by others to inhibit adrenergic neurotransmitter release causing loss of vascular tone.

INTRODUCTION

Severe hypovolemia results in hypotension and organ hypoperfusion, and if not corrected early by fluid replacement will progress to irreversible cellular damage and cardiovascular failure (1). The cardiovascular decompensation must result from either a cardiac (2) or peripheral vascular failure (3-7). A comprehensive cardiodynamic and substrate utilization study by our group argues against the former (4). Other studies by us (7) have indicated a rather significant loss of vascular tone (decompensation) in the hind limb skeletal muscle late in oligemia which appears only in those animals that entered into irreversible shock, while continued vasoconstriction (compensation) was noted in all animals surviving the hemorrhage procedure. The potential significance of this loss of vascular tone has been examined by Rothe and Selkurt (8) in studies in which they demonstrated a 40% fall in total peripheral vascular resistance between early and late oligemia. The order of magnitude of this decreased total peripheral resistance can be accounted for by the concurrent fall in skeletal muscle vascular resistance reported by us (3,5,6). Together, these studies suggest that the skeletal muscle vasculature may play a major role in the loss of total body vascular tone and eventual cardiovascular decompensation associated with prolonged hemorrhagic hypotension.

One possible explanation for the paradoxical loss of vascular tone could be due to an adrenergic inhibition resulting from excessive prostaglandin E release by the vasculature in the skeletal muscle during the high sympathetic tone induced by hemorrhage (9,10). Since free fatty acids (FFA) are known to be the precursors of these hormones (11-13), the objective of the present study was to determine whether or not a relationship exists between skeletal muscle FFA extraction and the onset of skeletal muscle vascular decompensation which is manifested as a loss of vascular tone.

METHODS

General

Thirteen healthy mongrel dogs of both sexes were anesthetized by intravenous administration of ca. 30 mg/kg pentobarbital sodium. All animals were placed in the supine position, tracheostomized and connected to a Bennett positive pressure respirator which was adjusted at the beginning of each experiment so that the arterial blood gases and pH were within acceptable values for normal animals (Table I). Once this adjustment was made for each animal, no further respirator adjustments were necessary during the experimental procedure.

Hind limb skeletal muscle vasculature was functionally isolated from the cutaneous vasculature by applying a tourniquet around the hock and ligating the lateral and medial saphenous veins (3). The femoral vein was cannulated distally, and muscle venous outflow directed through a hematocrit-insensitive precalibrated cannulation type electromagnetic blood flow probe with an inside diameter of 1/8 inch (14). Blood was returned to the central end of the femoral vein via a second cannula attached to the free end of the flow probe. The flow probe was coupled to a Model 501 flowmeter amplifier (Carolina Medical

[1]Presented at the fall American Physiological Society Meetings, St. Louis, MO, October 22-27, 1978.

TABLE I

Arterial and Skeletal Muscle Blood Chemistries during Selected Stages of Hemorrhagic Shock

Stage		I	IIa	IIb	IIc	III	IVa	V
		$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$
pO_2 (mm Hg)	Art.	91.9 ± 3.8	80.1 ± 3.9[a]	83.1 ± 4.1[a]	100.4 ± 6.1	108.7 ± 4.9	124.8 ± 3.4	93.8 ± 4.3
	Ven.	52.2 ± 2.7	19.9 ± 1.5[a]	15.8 ± 1.2[a]	19.9 ± 1.4[a]	24.8 ± 1.8[a]	84.6 ± 3.2	26.5 ± 2.2[a]
pCO_2 (mm Hg)	Art.	24.3 ± 1.9	20.4 ± 1.8	19.2 ± 1.5[a]	16.3 ± 0.9[a]	19.5 ± 1.8[a]	30.5 ± 1.4[a]	26.7 ± 2.5
	Ven.	27.8 ± 2.1	33.1 ± 2.3[a]	45.8 ± 3.7[a]	62.1 ± 4.7[a]	62.9 ± 4.5[a]	48.0 ± 2.1[a]	57.4 ± 2.5[a]
pH	Art.	7.496 ±0.002	7.488 ± 0.003	7.319 ± 0.003[a]	7.117 ± 0.005[a]	7.036 ± 0.004[a]	6.984 ± 0.003[a]	7.156 ± 0.003[a]
	Ven.	7.471 ± 0.022	7.395 ± 0.002[a]	7.191 ± 0.005[a]	6.905 ± 0.006[a]	6.860 ± 0.005[a]	6.910 ± 0.003[a]	7.038 ± 0.002[a]
Base (mEq/L)	Art.	-2.8 ± 0.5	-5.4 ± 0.7[a]	-13.8 ± 1.1[a]	-22.2 ± 1.6[a]	-24.4 ± 1.3[a]	-24.3 ± 1.1[a]	-18.3 ± 0.8[a]
	Ven.	-2.3 ± 0.4	-4.0 ± 0.6[a]	-11.4 ± 1.5[a]	-22.1 ± 2.2[a]	-24.4 ± 1.8[a]	-24.3 ± 1.3[a]	-16.8 ± 0.9[a]
Hb (gm %)	Art.	12.9 ± 0.6	13.8 ± 0.5	13.6 ± 0.5	13.1 ± 0.6	14.0 ± 0.6[a]	14.6 ± 0.5[a]	15.1 ± 0.6[a]
	Ven.	13.7 ± 0.7	13.0 ± 0.5	13.5 ± 0.5	12.5 ± 0.6	13.9 ± 0.5	14.6 ± 0.6	15.0 ± 0.6

[a]$P<0.05$ when compared to Stage I.

Electronics, Inc.). Skeletal muscle venous pressure was monitored from a port in the flow probe. Following completion of all surgical procedures, but prior to attachment of any of the extracorporeal circuitry, blood coagulation was inhibited by intravenous injection of 5 mg/kg heparin sodium with supplemental doses of 2.5 mg/kg administered hourly.

A system of catheters, T-tubes, low displacement P23Db Statham pressure transducers, and a blood reservoir bottle permitting the simultaneous monitoring of skeletal muscle venous pressure, systemic arterial pressure, and blood flow during hemorrhage and reinfusion has been described previously (3). All pressures, flows, heart rates and Lead II of the electrocardiogram were recorded on a Model VR6 Electronics for Medicine polygraph.

Hemorrhagic Shock Protocol

A slow hemorrhage was achieved by allowing 5 ml/kg body weight of blood to flow into a calibrated reservoir from a cannula in the femoral artery contralateral to the limb from which flow was being monitored. The animals were allowed to compensate for ca. 5 min and the procedure repeated until mean arterial blood pressures (ABP) had fallen to 35-40 mm Hg. The reservoir was then adjusted above the animals so that the open catheters and associated tubing resulted in a hydrostatic column of blood that exactly counterbalanced the animals' ABP. With this arrangement, blood flowed into the reservoir during the phase of compensation (i.e., peripheral vasoconstriction) and automatically returned to the animal during decompensation (i.e., peripheral vasodilation). After the animals had decompensated to the point of 25% uptake from the reservoir, the blood remaining in the reservoir was rapidly reinfused via a cannula in the jugular vein.

The shock protocol was divided into 5 primary stages (see Fig. 1). Stage I represents the prehemorrhage control state. Stage IIa occurred when the ABP first reached 35-40 mm Hg and the reservoir line was left open. Stage IIb represents the point of minimum vascular conductance (maximum vasoconstriction). Stage IIc is the point of maximum blood loss. Stage III occurred when the animal has spontaneously taken back from the reservoir 25% of the maximum shed blood. Stage IVa represents the phase of hemodynamic instability immediately following the rapid reinfusion of the blood remaining in the reservoir. Stage V occurred when the ABP had fallen below 50 mm Hg and death was imminent. This stage has been called normovolemic hypoten-

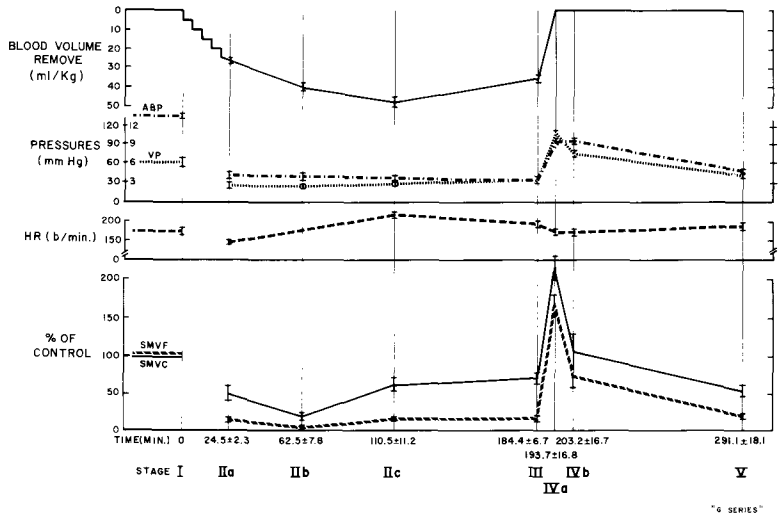

FIG. 1. The hemorrhage protocol and resulting hemodynamic data are presented in this figure. Each data point represents the mean of 13 observations and the associated bars describe ± 1 standard error of the mean. ABP - aortic blood pressure; VP - venous blood pressure; HR - heart rat; SMVF - skeletal muscle venous flow; and SMVC - skeletal muscle vascular conductance. The SMVC was calculated by dividing SMVF by the difference between ABP and VP. The prehemorrhage control or Stage I values for SMVF and SMVC were taken as 100%.

sion by some and irreversible shock by others. A pressure of 50 mm Hg was chosen because, below this point, animals not on respirators develop periodic breathing patterns that ultimately lead to respiratory failure.

Blood Chemistries

Approximately 3 ml of heparinized blood were taken from the femoral arteries and a second 3 ml sample from the extracorporeal tubing draining the muscle beds during Stages I, IIa, III, IVa and V (see Fig. 1). Approximately 1.5 ml of each of these samples were used to determine pH, pCO_2 and pO_2 using a Model 213 Instrumentation Laboratories, Inc. pH blood/gas meter. Hemoglobin (Hb) was determined photometrically by using an AO-Spencer Hb-meter and hematocrit (Hct) by centrifugation.

The remaining plasma was separated from the cells by centrifugation at 2,200 rpm, O C for 15 min in an International Centrifuge. One ml plasma samples were then taken for the determination of free fatty acids by the automated titration method of Lorch and Gey (15).

RESULTS

Hemodynamic Studies

The hemodynamic data obtained from the 13 animals described in the methods section are summarized in Figure 1. During Stage I, the ABP was 136 mm Hg; skeletal muscle venous pressure (VP) was 6 mm Hg; heart rate (HR) was 175 beats/min; skeletal muscle venous flow (SMVF) was 44 ml/min; and skeletal muscle vascular conductance (SMVC) was 0.32 ml/min •mm Hg. Both SMVF and SMVC are expressed as 100% control. Between Stage I and Stage IIa, ABP was reduced to 40 mm Hg by a series of 5 ml/kg hemorrhages, which resulted in significant reductions in VP, HR, SMVF and SMVC. With the bleeding line left open and the reservoir height adjusted to maintain an ABP between 35 and 40 mm Hg, the animals continued to compensate by reducing their SMVC to a minimum of 20% of control in Stage IIb. Stage IIc marked the point of maximum blood loss (48 ml/kg). It is significant to note that even though there was some evidence of cardiovascular compensation between Stages IIb and IIc (i.e., continued blood loss while maintaining a constant ABP and a significant increase in heart rate to 215 b/min), there was also a significant ($P<0.001$) increase in SMVC from 20 to 61% of control indicating a marked loss of skeletal muscle vascular tone (i.e., vascular decompensation). Between Stage IIc and Stage III, it was necessary for the animals to spontaneously take back from the reservoir ca. 25% of the maximum shed volume in order to maintain the ABP. Even when the right ventricle was stressed by reinfusion of the blood

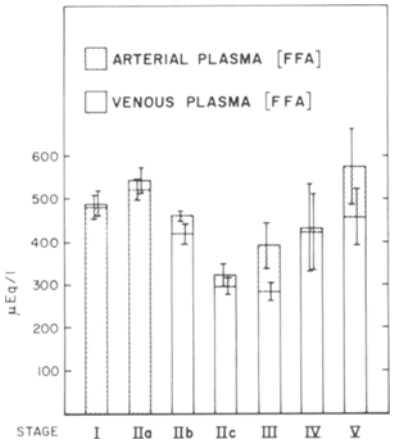

FIG. 2. The shaded bars on this histogram represent the arterial plasma levels of FFA while the open bars illustrate the venous plasma levels. The plasma samples were taken during the shock stages indicated along the abscissa. Each data point and its associated bar represents the mean ± 1 standard error of the mean for 13 observations.

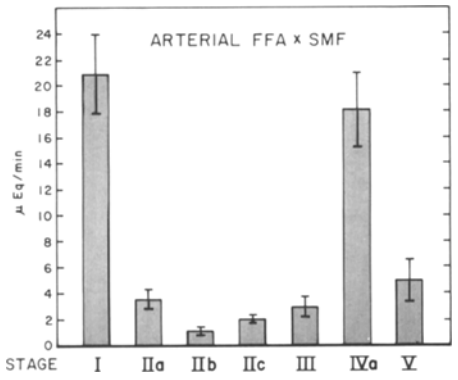

FIG. 3. This histogram illustrates the rate of FFA presentation to the skeletal muscle vascular bed during each of the protocol stages indicated on the abscissa. These data were determined by multiplying the arterial plasma FFA concentration (Fig. 2 – shaded bars) times the skeletal muscle venous flow (SMVF). Each data point and its associated bar represents the mean ± 1 standard error of the mean for 13 observations.

remaining in the reservoir between Stages III and IVa, the VP increased only transiently to 11 mm Hg providing additional evidence of adequate right ventricular function. The return of the shed blood resulted in an ABP increase to 95 mm Hg and SMVF to 171% of control, both increases represent a significant increase in left ventricular workload. Within 10 min (Stage IVb), however, SMVC had returned to pre-hemorrhage control values. Over the next one and one-half hr between Stages IVb and V, a progressive fall in all hemodynamic parameters except HR was noted. The Stage V VP was less than the prehemorrhage Stage I value suggesting that the terminal fall in ABP may be the result of a progressive reduction in effective circulating blood volume brought about by either a loss of intravascular fluid or an increased total vascular capacitance, or both. Therefore, no hemodynamic evidence is provided that would implicate myocardial decompensation as an explanation for the terminal hypotension.

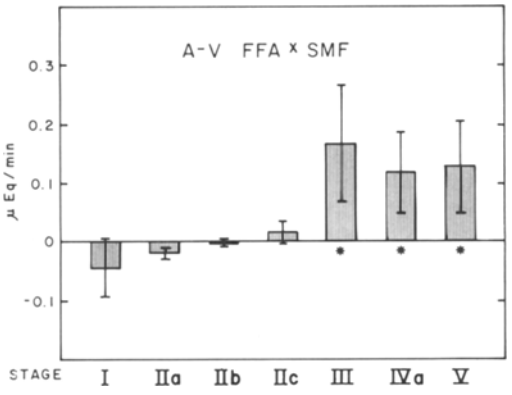

FIG. 4. This bar graph depicts the rate of FFA removal from the blood during the five stages indicated as it passes through the skeletal muscle vascular bed. These data were determined by multiplying the arterial/venous FFA difference times the skeletal muscle flow. Each data point together with its associated bar represents the mean ± 1 standard error of the mean for 13 observations. * indicates $P < 0.05$ when compared to Stage I.

Blood Analyses

Arterial and skeletal muscle venous blood gas/pH data taken from the 13 dogs during the 5 primary stages of the shock protocol (see Fig. 1) are presented in Table I. Arterial pO_2 was maintained essentially constant during all 5 stages using the respirator. The venous values, however, fell dramatically during the low flow Stages IIa-III. The pCO_2, pH and base values during these stages substantiate severe ischemia and metabolic acidosis in the skeletal muscle which was not corrected by the return of blood volume to normal in Stages IVa and V. The progressive increase in hemoglobin (Hb) between Stages IIc and V suggest that the terminal fall in pressure may be due to intravascular fluid loss. This hypothesis is consistent with the terminal fall in venous pressure described above.

The arterial and skeletal muscle venous

plasma concentration of FFA are presented in Fig. 2. Note that both the arterial and venous concentrations increase slightly between Stages I and IIa, after which both begin to fall, reaching their minimum arterial value by Stage IIc and venous value by Stage III. This was followed by a progressive increase until both the arterial and venous concentrations returned to values not significantly different from the Stage I controls.

The rate of FFA presentation to the skeletal muscle during the shock experiments is illustrated in Figure 3. These data were calculated by multiplying the arterial FFA concentration times the actual blood flow/min at the time of the blood sampling. The hemorrhage procedure resulted in a marked fall in FFA presentation from 21 ± 3 mEq/min in Stage I to a low point of 1.1 ± 0.3 mEq/min in Stage IIb. This was followed by a stepwise increase to 2.9 ± 0.8 mEq/min by Stage III. The increased flow occurring during restoration of blood in Stage IVa (see Fig. 1) resulted in a transient increase to nearly Stage I levels (18 ± 3 mEq/min). The subsequent fall in flow by Stage V resulted in a terminal presentation of only 4.9 ± 1.7 mEq/min.

Figure 4 illustrates the rate of FFA removal from the blood traversing the skeletal muscle. These data were calculated by multiplying the arterial-venous FFA differences times the skeletal muscle blood flows. These data suggest the occurrence of a borderline significant skeletal muscle release of FFA during Stages I and IIa followed by an absolute balance at Stage IIb. In Stage IIc, by which time skeletal muscle vascular decompensation has been manifested, a borderline significant FFA extraction was seen. The 3 subsequent stages all show significant ($P<0.05$) tissue uptake of FFA.

DISCUSSION

The data presented in Table I, Figure 1 and Figure 4 indicate the development of a sequential relationship between (a) severe metabolic acidosis coupled with ischemia; (b) the conversion from a negative to a positive FFA uptake; and (c) the loss of skeletal muscle vascular tone between compensatory (Stage IIa-IIb) and decompensatory oligemia (Stage IIc-III). These relationships exist despite a dramatic reduction in the rate of FFA delivery to the skeletal muscle tissue (Fig. 3) compared to prehemorrhage control or Stage I.

A positive correlation between the development of acidosis and the accumulation of FFA in mammalian cells has been reported by Mackenzie et al. (16). These authors demonstrated that the major lipid fraction that was increased as a result of a reduction in pH from 7.4 to 6.9 was the triglyceride component. In 1967, Spector (17) reported that a reduction in pH from 7.4 to 6.6 resulted in an increased oxidation of FFA and an increased esterification of FFA to both phospholipids and triglycerides, thus suggesting that pH plays a role in intracellular utilization as well as accumulation of FFA. Spector (17) also hypothesized that the binding of FFA to albumin is weakened when pH is lowered. This would allow the FFA to passively diffuse into the cells. The data and hypothesis of Mackenzie et al. (16) and Spector (17) provide a logical explanation for the data presented in Figure 4 which indicate FFA uptake coupled with acidosis (Table I) during Stages III-V.

Although the data presented in the current report do not provide verification of a cause and effect relationship between FFA utilization and the loss of vascular tone in the skeletal muscle during prolonged hemorrhagic hypotension, the following lines of evidence would support a hypothesis involving an augmented synthesis and release of the vasodilator agent prostaglandin E_1 (PGE_1):

1. Several independent studies have shown that PGE_1 causes a loss of vascular tone by inhibiting transmitter release at the adrenergic synapse (9-10,18), particularly under conditions of augmented adrenergic activity (11-12,19-20).

2. A 2.7-fold increase in the blood levels of PGE_1 have been shown by Flynn and Howard (20) to occur late in oligemic shock. The timing of this increase compares favorably with the advent of both the loss of skeletal muscle vascular tone reported by us previously (3-7,21) as well as in Figure 1, and the decrease in total peripheral vascular resistance reported by Rothe and Selkurt (8).

3. The loss of vascular tone occurs at a predictable time during the hemorrhage protocol when adrenergic activity has been shown by Gonzalez and Bond (21) to be 4 times higher than prehemorrhage control. The same report indicates a similar loss of vascular tone during persistent electrical stimulation of the autonomic chain supplying the vascular bed under study.

4. Evidence favoring a tissue release of PGE_1 during autonomic stimulation has been presented by Brody and Kadowitz (9) and Davis and Horton (19). Furthermore, De La Lande et al. (11) have reported a reduction in the vascular tone, initiated by either sympathetic nerve stimulation or extraluminal norepineph-

rine, by either PGE_2 or its precursor arachidonic acid.

5. PGE_1 is not stored in tissues but rather requires a constant supply of precursor substrates which include the FFA (13,22). A comparison of the data presented in Figure 1 and 4 indicate that the loss of vascular tone and conversion from no FFA uptake to significant uptake occur during the same time frame (Stages IIc-III).

6. The synthesis and release of PGE_1 is stimulated by tissue ischemia (18) and high sympathetic tone (9,12,19,22), both of which have been shown to occur during hemorrhagic hypotension. The degree of tissue ischemia and metabolic acidosis is seen in Table I when one compares the Stage I data with Stages IIc and III.

In conclusion, we suggest that the high sympathetic tone which occurs during hemorrhagic hypotension together with tissue ischemia and metabolic acidosis provide the optimal conditions for FFA extraction from the blood which then may initiate an increased synthesis and release of PGE_1. The increased levels of PGE_1 are then available to inhibit adrenergic neurotransmitter release causing the vasoconstricted vessels to relax or lose some of their pre-existing high tone. The resulting vasodilation is interpreted by us as skeletal muscle vascular decompensation which could contribute to the fall in total peripheral vascular resistance seen by Rothe and Selkurt (8). This vascular decompensation may then progress to a condition known as irreversible shock.

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Lack of Regioselectivity in Formation of Oxohydroxyoctadecenoic Acids from the 9- or 13-Hydroperoxide of Linoleic Acid[1]

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ABSTRACT

Either 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid or 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid was treated with the catalyst, cysteine-$FeCl_3$, in the presence of oxygen. Oxohydroxyoctadecenoic acids were among the many products formed as a result of hydroperoxide decomposition. A mixture of 9(13)-oxo-13(9)-hydroxy-*trans*-11(10)-octadecenoic acids (δ-ketols) was produced from either isomeric hydroperoxide. The formation of isomeric δ-ketols from 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid (epoxyol), a known product of 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid decomposition, implies that the epoxyol is an intermediate. The mechanism was elucidated by the facile conversion of the epoxyol (methyl ester) to methyl 9(13)-oxo-13(9)-hydroxy-*trans*-11(10)-octadecenoates with a Lewis acid, BF_3-etherate.

INTRODUCTION

The formation of 9(13)-oxo-13(9)-hydroxy-*trans*-11(10)-octadecenoic acids (δ-ketols) from linoleic acid hydroperoxides (LOOH) has been observed in a few investigations (1,2). According to Gardner et al. (1), ferrous (cysteine-$FeCl_3$) catalyzed decomposition of LOOH to δ-ketols among a number of other products; however, in that investigation, there was no indication as to whether δ-ketols were formed by a regiospecific route. By gas chromatography-mass spectroscopy (GC-MS), Sessa et al. (2) identified δ-ketols isolated from oxidized phosphatidylcholine obtained from hexane-defatted soybean flakes. Because of insufficient sample, no other data was obtained.

Since there is ample evidence for the regiospecificity of epoxide formation from specific isomers of LOOH (1,3-9), it was of interest to determine if δ-ketols were formed by a regiospecific pathway.

MATERIALS AND METHODS

Specific isomers of LOOH were prepared as described (10). The LOOH isomers were decomposed with the cysteine-$FeCl_3$ catalyst (1), except that the reaction solvent was methanol/water, 8:2, instead of ethanol/water, 8:2. Thorough oxygenation was ensured by bubbling pure oxygen into the reaction mixture. Product δ-ketols were isolated by column chromatography (1). 9-Hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid and its methyl ester were prepared from 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid as before (3).

BF_3-etherate (Eastman Organics) diluted with ether [$BF_3O(C_2H_5)_2$-ethyl ether, 1:19] was used to convert methyl 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoate to δ-ketols (methyl ester) at room temperature for 30 min.

For analysis by GC-MS, the δ-ketols were converted to methyl esters with diazomethane and trimethylsilyloxy ethers (OTMS) with hexamethyldisilazane/trimethylchlorosilane/pyridine, 2:1:1 (OTMS reagent). Also, hydrogenated δ-ketols were analyzed by GC-MS. Methyl oxo-OTMS-octadecenoate was hydrogenated after removal of the OTMS reagent. Conditions were 30 min at 25 C with H_2-Pd (10% Pd on charcoal, Matheson Coleman and Bell) in methanol. The hydrogenated sample was reacted again with OTMS reagent before GC-MS analysis. GC-MS was employed essentially as described before (8).

Thin layer chromatography (TLC) plates were spread with 250 μ thick layers of Silica Gel G. Fatty acids were separated with hexane/ether/acetic acid, 50:50:1 (R_f of δ-ketol, 0.13; R_f of 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid, 0.19) and fatty methyl esters with hexane/ether, 6:4, double development (R_f of δ-ketol methyl ester, 0.11; R_f of methyl 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoate, 0.23). Separation of isomeric δ-ketols or their methyl esters was not accomplished by TLC.

RESULTS AND DISCUSSION

The δ-ketols produced by decomposition of 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic

[1]Presented at the 14th World Congress, International Society for Fat Research, Brighton, U.K., September 17-22, 1978.

[2]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

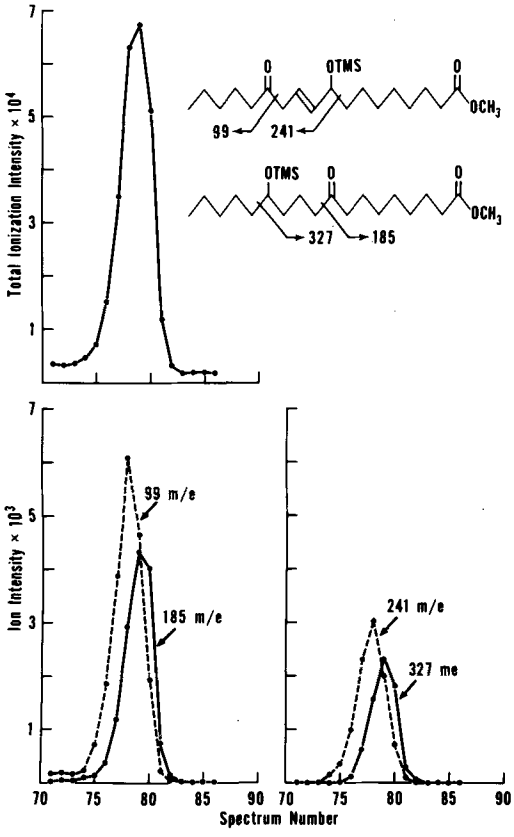

FIG. 1. Mass chromatography of 9(13)-oxo-13(9)-hydroxy-*trans*-11(10)-octadecenoic acids (δ-ketols) obtained from decomposition of the 13-hydroperoxide of linoleic acid. Samples were analyzed as their trimethylsilyloxy ether, methyl ester derivatives, and were separated by a 4 ft GC column packed with 3% OV-1 on Gas-Chrom Q with temperature programming from 185 to 245 C at 1-½ C/min. Spectrum numbers are arbitrary because they depend on the start of scanning.

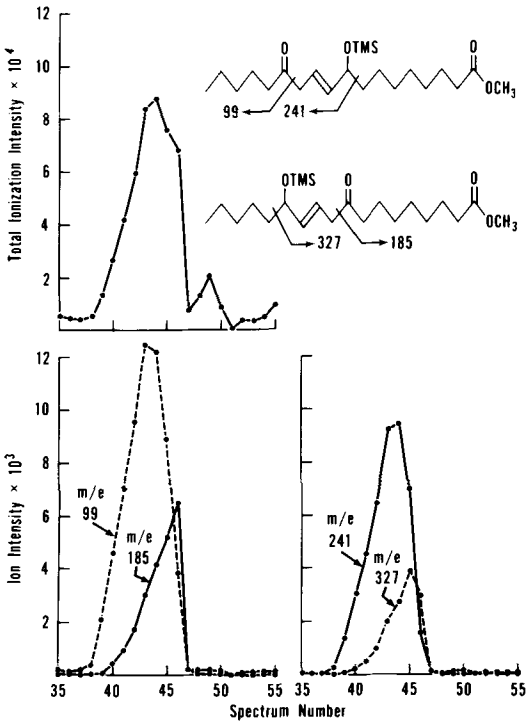

FIG. 2. Mass chromatography of δ-ketols obtained from decomposition of the 9-hydroperoxide of linoleic acid. Conditions were the same as used in Figure 1.

acid (13-LOOH) with the cysteine-$FeCl_3$ catalyst were only a part of the numerous products that can be isolated from this reaction (1,3). The δ-ketols were isolated and identified by nuclear magnetic resonance spectroscopy (NMR) and GC-MS according to Gardner et al. (1). As ascertained by GC-MS, the δ-ketols from 13-LOOH were an isomeric pair (Fig. 1). The most characteristic fragment ions of methyl 9-oxo-13-OTMS-*trans*-11-octadecenoate, 185 and 327 m/e, were plotted and compared with the most characteristic ions of methyl 13-oxo-9-OTMS-*trans*-10-octadecenoate, 99 and 241 m/e. The "mass chromatogram" shown in Figure 1 and elsewhere in this communication demonstrated that methyl 13-oxo-9-OTMS-*trans*-10-octadecenoate eluted slightly prior to methyl 9-oxo-13-OTMS-*trans*-11-octadecenoate. The decomposition of 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (9-LOOH) also resulted in a pair of δ-ketols (Fig. 2). Although there may be some variation in the isomeric composition of the δ-ketols as surmised by some differences in the mass chromatograms, these differences are of questionable significance. Until a method is developed to separate the isomeric δ-ketols, subtle anomalies in isomeric distribution cannot be assessed.

Olefinic compounds often give rise to rearrangements under GC-MS conditions that lead to ambiguous interpretation of the mass spectral data. To eliminate this possibility, the δ-ketols obtained from 9-LOOH were hydrogenated and then analyzed by GC-MS (Fig. 3). A small amount of unhydrogenated δ-ketols preceded the main peak of saturated δ-ketols. The data again confirmed the mixture of δ-ketols.

One possible route to an isomeric product mixture from a specific hydroperoxide isomer is through rearrangement of the hydroperoxide itself to a mixture of 9-LOOH and 13-LOOH as described by Chan et al. (11). Since cysteine-

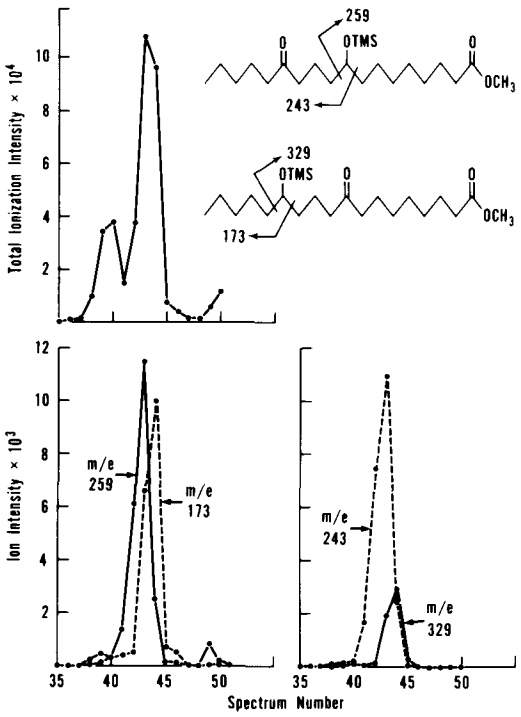

FIG. 3. Mass chromatography of hydrogenated δ-ketols obtained from decomposition of the 9-hydroperoxide of linoleic acid. Conditions were the same as used in Figure 1, except temperature programming was from 200-250 C at 4 C/min.

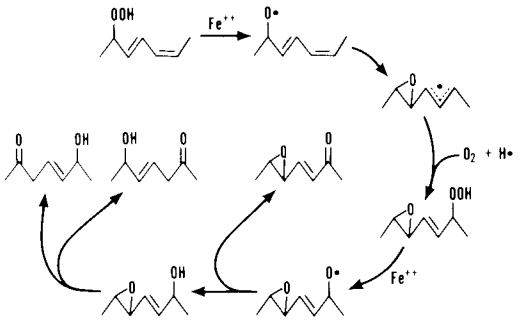

FIG. 4. Proposed pathway of formation of δ-ketol isomers from a specific isomer of linoleic acid hydroperoxide. Structures are abbreviated.

$FeCl_3$ catalyzed formation of 12,13-epoxides from 13-LOOH and 9,10-epoxides from 9-LOOH with a high degree of specificity (3,9), hydroperoxide rearrangement was discounted. Thus, the specific LOOH isomers undoubtedly were converted to products before they could rearrange. According to Chan et al. (12), a peroxy radical precedes hydroperoxide rearrangement, but cysteine-$FeCl_3$ catalyst appeared to degrade LOOH through an alkoxy radical (3).

We propose that the δ-ketols are formed from a hydroxyepoxyoctadecenoic acid intermediate. Isomeric δ-ketols were produced by treatment of 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid (epoxyol) with the cysteine-$FeCl_3$ catalyst in methanol/water, 8:2. The concentration of the epoxyol was 1 mg/ml, and the other reagents and conditions were the same as used to decompose LOOH to δ-ketols. At the end of the reaction, the major compounds detected were the solvolysis products, dihydroxymethoxyoctadecenoic acid and trihydroxyoctadecenoic acid, but about 20% of the products were δ-ketols. When a spray containing 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl was used, the δ-ketols were detected readily by TLC at R_f 0.13. The spray also revealed the epoxyol (R_f = 0.19), but color development was slower than with the δ-ketols. Subsequently, the δ-ketols were methyl esterified, isolated by preparative TLC, and analyzed by NMR as reported before (1). GC-MS of the δ-ketols (OTMS ether, methyl ester) gave essentially the same results as shown in Figure 1. Isolation of the δ-ketols prior to GC-MS was mandatory because the epoxyol (OTMS ether, methyl ester) eluted too close by GC to the δ-ketols (OTMS, methyl ester). Also, the mass spectrum of the epoxyol derivative was very similar to the mass spectrum of one δ-ketol isomer (methyl 13-oxo-9-OTMS-*trans*-10-octadecenoate). The only significant difference noted in the two spectra was that the epoxyol derivative yielded a more intense 103 m/e fragment ion.

The above observation argues that the epoxyol may be a precursor of δ-ketols. Hamberg (6) detected the epoxyol as a product of 13-LOOH degradation by hemoglobin, but Gardner et al. (1) did not detect epoxyol when cysteine-$FeCl_3$ was used as a catalyst. In a subsequent investigation, Gardner et al. (3) identified the apparent precursor of the epoxyol by terminating the reaction early. The isolated precursor, 9-hydroperoxy-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid, was degraded readily to epoxyol with the cysteine-$FeCl_3$ catalyst. Apparently, the epoxyol is also an intermediate that eventually disappears during a full 1-hr reaction or during work-up. Thus, the pathway shown in Figure 4 is suggested, part of which has been proposed before (3). Also, it is implied, but not directly demonstrated, that a product of 9-LOOH degradation, 13-hydroxy-*trans*-9,10-epoxy-*trans*-11-octadecenoic acid, will also produce isomeric δ-ketols.

The mechanism by which the epoxyol is transformed into δ-ketols was elucidated

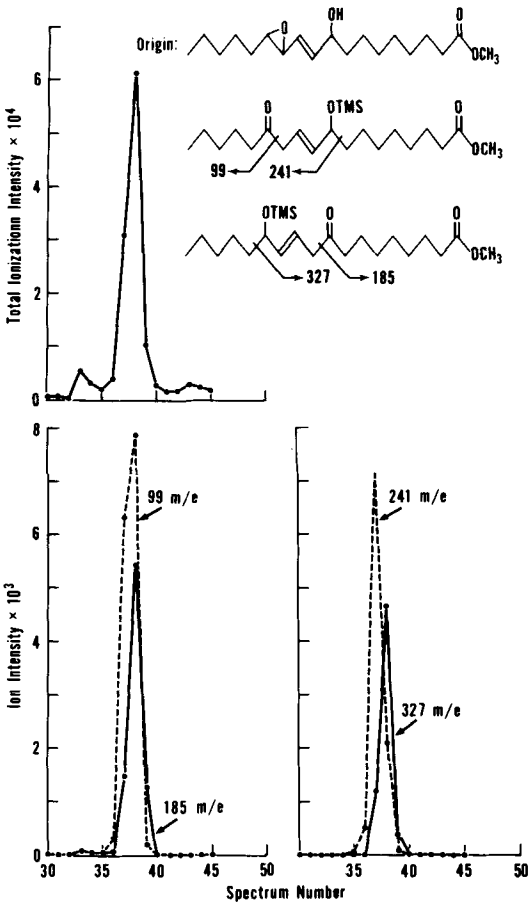

FIG. 5. Mass chromatography of δ-ketols obtained from treatment of methyl 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoate with BF_3-etherate. Conditions were the same as used in Figure 3.

through use of a Lewis acid, BF_3-etherate, as a catalyst. After treatment of the epoxyol (methyl ester) with BF_3-etherate, TLC demonstrated that the major products were δ-ketols (methyl ester). A mass chromatogram of these δ-ketols (OTMS ether, methyl ester) shown in Figure 5 compared with the results obtained by decomposition of 13-LOOH to δ-ketols (Fig. 1). Essentially, BF_3-etherate could cause the formation of a regioequivalent allylic carbonium ion (Fig. 6). A hydride could shift from either the 9- or 13-carbon to the charged vicinal carbon analogous to the "NIH Shift" observed in some biochemical reactions (13). It is of some interest to note that the plant enzyme, linoleic acid hydroperoxide isomerase, catalyzed the formation of α and γ-ketols from LOOH. Gardner (14) proposed that a similar "NIH Shift" type reaction may be involved in the enzymic transformation as well.

One other regioequivalent intermediate is

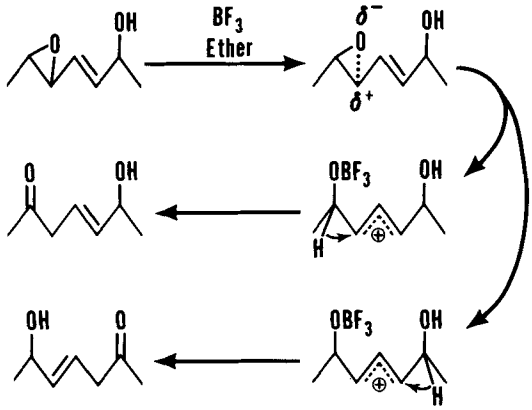

FIG. 6. Proposed mechanism of conversion of methyl 9-hydroxy-*trans*-12,13-epoxy-*trans*-10-octadecenoate to δ-ketols by BF_3-etherate. Structures are abbreviated.

possible, but we have no basis to assess its importance nor any evidence of its existence. It is mentioned here because we wish to stress that other pathways could participate. If a second molecule of oxygen were added to LOOH at the olefinic carbon δ to the hydroperoxy carbon, a dihydroperoxy allylic radical would result. Decomposition of the dihydroperoxide conceivably could disproportion into isomeric δ-ketols.

ACKNOWLEDGMENT

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Membrane Lipids in Bromodeoxyuridine-Differentiated Astroglial Cells in Culture

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ABSTRACT

Embryonic hamster astroblasts (NN strain) grown in continuous line were cultivated in the presence of bromodeoxyuridine (BrdU). A decrease in the growth rate of the cells and striking changes in their morphology were observed, the morphology of the cells resembling that of mature astrocytes. Membrane lipids of BrdU-differentiated and standard cells were compared. No modification of the lipid/protein ratio was observed. Phospholipids and cholesterol were increased in the same proportions in the cells, and no modification of the phospholipid distribution was observed. Ganglioside sialic acid remained at the same level, but the ganglioside distribution was highly modified. Complex gangliosides appeared (G_{M1} and G_{D1a}), while the proportion of simple gangliosides (G_{M3} and G_{D3}) decreased. However, neither G_{T1} nor G_{Q1} were detected in differentiated cells. The distribution of phosphoglyceride acyl groups was highly modified, the proportion of arachidonic and docosapentaenoic acids being 2 to 3 times higher in BrdU-treated cells than in proliferating ones. These results were compared to those obtained with another clonal line of glial cells (C6) which exhibited no morphological differentiation in the presence of BrdU; the lipids of these cells were not modified by such a treatment.

INTRODUCTION

Differentiation of cultured cells is a phenomenon generally characterized by a change in cell morphology or in some physiological or biochemical properties of the cells, such as synthesis of melanin by melanocytes (1,2) or of collagen by bone fibroblasts (3) or tendon cells (4). Differentiation can be a physiological event in vivo, as observed in intestinal mucosa cells (5); it can also be induced in some cell types by a treatment with various compounds. 5-Bromo-2′-deoxyuridine (BrdU) is well known as an inhibitor of cellular differentiation (6). However, Schubert and Jacob (7) have shown that BrdU can induce a morphological differentiation of neuroblastoma cells. We have observed that a strain of embryonic astroblasts (NN cells) could take the morphology of mature astrocytes when grown in the presence of BrdU. One can expect that a morphological change of a cell can be associated with changes in membrane structure. Only few data exist on the changes occurring in membrane lipids during cell differentiation. Striking variations in the glycolipid patterns have been observed during physiological (5), spontaneous (8), or chemically induced (9-16) differentiation, but the only available indications existing to our knowledge about the other membrane lipids concern plasma membrane cholesterol and phospholipid changes during myoblast differentiation (17), sphingomyelin changes during lens fiber differentiation (18), and phospholipid synthesis in differentiating intestinal epithelium (19). In previous papers (20,21), we have reported the lipid composition of NN glial cells grown in standard conditions. We present here the changes observed when these cells were morphologically differentiated with BrdU. We compare these results to those obtained with the C6 strain, which does not exhibit morphological changes when grown in the presence of BrdU (22).

MATERIALS AND METHODS

Cell Culture

NN cells (23) and C6 cells (24) were routinely cultivated in 75 cm2 plastic dishes with Eagle-Dulbecco synthetic medium supplemented with 10% fetal calf serum, in a wet atmosphere containing 5% CO_2 and 95% air. A morphological differentiation was obtained by growing cells for 14 days in the presence of 10^{-5}M BrdU. The cells were harvested by scraping with a rubber policeman, washed in buffered saline and pelleted.

Lipid Analysis

Lipids were extracted and partitioned according to Benda et al. (24) and Folch et al. (25). In the lower phase, lipid phosphorus was assayed with the technique of Macheboeuf and Delsal (26), and cholesterol was assayed according to Idler and Baumann (27). Phospholipid distribution was obtained after a two-direction, thin layer chromatography in the system of Nussbaum et al. (28). Ethanolamine

plasmalogens were separated from other ethanolamine phosphoglycerides by an acidic hydrolysis performed directly on the place between the two migrations (29). Fatty acids were obtained after an alkaline methanolysis of the lipid extract (30). They were then separated by gas liquid chromatography on a column of 10% EGSS-X on chromosorb W-HP. The identification of the peaks was performed as already described (31). Neutral glycolipids were estimated according to Robert and Rebel (32).

Gangliosides of the Folch's upper phase were purified by dialysis, assayed with the technique of Miettinen and Takki-Lukkainen (33), and separated in the chromatographic thin layer system described by Van den Eijnden (34), using as solvent the mixture chloroform/methanol/water with 20% KCl (60:35:8, v/v/v). The distribution of sialic acid among gangliosides was obtained by densitometry according to Smid and Reinisova (35) after visualization of the plate with Bial's orcinol reagent. Gangliosides were eluted from plates, two times with chloroform/methanol (1:2, v/v) containing 5% water than with methanol/water (1:1, v/v).

Other Techniques

Cell counts were performed with an hemocytometer. Proteins were assayed according to Lowry et al. (36) using bovine serum albumin as a standard.

RESULTS

The morphology of normal and BrdU-differentiated NN cells is presented in Figure 1. Striking changes could be observed in the size and shape of the cells; the treated cells were much bigger and contained much more expansions and processes than the control cells.

Table I shows that there was a considerable reduction in cell number per culture dish. The remaining cells contained about ten times more proteins and lipids than control cells. However, the lipid/protein ratio remained unchanged, and the cholesterol/phospholipid molar ratio was the same in both conditions of culture.

The proportion of individual phospholipids (Table II) did not differ significantly in the two types of cells. The percentage of ethanolamine plasmalogens seemed slightly higher in the BrdU-treated cells.

The level of polyunsaturated fatty acids was notably increased in BrdU-differentiated cells total phosphoglycerides (Table III). This was correlated with a decrease in the monounsaturated series proportion. A 2-3 fold increase of the percentages of arachidonic and docosapentaenoic acids could be observed in BrdU-differentiated cells, while no or little changes occurred in other polyunsaturated fatty acids.

The amount and distribution of the neutral glycolipids were similar in proliferating and BrdU-differentiating NN cells (Table IV). Although the total amount of lipid sialic acid did not change, the ganglioside pattern of BrdU-treated cells was strikingly different from that of proliferating cells (Table IV and Fig. 2). Our previous structural analysis of the glycolipids in proliferating NN and C6 cells have shown that these cells contain glucosylceramide, lactosylceramide, G_{M3} and G_{D3} (21). After growth in presence of BrdU, high amounts of G_{M1} and G_{D1a} could be detected, while the proportions of G_{M3} and G_{D3} decreased. Traces of G_{D1b} could be detected, but not G_{T1} or G_{Q1} could be found. Figure 2 shows that each ganglioside species gives a double spot when run in the van den Eijden's system. This doubling was also observed, using other solvent systems, by Duffard et al. (39) and Manuelidis et al. (40) in their study of gangliosides obtained from NN or other glial cells. More recently Van Dessel et al. (41) have also shown that each ganglioside species (characterized by their sugar composition) obtained from bovine thyroid gives two or even three spots when submitted to high performance thin layer chromatography. Since not enough of each ganglioside was available for a structural analysis, the identity of the different spots was confirmed using high performance thin layer chromatography, using the following solvents: chloroform/methanol/water (60:35:8) + KCl (34), methyl acetate/isopropanol/water (45:30:20) + KCl (38). Compounds 1, 2 and 3 (Fig. 2a) were eluted separately and run again with known markers (Fig. 2c). This shows that the new gangliosides detected in differentiated NN cells are mainly G_{M1} and G_{D1a}. This observation agrees well with the report of Duffard et al. (39), who have found in NN cells a notable activity of the two enzymes involved in the synthesis of G_{M1} and G_{D1a}.

The morphological changes began to appear in NN cells after 10-12 days of culture in the presence of BrdU; the modification of the ganglioside pattern of these cells occurred simultaneously with the morphological changes (Table V and Fig. 2b).

In contrast, no significant changes in the membrane lipid composition of C6 cells were observed after a similar treatment with BrdU (Tables I, II, III, and IV).

DISCUSSION

At present time, morphological changes of

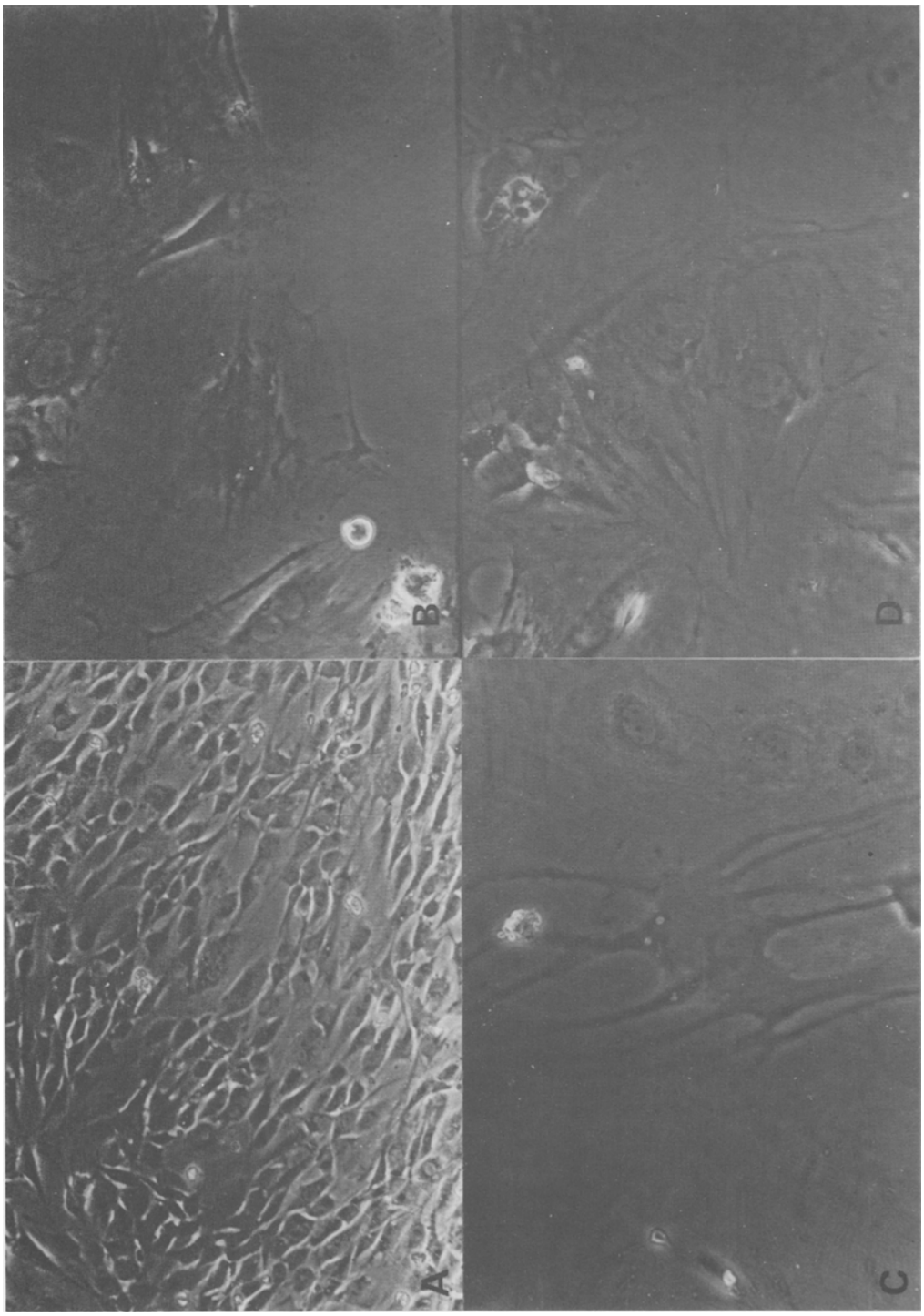

FIG. 1. Phase-contrast micrographs of NN cells (original magnification: X 250). A. Cells grown in standard conditions; B, C and D, cells grown 14 days in the presence of 10^{-5}M BrdU.

TABLE I

Cell Number and Protein, Cholesterol and Phospholipid Content of NN and C6 Cells Grown in the Presence of BrdU

	NN Cells		C6 Cells	
	Control	BrdU-treated	Control	BrdU-treated
Cell number X 10^{-6} per plastic dish	13.5	0.144	30.4	29.0
Proteins (mg per plastic dish)	3.63	0.331	6.21	5.88
Proteins (ng per cell)	0.27	2.30	0.20	0.20
Cholesterol (μmol/mg protein)[a]	0.130	0.137	0.156	0.171
Lipid phorphorus (μmol/mg protein)[a]	0.288	0.291	0.392	0.407

[a]Calculated on a cell basis, cholesterol amounts to 0.035 and 0.31 pmole in control and treated NN cells, and lipid phosphorus to 0.077 and 0.66 pmol.

TABLE II

Phospholipid Distribution in NN and C6 Cells Grown in the Presence of BrdU[a]

	NN Cells		C6 Cells	
	Control	BrdU-treated	Control	BrdU-treated
Phosphatidylserine	6.9	5.7	4.5	5.6
Phosphatidylinositol	7.2	5.8	3.8	4.7
Sphingomyelin	8.6	7.9	8.1	8.5
Phosphatidylcholine	47.4	49.2	52.2	53.8
Phosphatidylethanolamine	16.7	15.5	18.0	16.5
Ethanolamine plasmalogens	10.0	12.6	10.5	10.7
Phosphatidic Acid	1.6	2.7	1.0	tr.
Cardiolipin	2.6	2.5	1.9	0.2

[a]Phospholipids are expressed as weight percentages of total lipid phosphorus; each value is the mean of four independent experiments.

glioblastoma cells have been obtained with dibutyryl cyclic AMP (42-44), dibutyryl cyclic GMP or butyrate (43), prostaglandin E1 (45), epinephrine (46), norepinephrine (47), amethopterin (48,49) or BrdU (48-50). However, the reported changes were never drastic and did not provide the morphology of mature glial cells. Furthermore, these changes were obtained very soom after the addition of the differentiating agent in the culture medium. In contrast, the differentiation of NN cells was much slower; the morphological differentiation of the cells was observed after two weeks of culture in the presence of BrdU.

The morphological differentiation of cultured cells of nervous origin is characterized by a striking increase of the cell expansions; the area of the plasma membrane is, therefore, increased. This explains the increase in protein, cholesterol and phospholipid we observed. It is difficult, however, to assume that the newly formed membranes of BrdU-treated NN cells have the same lipid composition as the plasma membranes of proliferating cells since we could not evaluate the increase in cell surface area which occurred during differentiation. Such a similarity is suggested by the fact that the cholesterol/phospholipid molar ratio was unchanged, as well as the phospholipid distribution, in BrdU-treated and untreated cells. One cannot exclude, however, that BrdU induced changes in each type of cell membrane, these changes being compensated in the whole cells.

Our finding that no changes were observed in the phospholipid pattern and phospholipid/cholesterol ratio of BrdU-treated NN cells is in

TABLE III

Phospholipid Fatty Acid Distribution in NN and C6 Cells Grown in the Presence of BrdU[a]

	NN Cells		C6 Cells	
	Control	BrdU-treated	Control	BrdU-treated
14:0	1.3	1.1	0.7	0.7
16:0	18.7	18.0	23.0	25.1
16:1	7.2	3.5	4.7	3.9
18:0	10.5	14.4	13.0	13.5
18:1	40.3	27.5	40.0	37.4
18:2ω6	2.5	2.7	1.1	1.2
18:3ω3 + 20:1	1.9	1.2	1.3	1.2
20:2ω6	0.8	0.1	0.8	0.6
20:3ω9	1.0	1.9	1.7	1.7
20:3ω6	0.8	0.7	0.5	0.6
20:4ω6	5.4	15.2	6.1	7.0
20:5ω3	2.2	2.8	0.8	0.8
22:4ω6	0.8	1.6	1.0	1.0
22:5ω6	0.2	0.2	tr.	tr.
22:5ω3	3.1	6.2	2.2	2.1
22:6ω3	2.8	4.3	3.1	3.2

[a]Results are expressed as weight percentages. Fatty acids are abbreviated in the usual manner; i.e., a number indicating chain length followed by the number of double bonds per molecule; the ω refers to the number of carbon atoms between the methyl end of the molecule and the first double bond on this side. Each value is the mean of two independent experiments.

TABLE IV

Glycolipids of NN and C6 Cells Grown in the Presence of BrdU

	NN Cells		C6 Cells	
	Control	BrdU-treated	Control	BrdU-treated
Neutral Glycolipids hexose (nmol/mg protein)	4.47	4.31	0.92	n.d.
Glucosylceramide[a]	12.3	14.7	66.3	n.d.
Lactosylceramide	87.7	85.3	33.7	n.d.
Gangliosides Sialic Acid (nmol/mg protein)	3.92	4.04	3.40	3.33
G_{M3}[b]	66.8	30.0	95.2	95.8
G_{M2}	---	2.8	1.8	1.9
G_{M1}	1.3	23.5	---	---
G_{D3}	30.6	21.4	2.3	---
G_{D1a}	2.3	20.6	0.7	2.3
Other (G_{D1b} ?)	---	1.6	---	---

[a]Individual neutral glycolipids are expressed as mole percent of total lower phase lipid hexose.

[b] Individual gangliosides are expressed as mole percent of total lipid sialic acid. The nomenclature of Svennerholm was used (37).

accord with the observation of Walther et al. (51) who found that this nucleoside has only a slight, if any, action on phospholipid and sterol synthesis in cultured pancreas cells. Studying the differentiation of intestine cells, O'Doherty (19) has also found no changes in the activity of the enzyme synthesizing the main phospholipids. The results obtained with NN cells contrast with those given by myoblasts where a net change in the plasma membrane lipids is observed after differentiation (17).

The fatty acid pattern of phospholipids extracted from NN or C6 cells grown in standard conditions showed very low amounts of

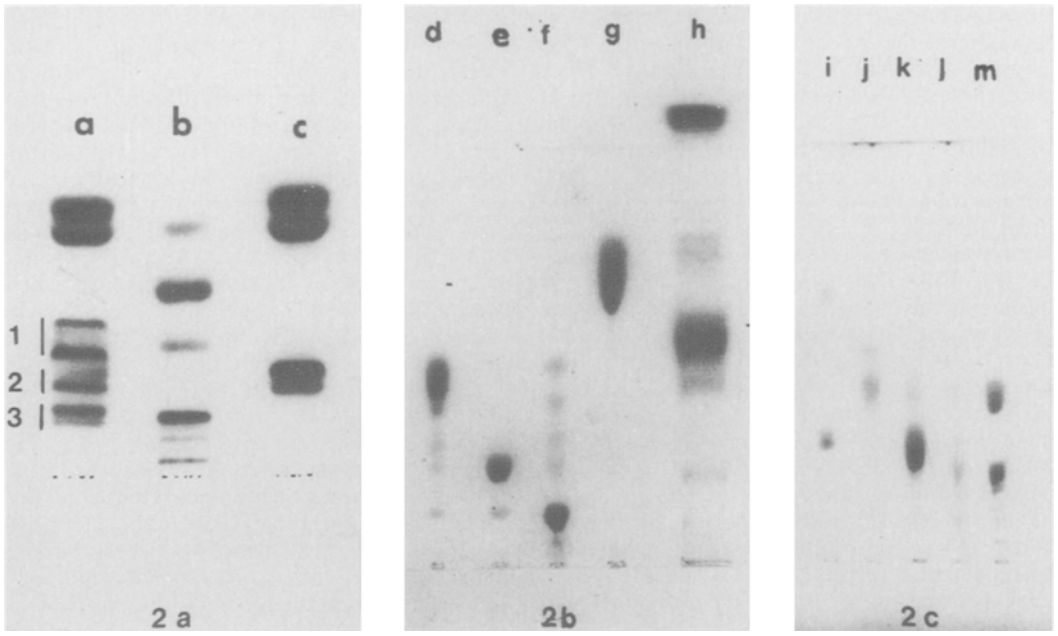

FIG. 2. Thin layer chromatography of gangliosides isolated from control and BrdU-treated NN cells.
2a: Precoated plate – Solvent system: chloroform/methanol/water (60:35:8) + KCl (34).
a: gangliosides from differentiated cells; b: mixture of pig brain gangliosides + G_{M3} and G_{M2}; c: gangliosides from control cells.
2b: High performance TLC plate – Solvent system: methylacetate/isopropanol/water (45:30:20) + KCl (38).
d: G_{D1a}; e: G_{D1b}; f: G_{T1}; g: G_{M1}; h: Gangliosides from NN cells at the beginning of the differentiation (11 days culture with BrdU).
2c: High performance TLC plate – Solvent system as for 2a.
i: G_{D3}; j,k,l: fractions 1-2-3 eluted from 2a; m: G_{M1} + G_{D1a}. G_{M3} was obtained from beef spleen. G_{D3} was a generous gift from Dr. J. Portoukalian (Lyon, France). Other gangliosides were purchased from Supelco. Inc., Bellefonte, PA.

TABLE V

Evolution with Time of the Ganglioside Pattern of NN Cells Grown in the Presence of BrdU

Ganglioside[a]	Control	3 days	10 days	12 days	14 days	18 days
G_{M3}	67.8	70.0	73.0	70.1	30.0	33.8
G_{M2}	---	tr.	---	0.2	2.8	2.8
G_{M1}	1.3	1.0	1.5	3.0	23.5	23.0
G_{D3}	30.6	29.5	25.5	19.3	21.4	21.9
G_{D1a}	2.3	tr.	tr.	7.5	20.6	18.5

[a]Gangliosides are expressed as mole percent of total lipid sialic acid.

polyunsaturated fatty acids. We have already shown this was due to a deficiency of the fetal calf serum in linoleic and linolenic acid (31). A lack of the Δ4 desaturase activity was also observed (31, 52). BrdU differentiation of NN cells provides a net increase of the polyunsaturated fatty acid content of the cell lipids. Comparison of these results with those obtained with NN cells growing in a medium enriched in linoleic and linolenic acids (31) led us to conclude that the effect of BrdU was not to increase the uptake of linoleic and linolenic acids by the cells, but to increase their transformation into arachidonic and docosapenta- or

docosahexaenoic acids. It is difficult to speculate about the effect of this change on the physical properties of cell membranes. Since the cholesterol/phospholipid molar ratio of mammalian cells plasma membranes is enough to maintain the membranes in a fluid state even if changes in the fatty acid pattern occur, the changes observed in NN cells have probably no great influence on the physical properties of their membranes, as it was shown by Schroeder et al. (53). The McElhaney concept of homeoviscous adaptation (54-57) is probably also valuable for mammalian cells (53) when we consider the general membrane fluidity. Thus, the changes observed during NN cells BrdU differentiation could be compensated by changes in some parameters involved in the regulation of membrane physical properties. That does not exclude, however, the possibility of physiological changes in the cells, such as the activities of some lipid-dependent membrane-bound enzyme (58).

The only other noticeable change in the membrane lipids occurring during NN morphological differentiation concerned the ganglioside distribution. Langenbach et al. (8) have shown that morphological changes of mouse embryo cells are accompanied with modifications of the ganglioside pattern. Similarly, differentiation of intestinal mucosa cells (5), HeLa, KB or NRK cells (9-11,14,15), myoblasts (12), lymphoblasts (13) or neuroblastoma cells (16) is also characterized by changes in the relative concentrations of cell glycolipids. Morphological differentiation of NN cells provides similar observations, and is characterized by a net increase in the content in two gangliosides (G_{M1} and G_{D1a}) which were present at low amounts in the standard cells. Duffard et al. (39) have recently reported that NN cells presented high activities of UDP-galactose:G_{M2} galactosyltransferase and of CMP-N-acetylneuraminic acid:G_{M1} sialyl-transferase, but did not exhibit the activity of the G_{M2}-synthetizing enzyme (UDP-N-acetyl-galactosamine:G_{M3} N-acetylgalactosaminyltransferase). It seems, therefore, that the morphological differentiation of NN cells must be accompanied by an increase of the G_{M2}-synthetizing enzyme activity.

However, it seems that in differentiated NN cells the activity of the G_{M2} synthetizing enzyme probably remains relatively low. Indeed, the amount of G_{M2} in these cells is not greatly increased comparatively to those of G_{M1} and G_{D1a}. This contrasts with the pattern observed in neuroblastoma cells where notable amounts of G_{M2} are present (16,59,60) and where a high activity of the G_{M2} synthetizing enzyme is observed (39, 67).

Similar increase of the activity of a ganglioside-synthetizing enzyme was observed by Glickman and Bouhours during intestinal mucosa cells differentiation (5), and by Fishman et al. (9,10) and Macher et al. (14), during the butyrate-induced morphological changes of HeLa or KB cells. The role of gangliosides in cells is not exactly known at the present time. Since these lipids could be involved in the reception of some hormones (61) or neurotransmitters (62,63), one could expect some changes in cell physiology when the ganglioside pattern is greatly modified.

Comparing our results with those of Manuelidis et al. (40), the ganglioside pattern of our undifferentiated NN is not very different from the pattern found by these authors with their highly dedifferentiated glial clones, TC 178 and TC 501. Furthermore, gangliosides of BrdU-treated NN cells became similar to those of TC 526 and TC 593 cells which had maintained their morphology of normal glial cells. This observation is in accord with our finding that the changes in ganglioside occur with the morphological differentiation.

Although the morphology of the BrdU-differentiated NN cells is somewhat similar to that of mature astrocytes, the ganglioside pattern of these cells remains very different from the pattern of glial cells fractions prepared from brain by gradient centrifugations (64-66). Tri- or tetrasilogangliosides are absent from BrdU-treated cells, while they account for a large part of gangliosides of glial fractions isolated from brain. However, the possibility of a contamination of the brain glial fractions by synaptosomes (which contain very large amounts of gangliosides) has never been excluded. Therefore, the exact pattern of glial cells gangliosides in situ remains unknown.

When C6 cells were cultured in the presence of BrdU in the same conditions as NN cells, no changes in the morphology could be detected, except a slight flattening of the cells. No difference in lipid composition was observed between the control and the treated cells. One can, therefore, presume that the changes observed in NN cells are related to the morphologic differentiation. This agrees with the synchronization observed between the morphological changes of the cells and the modification of their ganglioside pattern.

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METHODS

A Simplified Procedure for the Determination of Betaine in Liver

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ABSTRACT

A convenient procedure for the determination of hepatic betaine levels is described. The method takes advantage of ethanol precipitation to rid acidified tissue extracts of interfering substances. Betaine is reacted with potassium triiodide to form betaine periodide, which is selectively precipitated via pH adjustment. The precipitate of betaine periodide is dissolved in ethylene dichloride and measured spectrophotometrically. The method is specific, accurate, and simple and showed recoveries of from 97 to 103% at two different levels of added betaine. The applicability of the method was shown when it was demonstrated that diets containing different amounts of choline influenced levels of hepatic betaine.

INTRODUCTION

When choline is taken up by the liver, it is subject to two main metabolic fates. It can be converted via choline kinase to phosphorylcholine, an intermediate in the synthesis of phosphatidylcholine, or it can be readily oxidized by choline oxidase to form betaine.

From an investigative standpoint, a method for hepatic betaine analysis is important since betaine (a) is an important metabolite of choline, (b) is believed to be an important methylating substance in liver (1), and (c) is greatly reduced in liver during a choline deficiency (2). Results from this laboratory (3-5) and confirmed by others (6) have shown that ethanol ingestion increases the oxidation of choline in liver. These studies should stimulate an even greater interest in the measurement of hepatic betaine levels.

The methods presently used for the analysis of betaine in biological tissue are quite cumbersome, and involve either paper (7) or column chromatographic (8) isolation before analyses can be carried out. Friedman et al. (9) have discussed some of the difficulties in employing some of the existing chromatographic methods of analysis for quaternary nitrogen compounds in general and have emphasized the need for simple and better methods in this area.

The current method involves the extraction of betaine from liver, essential precipitation procedures to rid the extract of interferring substances and the spectrophotometric analysis of betaine by a modification of the Appleton et al. (10) technique.

MATERIALS AND METHODS

Reagents

Potassium triiodide. Iodine (15.7 g) and 20 g of potassium iodide were dissolved in 100 ml of water, shaken for 45 min on a mechanical shaker to affect solution and stored at 4 C.

Standard Betaine Solution. Betaine – HCl (Eastman Kodak Co., Rochester, NY) was dried over concentrated sulfuric acid in vacuo. A standard solution was prepared containing 10 mg of base per 100 ml of water. Working standards were prepared by suitable dilution of the stock solution with water.

Other reagents: (a) 15% trichloroacetic acid, (b) ethyl ether, (c) ethylene dichloride, (d) concentrated sulfuric acid, and (e) ethyl alcohol 95%.

Betaine Standard Curve

Varying amounts of pure betaine hydrochloride (10 to 50 μg of base) in 0.5 ml of solution were placed in a narrow tipped centrifuge tube to which was added 0.2 ml of potassium triiodide. To this mixture was added 10 drops of concentrated sulfuric acid and the tube cooled in ice for 2 hr. The tube was centrifuged for 15 min at 3000 rpm and the supernatant drawn off with care not to disturb the dark precipitate at the bottom of the tube. A linear standard curve was obtained after the precipitate was dissolved in 10 ml of ethylene dichloride and absorbance measured at 365 nm using ethylene dichloride as a blank.

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Preparation of Liver Extract

Male, Sprague-Dawley rats were anesthetized with ether and their livers freeze-clamped in situ. The livers were stored at -70 C until analyzed. Two grams of frozen liver were placed in 8 ml of cold 15% trichloroacetic acid and homogenized. The homogenate was transferred to a centrifuge tube, and the homogenizing flask was rinsed with 5 ml of cold 15% trichloroacetic acid which was also added to the centrifuge tube. This mixture was centrifuged at 12,000 rpm for 10 min and the supernatant solution placed in a 40 ml graduate test tube and the volume noted. The supernate was then extracted 3 times with 10 ml of ethyl ether to remove the trichloroacetic acid and then subjected to a stream of air for 60 min at room temperature to remove the residual ether. This solution was adjusted to between pH 7 and 8 with dilute NaOH and brought back to its original volume (ca. 13 ml) with water.

Ten ml of the extract was placed in a 40 ml centrifuge tube to which was added 15 ml of ethyl alcohol. This mixture was chilled for 30 min and centrifuged. The supernatant was then decanted into a large test tube and the precipitate discarded. The supernatant was evaporated to dryness under a stream of air at room temperature. The resultant residue was subsequently dissolved in 10 ml of water.

Determination of Betaine

Two-tenths of a milliliter of the potassium triiodide solution was added to 2 ml of the prepared extract, covered and refrigerated overnight. The mixture was then centrifuged at 3000 rpm for 10 min to sediment interfering periodides. Aliquots of supernatant (0.55 ml) were added to narrow-tipped 15 ml centrifuge tubes. While chilled in ice, 10 drops of concentrated sulfuric acid was added to each tube and the sample refrigerated for 2 hr. The tube was centrifuged at 3000 rpm for 10 min and the supernatant aspirated with care taken not to disturb the dark precipitate. This precipitate was dissolved in 10 or 20 ml of ethylene dichloride and the absorption read in a spectrophotometer at 365 nm using ethylene dichloride as a blank. The betaine content of the liver was estimated from the standard curve and the level expressed as μmoles of betaine base per gram liver.

EXPERIMENTAL

To demonstrate the influence of dietary choline on hepatic levels of betaine, male Sprague-Dawley rats (250 g) were divided into three groups. One group was fed the choline-deficient diet of French (11) for 5 days, and another group was fed the French control diet for the same period. A third group was fed a 50-50 mixture by weight of the choline deficient and choline control diets. The daily intake of choline of the animals on the deficient diet was negligible, where with the control diet the average daily choline intake was 115 mg and with the mixed diet 57 mg. Nonfasted animals were killed and their livers promptly assayed for betaine. Results were expressed as μmoles per g tissue as well as μmoles per total liver per 100 g of body weight for proper comparison, since the choline deficiency produced a fatty liver (12). In addition, rats were fed Purina rat pellets for 30 days and the livers assayed for betaine in order to establish betaine levels in animals fed a commercial rat chow diet.

RESULTS

Figure 1 shows that with the present procedure betaine estimation is linear over a wide range of tissue and indicates the range of betaine concentrations which are detectable by this method.

Betaine recovery studies were conducted with the present procedure using livers from

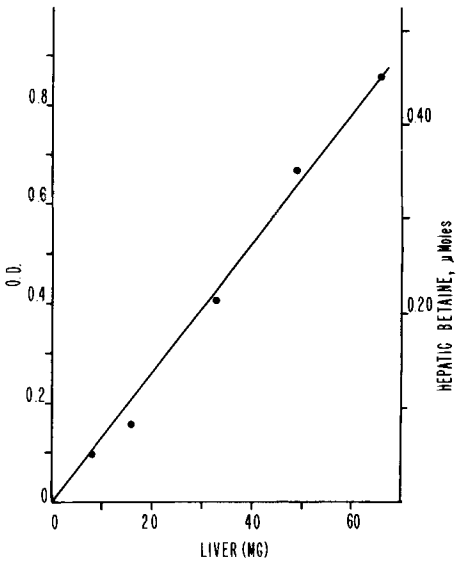

FIG. 1. Relationship of optical density to tissue betaine in various quantities of liver from a rat fed Purina rat chow.

TABLE I

Recovery of Betaine from Rat Liver[a]

Diet	Endogenous[b] betaine μg	Amount betaine added μg	Betaine recovered μg	Percent recovery
Purina rat pellets	34	15	14.5	97
Purina rat pellets	50	30	30.9	103
French choline control	146	20	19.1	104

[a]Recoveries were conducted in duplicate on separate animals fed the respective diets for 30 days.

[b]Recoveries were made from 66 mg liver in each case.

TABLE II

Effect of Dietary Choline on Betaine Level in Rat Liver

Diet	No. animals	Average daily intake, mg	μmol/g[a]	μmol/liver/ 100 g body weight[a]
Choline deficient (5 days)	5	0	0.79 ± 0.04	4.15 ± 0.23
Mixed deficient and control (5 days)	5	57	12.3 ± 1.02[b]	63.3 ± 6.30[b]
Control (5 days)	5	114	18.8 ± 1.00[b]	95.4 ± 6.30[b]
Rat Chow (30 days)	5	34	3.95 ± 0.36	13.8 ± 1.14

[a]Values are means ± S.E.M.

[b]When compared to choline deficient diet, $p < 0.001$.

rats fed both Purina rat pellets and the choline control diet of French (11). Results from these recoveries are shown in Table I and indicate that when varying amounts of betaine were added to homogenates of livers containing various levels of endogenous betaine, good recoveries were obtained in each case.

As shown in Table II, the hepatic pool size of betaine varies with the level of dietary choline. Feeding rats the French control diet (0.70% choline) for 5 days produced a betaine level of 95.4 μmoles per liver per 100 g body weight. Reducing the dietary choline level by one half (0.35% choline) lowered the hepatic betaine level 33% and animals placed on the choline-deficient diet for the 5 day period showed a 95% lowering in liver betaine. These results are in agreement with the findings of Wong and Thompson (2), who also reported a large reduction of hepatic betaine levels in a choline deficiency.

Rats fed Purina rat chow (0.225% choline) had hepatic betaine levels of 13.8 μmol/liver/ 100 g body weight. This value compares with that (24.5 ± 6.1 μmol/liver/100 g body weight) reported by Wong and Thompson (2) who used the diet formulated by Young et al. (13) which was similar in choline content.

DISCUSSION

The current method for measuring hepatic levels of betaine is a simplified procedure compared to those chromatographic methods that have been reported. The ethyl alcohol precipitation step, which retains betaine and choline in solution, eliminates large amounts of interfering material from the liver extract and allows precipitation of betaine periodide at pH 0 to 1 which is essential to the method. Without the removal of these substances, betaine periodide is not crystallized, and a false precipitate forms which is not soluble in ethylene dichloride.

Any procedure used for the determination of betaine involving the formation of betaine periodide requires the separation of betaine from choline. In the development of the present method, advantage was taken of the earlier finding by Wall et al. (14), using solutions of pure betaine and choline, that choline periodide could be precipitated completely at any pH between 0 and 11, and betaine periodide was only fully precipitated between pH 0 and 1. Thus, in the present method, choline is eliminated by precipitation at pH 7 to 8, and betaine is precipitated for measurement between pH 0 and 1.

As demonstrated in Table II, hepatic betaine levels vary with the amount of choline in the diet. This relationship may be more evident in the rat than in other species because of the high level of choline oxidase in rat liver (15). In studies of choline metabolism in the liver, the ability to measure betaine is essential to the understanding of the overall schema of hepatic choline utilization. Choline is a pivotal substance acted upon by several metabolic pathways, and betaine levels would reflect the degree of substrate flux via the choline oxidative pathway and the subsequent influence of experimental conditions on this pathway.

The procedure described is an accurate, specific and simplified measurement of hepatic betaine and allows a convenient means of studying hepatic choline metabolism and its role in both the normal and diseased states.

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pH Gradient Electrophoresis and Isoelectric Focusing of Lipoproteins on Agarose Bead Thin Layers[1]

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ABSTRACT

A new method of isoelectric focusing (IEF) and pH gradient electrophoresis, using thin layers of agarose gel beads, was devised to investigate chylomicrons and very low density lipoproteins (VLDL). pH gradient stability and cathodal gradient drift were similar to those of polyacrylamide gel IEF, and linearity of gradients was maintained for 23 hr. Chylomicrons and VLDL were detectable without staining. Chylomicrons from human serum and from rat lymph migrated in this system. Rat lymph chylomicrons, obtained by ultracentrifugation, migrated in several discrete bands, and this heterogeneity of rat chylomicrons was confirmed by electron microscopic demonstration of chylomicrons in each band. This new technique has permitted the first measurement of isoelectric points of some lipoproteins in the ultracentrifuged fraction of human serum chylomicrons and the first separation of multiple discrete fractions of ultracentrifuged lymph chylomicrons.

INTRODUCTION

Chylomicrons transport dietary long chain fatty acids from intestinal cells via intestinal lymph to the plasma and peripheral tissues. Intestinal very low density lipoproteins also transport lipid, but chylomicrons transport the major mass of absorbed fatty acids following a fat meal (1). Possible heterogeneity of chylomicrons is potentially of major importance in studying dietary lipid transport and metabolism. Chylomicrons have been investigated for heterogeneity by methods which provide chylomicron subfractions on the basis of differing particle diameter, e.g., ultracentrifugation (2) and agarose bead column chromatography (3), but distinct subpopulations have not been demonstrated. In electrophoretic systems, both lymph and plasma chylomicrons tend to adhere to the support mediums and remain at the origin in paper (4), cellulose acetate (5) and agarose gel electrophoresis (6). Both lymph and plasma chylomicrons migrate as a single peak in the α_2 globulin region in starch block electrophoresis (7). There has been no evidence for heterogeneity of lymph chylomicrons from any electrophoretic techniques.

Isoelectric focusing (IEF) (the abbreviation or phrase refers to (a) isoelectric focusing, a state in equilibrium, and, in the discussion, also refers to (b) pH gradient electrophoresis [where equilibrium has not been reached or cannot be demonstrated].) has been widely used in protein analysis since synthetic ampholytes became available in 1966 (8). In lipoprotein analysis, IEF has given excellent resolution of apoproteins (9), but its applications to intact lipoproteins have been less successful, because they precipitate very readily at their isoelectric points (pI) (10) and because certain larger lipoproteins cannot enter conventional polyacrylamide gel IEF systems (11). However, prestained lipoproteins in human fasting serum have been resolved into 7 or 8 distinct components using polyacrylamide gel IEF (12). pI values for the intact lipoproteins, high density lipoproteins (HDL) (13,14), low density lipoproteins (LDL) (10) and very low density lipoproteins (VLDL) (15), have been obtained from sucrose column focusing, but the stability of the pH gradients was not measured and resolution of mixtures of intact lipoproteins, eluted from sucrose columns, is poor.

Recently, anticonvective stabilization with Sephadex or polyacrylamide granules in thin layers has provided IEF pH gradients (16) in which stability can be directly measured and resolution of proteins is excellent.

A translucent gel bead support medium permitting migration of chylomicrons and other VLDL, in the extra-bead volume, in a pH gradient, would allow detection of unstained lipoproteins and their ready elution for analysis. The present work describes such a method of isoelectric focusing, using agarose gel beads. Its particular application to the analytical separation of chylomicron subfractions is discussed.

MATERIALS AND METHODS

Human blood was collected in EDTA (2 mg/ml) and plasma stored under N_2 at 2 C - 6 C. Human hyperlipoproteinemic subjects and their plasma were phenotyped by W.H.O.

[1]This work was presented, in part, at the annual 51st Scientific Sessions of the American Heart Association at Dallas, Texas in November 1978.

criteria (17). Lymph was obtained from male Wistar rats (300-350 g) with cannulated thoracic ducts and was stored coagulated (18). In rat experiments, either safflower oil (300 μl) or a mixture of triolein, trilinolein and trilinolenin (100 μl of each) (Serdary Research Labs, London, Ontario) were emulsified with 7.5 mg sodium taurocholate and saline and fed as bolus dose via an intragastric polyethylene tube. Lipoproteins were obtained by ultracentrifugation (19) in a B60 ultracentrifuge (International Equipment Co., Needham, MA) using a swinging bucket rotor (SB-283). Plasma and defibrinated chyle were overlayered with 5 cm of saline (d=1.006) for the first centrifugal run, and the floating lipoproteins were removed by tube slicing and recentrifuged under the same conditions. The ultracentrifuge runs for human and rat chylomicrons were for 3×10^5 and 1.6×10^6 g/min, respectively, at 14 C. In experiments with the feed of triple triglyceride, ultracentrifugation was modified to obtain very large chylomicrons (>2,500 Å) and to reduce VLDL contamination. Smaller 4 ml polyallomer tubes in an SB 405 swinging bucket rotor were used; lymph, to a height of 3.5 cm, was overlayered with saline (d 1.006) to a height of 5 cm and centrifuged for 10^6 g/min. In the second run, 1.5 ml of chylomicrons were mixed with 1 ml of d 1.342 sodium bromide solution and overlayered with 2 cm of EDTA-saline (d 1.006). Lipoproteins were dialyzed in cellulose tubing (Fisher Scientific Co., Montreal) against saline (4.5 g NaCl/l) containing sodium azide (100 mg/l) for 18 hr at 8 C.

Lipoproteins, for electron microscopy, were eluted from agarose with distilled H_2O, negatively stained with sodium phosphotungstate (20) and examined on carbon-coated copper grids in a Philips 200 C electron microscope (Philips Electronics Ltd., Montreal). Particle magnifications were calculated from grids of standardized width (Philips Ltd.), and chylomicron diameters were measured on positive photographic prints with a particle analyzer (Model TGZ3, Carl Zeiss Ltd., Montreal).

Isoelectric Focusing

Isoelectric focusing and pH gradient electrophoresis were both performed in the same system of thin layers of agarose beads (A5m, 200-400 mesh, Bio Rad Labs., Richmond, CA); 110 ml of 6% agarose bead suspension was stirred with 100 ml deionized water. Excess water was removed by filtration in a Buchner funnel and agarose was washed twice with 75 ml water. The volume was brought to 100 ml to give the viscosity of a thin syrup ($\eta \simeq 0.3$). To prepare thin layers, 30-2 ml of agarose were stirred for 10 min with lysine (1 mg/ml) and 1.7 ml of pH 2-10 ampholytes (40% W/V) or 1.1 ml of pH 3-5 (20% W/V) and 1.1 ml of pH 2-10 ampholytes (20% W/V) (Physolytes, Brinkmann Inst. Inc., Rexdale, Ontario). The suspension was poured on to 20 x 20 cm glass plates and dried in room air for 1½ - 2 hr depending on humidity and temperature. The drying surface was flat and no surface water was visible for at least 30 min before the plates were placed in a double isoelectric focusing chamber (Brinkmann Instruments), humidified by wet gauze and cooled by recirculated iced water. Anodal and cathodal solutions of 0.30 M H_2SO_4 and 0.056 M ethylene diamine, respectively, were absorbed by filter paper strips, 8 mm x 20 cm. These strips formed the contact between platinum ribbon electrodes and agarose. Plates were cooled for 15 min and then prefocused for a minimum of 60 min at 200 V (power supply, E.C. Apparatus Corp., St. Petersburg, FL). Samples were applied with glass microscope cover glasses (18 mm) at 4 cm from the cathode or at 1½ - 2 cm if chylomicrons were present. The chamber was closed and flushed with N_2. A 16-18 hr electrophoresis at 100 V with amperage commencing at 10 mA was convenient for both pH gradient electrophoresis and IEF. In contrast to focusing of small proteins in other systems, high voltage (400-600 V) failed to improve focusing of chylomicrons and VLDL.

Detection of Lipoproteins

Chylomicrons and VLDL were visualized directly, without staining, as white bands in the translucent agarose layer. This was achieved by oblique transillumination or by oblique illumination over a black surface. Rat and human LDL were prepared from fasting plasma by ultracentrifugation (19), but neither was directly visible on agarose layers. R_fs of larger lipoproteins were measured directly on the thin layers and results recorded by photography with high contrast microfilm (HCF 5369, Eastman Kodak Co., Rochester, NY) using oblique illumination (Figs. 1-4). The lipoprotein migrating farthest was assigned an R_f of 1.0. The photography of unstained lipoproteins, on a grey translucent background, was difficult since a single film exposure did not necessarily give correct exposure on all sections of the electrophoresis. Consequently, the resolution on some photographic prints was inferior to that of direct vision or densitometry of the thin layers. Chylomicrons, VLDL and LDL were detectable by absorption to print

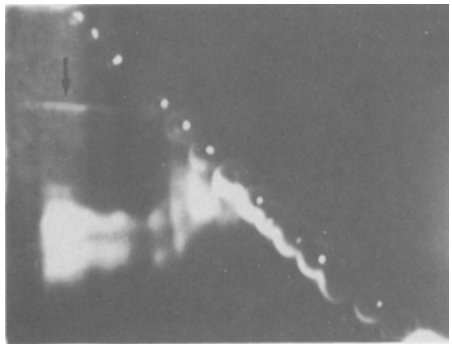

FIG. 1. Well isoelectric focusing method. Lymph from rat absorbing triolein was applied in wells placed obliquely between the cathode (top) and anode (bottom) and electrophoresed for 16 hr. The photograph of unstained lipoproteins was taken from the cathodal half of the plate using oblique illumination. Triglyceride-rich lipoproteins appear white. The upper three wells are empty and show only a light reflex. Lower wells show curved white lipoprotein bands on their circumference; these are precipitated lipoproteins. A focused lipoprotein intersects the line of wells, 4 wells from the top (arrow).

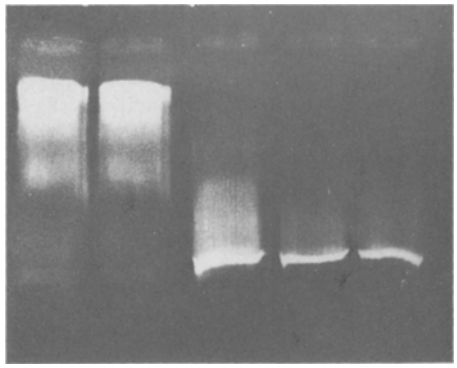

FIG. 2. pH gradient electrophoresis of whole rat lymph with pH 2-10 and pH 3-5 ampholytes for 17 hr. The cathode is at the top and samples were applied 1½ cm below it. Safflower oil fed lymph on the left and chow fed lymph on the right were obtained from the same rat as described in the text. Photography was by oblique illumination, as in Figure 1.

paper (Brinkmann Inst. Inc.) which was placed on the agarose for 3-5 min, air dried at 125 C and stained overnight with Oil Red 0 in ethanol at 37 C (21). Such prints were of limited value since they gave qualitative but not quantitative absorption and the horizontal migration of chylomicrons during print absorption frequently obscured resolution obtained in lipoprotein mixtures containing chylomicrons.

Determination of Isoelectric Points

Lipoproteins tested in this and other systems

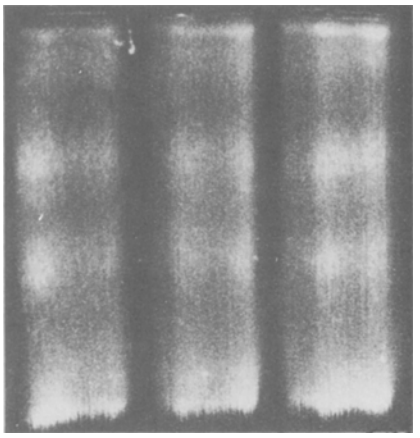

FIG. 3. pH gradient electrophoresis of chylomicrons obtained after a feed of triple triglyceride. Running time 17 hr. The cathode is at the top and the origin is visible above the uppermost thin dense chylomicron band.

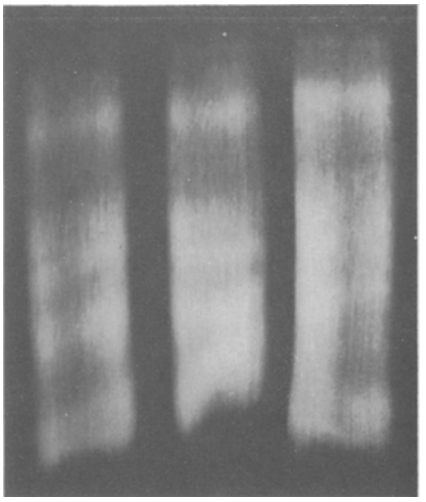

FIG. 4. pH gradient electrophoresis of chylomicrons obtained after a trilinolenin feed. The origin is at the top. The conditions are as described in text but with increase in ethylenediamine concentration to 0.2 M. Illumination of unstained chylomicrons was from the side.

tend to precipitate at and below their pI (10); therefore, it was not possible to detect the pI by combined anodal and cathodal migrations to the pI. To detect the limiting pH of anodal migration, a new technique, which will be called IEF, was used. Lipoproteins were placed, in a series of wells, diagonally across the plate (Fig. 1) using a 10 μl pipette. A focused protein was detected by formation, parallel to the

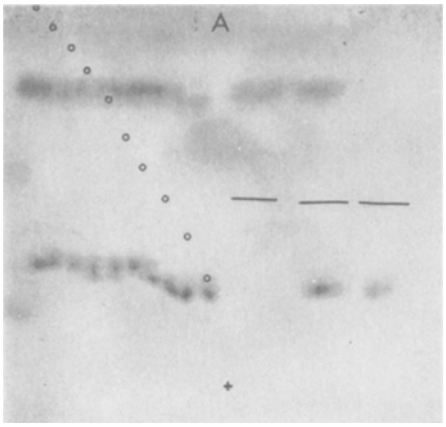

FIG. 5a. Print paper of well isoelectric focusing of mixed ovalbumen (0.3%) and horse myoglobin (0.3%) after 16 hr electrophoresis with 1.1 mg of pH 2-11 and 0.6 ml of pH 4-6 ampholines. Proteins were stained by Coomassie Blue, and the position of wells, slots and the orientation of electrodes are indicated. Myoglobin and ovalbumen were also applied as single proteins to the left and right slots, respectively.

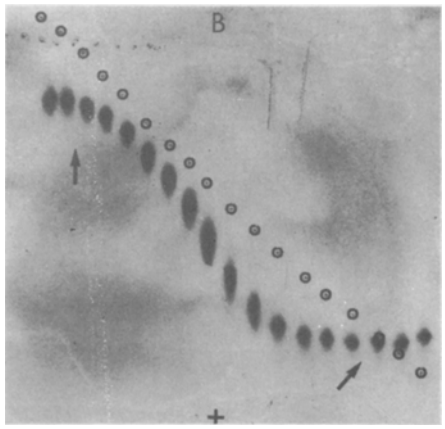

FIG. 5b. Print paper of well focusing of ovalbumen taken before complete equilibration of protein migration after 4 hr electrophoresis. The oblique arrow indicates the isoelectric point (pI = 4.85), and the vertical arrow indicates horizontal line formation but without well line intersection.

electrodes, of a straight line that intersected the diagonal well line (Fig. 1). Multiple pH measurements were taken on such lines, at least 1 cm out from the well line, since ampholyte zones were disturbed around the wells. Lipoproteins, in wells at pH less than their pI, precipitated in the wells. Lipoproteins and smaller proteins sometimes formed lines with a straight component with one pH when the distance from their pI was great, but these lines curved toward the anode before intersecting the well line at the pI of the protein. The classical narrowly focused lines seen with smaller proteins were rarely observed with chylomicrons and VLDL. When lipoprotein lines were >1 mm wide, pHs were measured in their leading anodal edge. pH was measured with a flat surface, combination, probe electrode (Desaga-Brinkmann, Rexdale, Ontario), or miniature pH and reference electrodes (Bio Rad. Labs., Richmond, CA).

The validity of the well technique was tested with smaller proteins of known pI, i.e., sperm whale myoglobin, horse myoglobin, limpet hemocyanin and conalbumen (Sigma Chemical Co., St. Louis, MO). Duplicate experiments were run for each protein and the pI of the major protein band was determined from triplicate readings. Focusing conditions were identical to those used for lipoproteins with the addition of continuous humidified N_2 to the chamber in the final hour of focusing. The pI values of the four proteins did not differ significantly from expected values having pIs 0.4 ± 0.23 less than those reported for IEF on Sephadex (16). The omission of N_2 in 3 experiments gave pI readings 1.09 ± 0.28 less than expected values for 3 proteins. Unlike lymph lipoproteins which did not migrate at pHs below their pI, the smaller proteins showed both cathodal and anodal migration to form straight lines intersecting the diagonal well line and visible on both sides of it (Figs. 5a and 5b). *Comparison between well and slot application* was made with equine myoglobin and ovalbumen (Sigma Chemical Co., St. Louis, MO) electrophoresed for 16 hr; equilibrium was reached since the proteins in wells migrated varying distances to form straight lines intersecting the well line (Fig. 5a). Where equilibrium has not been reached, the isoelectric point can be detected by the well technique as shown for ovalbumen electrophoresed for 4 hr (Fig. 5b). A relatively straight line, which does not intersect the well line, is shown and should not be confused with the pI line. These results indicate that the well technique and agarose gave pI results for small proteins similar to those from conventional methods. The well technique had the specific advantage for large lipoproteins of indicating the pI with only unidirectional migration; with small proteins, the method is inferior to Sephadex thin layer at high voltage (16) since protein bands are more diffuse, less regular, and equilibration is prolonged.

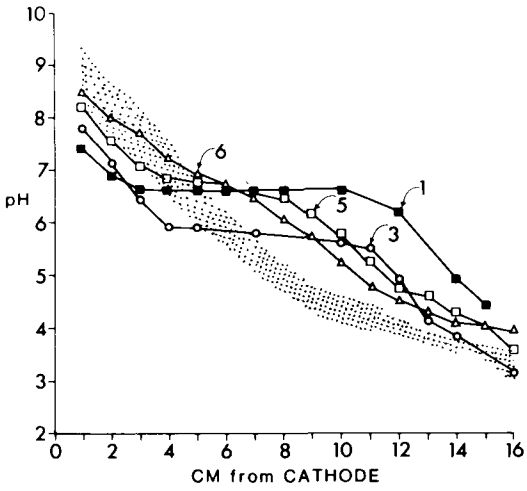

FIG. 6. pH gradients were measured in 4 agarose thin layers containing mixed ampholytes (pH 2-10, pH 3-5). The number of hours of electrophoresis is indicated for each experiment. The stippled area represents the limits of measurements after 11 hr and 17 hr in experiments shown in Figure 7.

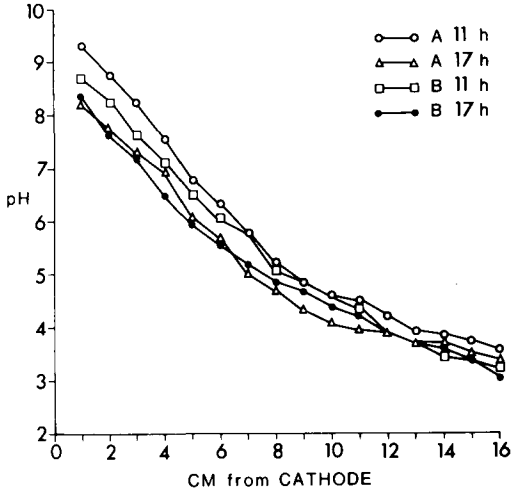

FIG. 7. pH gradient in 2 mixed ampholyte (pH 2-10, pH 3-5) agarose layers (A and B) were measured first after 11 hr and subsequently at 17 hr. The outer limits of the pH readings are shown in shaded areas in Figures 6 and 8.

RESULTS

The time taken to establish linear pH gradients in agarose bead thin layers was obtained by measuring pHs in 5 consecutive experiments after 1 hr of electrophoresis and at one subsequent time. The results during the early phase of focusing, up to 6 hr, are shown in Figure 6. For clarity, only 4 gradients are shown, but the omitted 4 gradients measured at 1 hr and a gradient measured at 6 hr were almost identical to those shown. The central plateau, showing pH changes of <0.40, were 9 ± 0.9 cm (n=5) long at 1 hr but decreased progressively to 4 cm at 5 hr and 0 and 2 cm at 6 hr. In these conditions, it required ca. 6 hr to establish a linear pH gradient.

Gradient stability was measured by comparing 11 hr and 17 hr pHs in each of two experiments. The results are shown in Fig. 7. In both experiments, the parallelism of gradient was maintained, but there was a drift of any given pH point to the cathode.

Gradient linearity was examined at later times up to 24 hr, and the results from 4 different experiments are shown in Figure 8. In comparison with the 11 hr and 17 hr experiments, there was further cathodal drift in the cathodal half of the gradient, but this was no longer progressive with increasing times. By 24 hr, linearity was lost in the anodal 5 cm of the gradient.

These results indicate that agarose gel beads provide a suitable anticonvective medium for

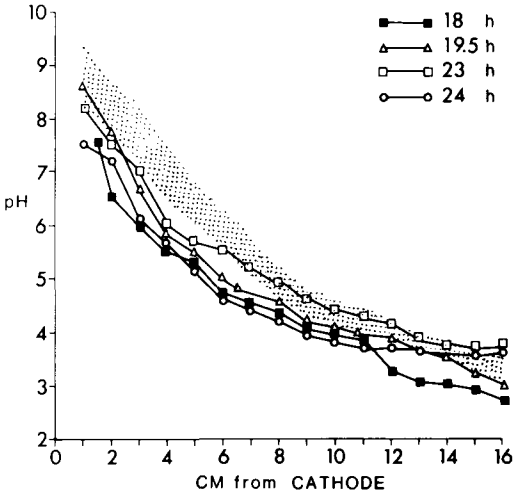

FIG. 8. pH gradients were measured after 17 hr in 4 experiments with mixed ampholytes (pH 2-10, pH 3-5). The shaded area shows the outer limits of pH readings in Fig. 7.

pH gradient electrophoresis and IEF, in the pH range from 3-9. Linearity was established in 6 hr and was maintained for almost 24 hr.

Isoelectric Focusing of Lipoproteins

Results of isoelectric focusing by the well technique are illustrated in Figure 1. For photographic purposes, the wells were overloaded with undialyzed thoracic duct lymph

from a rat fed intraduodenal triolein emulsified with sodium taurocholate. The unstained triglyceride-rich lipoproteins are readily visualized as white bands. A sharply focused lipoprotein band (VLDL, particle diameters 300-650 Å) marked by an arrow, intersects the well line horizontally. The wells cathodal to (above) this line are empty, indicating complete migration of visible lipoproteins at the pHs of these wells. In the wells anodal to the heavily overloaded bands, minimal migration to the anodal side of each well is due to osmotic flow of precipitated material early in electrophoresis. This is recognized by a uniform migration length in a series of wells of different pH and by failure of band formation.

To determine if the agarose method had the capacity to resolve and focus human chylomicrons, ultracentrifugally prepared plasma chylomicrons were examined and a sharply focused lipoprotein was found at the cathodal end of the separated lipoproteins after 17 hr electrophoresis; pIs of this lipoprotein band in three individuals with normal, Type I and Type V primary hyperlipoproteinemia were, respectively, 5.93, 5.78 ± 0.08 (± SEM, n=4) and 5.88 ± 0.17 (n=4) where n is the number of focusing experiments.

These results demonstrate that agarose IEF (a) permits direct visualization and pI measurement of unstained lipoproteins; (b) permits migration of all visible triglyceride-rich lipoprotein in rat lymph; (c) is unique in focusing at least one component of ultracentrifuged human serum chylomicrons with reproducible pH results; and (d) indicates the limiting pH of anodal migration of lipoproteins with the well technique, avoiding the uncertainty, inherent in the standard IEF polyacrylamide and Sephadex techniques, that a protein may still be migrating.

pH Gradient Electrophoresis

The capacity of agarose bead pH gradient electrophoresis to resolve mixed triglyceride-rich lipoproteins was investigated with rat thoracic duct lymph.

Lymph was collected from cannulated rats during absorption of safflower oil and 12 hr later during chow feeding. Aliquots of the two collections were electrophoresed simultaneously. The triglyceride-rich lipoproteins migrated as discrete white bands and little visible lipoprotein remained at the origin. There were major differences in mobility between the principal lipoprotein bands in the two nutritional states from each of five animals. A typical result is shown in Figure 2. In the oil-fed state, the R_fs of the major bands were <0.62 (n=5), but in the chow-fed, the major bands had an R_f of 0.96 ± .01. In two experiments, the major band from each nutritional state was eluted for electronmicroscopy; in the oil-fed state, 96% of the particles were chylomicrons (particle diameter range, 650-1950 Å); in the chow-fed state, 94% of the lipoproteins were VLDL (particle diameter range, 300-650 Å) and 6% chylomicrons. These experiments demonstrated that in this system chylomicrons migrate in discrete bands and that, in the conditions of these experiments, most chylomicrons were readily separated from intestinal VLDL from the same animal.

To determine if the method was useful in investigation of chylomicron heterogeneity, large chylomicrons were obtained ½ to 4 hr after feeds of the three unsaturated triglycerides, a condition favoring the production of larger chylomicrons (22). In initial experiments, triple triglyceride feeding produced lymph with a chylomicron fraction in which 4 distinct bands migrated in addition to a thin, dense band just below the origin (Fig. 3). Similar findings were obtained with feeding of a single triglyceride, trilinolenin and, by increasing the cathodal electrolyte, five components showed unequivocal migration from the origin (Fig. 4).

In 3 further experiments, the chylomicron nature of the separated bands was confirmed by electron microscopy. In these 3 experiments, each animal was fed a mixture of triolein, trilinolein and trilinolenin and, to reduce potential contamination with VLDL, the centrifugal preparation was altered as described above. The results of pH gradient electrophoresis were similar to the previous experiments in that 5, 4 and 5 migrating bands, respectively, were found in each of 3 experiments. Each band, by electron microscopy, was composed of chylomicrons with a minimum diameter of 2,500 Å and the upper limits of the range were 12, 15 and 18 x 10^3 Å for each of the 3 experiments. Smaller particles in the size range of VLDL (300-700 Å) were seen occasionally, but their contribution to lipid mass was negligible (<1%). Material remaining at the origin consisted of oil droplets, nonsperical particles and chylomicrons. These experiments demonstrate that this method has the unique capacity to separate multiple components of the chylomicron "fraction" obtained by ultracentrifugation. More detailed characterization of these chylomicron bands is beyond the scope of this methodology report.

DISCUSSION

Isoelectric focusing of proteins has been

greatly improved by the introduction of IEF in polyacrylamide gel tubes and thin layer IEF on a granular support bed such as Sephadex or polyacrylamide; the latter method gives resolution superior to IEF in sucrose columns, and the separated proteins are readily accessible to direct pH measurement and to elution for analysis.

In an attempt to find IEF systems suitable for chylomicron and VLDL analysis, we rejected: (a) polyacrylamide gels, since chylomicrons and large VLDL do not enter the gel at the usual gel concentrations (11) although chylomicrons enter spacer gel (23); (b) Sephadex, which, in our hands, caused precipitation of chylomicrons; and (c) agarose gels, which are unsuitable for IEF since high osmotic flows rupture gels (24). Agarose gel beads do not rupture osmotically and permit use of agarose as a thin granular layer. Agarose beads (A5m) exclude chylomicrons and VLDL and provide a support medium from which recoveries of lipoprotein can be high (3).

Agarose bead layers have a number of other advantages for IEF; they provide a translucent layer on which the triglyceride-rich lipoproteins can be visualized directly, permitting pH measurement on and elution of unstained lipoproteins. IEF with agarose beads also has potential value in separating protein mixtures by both pH gradient effects and molecular sieving prior to equilibrium, i.e., during pH gradient electrophoresis. Commercial agarose has ionizable groups with K_ms which are probably in the range of 10^{-2} to 10^{-4} (24), but pH gradient anomalies were not observed in the pH range (3-9) reported here.

The stability of pH gradients in agarose beads is comparable to that in polyacrylamide gel (25). The cathodal drift observed in the first 17 hr creates difficulty in determining the precise isoelectric point of large lipoproteins which migrate slowly. For this reason, the term "apparent pI" should be used until the shortest focusing time for a given lipoprotein is known (26).

This new method appears particularly suitable for chylomicron and VLDL preparation and analysis. Chylomicrons are generally defined as lipoproteins of $S_f > 400$, produced in the intestine with lipid derived largely from dietary fat precursors. The size range of chylomicrons is very wide, and attempts have been made by serial ultracentrifugation to separate chylomicrons into 3 groups of diminishing diameter (27), but there is no evidence that these represent separate classes of chylomicrons since they overlap, have no definable medians, and have no unique chemical or electrophoretic characteristics. Chylomicrons migrate on some electrophoretic systems such as starch block (7) and, to a limited extent, on paper, but in neither is heterogeneity of chylomicrons detectable. It is probable that some heterogeneity of chylomicrons is produced by preparative methods, and ultracentrifugation is known to reduce the apoprotein content of VLDL (28) and chylomicrons (29). The sensitivity of agarose IEF for lipoprotein separation was established by the demonstration of considerable heterogeneity in ultracentrifuged lymph chylomicrons, previously considered homogeneous.

ACKNOWLEDGMENT

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COMMUNICATIONS

Composition of Wax Esters and Triacylglycerols in the Melon and Blubber Fats of a Young Sowerby's Whale *Mesoplodon bidens*

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ABSTRACT

The blubber fat of a yearling Sowerby's Whale, *Mesoplodon bidens*, stranded on the Dutch coast, contained 59% triacylglycerols. This is quite unexpected because low levels (0-6%) of triacylglycerols are characteristic of *Ziphiidae* whales. In addition, the chain lengths of the fatty acids of the melon were longer than those of previously studied related species. As young Sowerby's Whales undergo a change in diet from mainly triacylglycerols in milk to wax ester containing food during their development from infancy to independence, these findings could reflect the age of the animal.

INTRODUCTION

It has been noted before that the composition of the blubber fats of the *Odontoceti* (toothed whales) often differs markedly from that of the fats of the melon and jaw (1). It has been postulated that the lipids of the melon and jaw tissues are responsible for the special acoustical properties of these organs, which make them suitable for echolocation (2). Especially the unusual isovalerate lipids in many of these "acoustic" oils have received much attention (3-5).

In a recent comparative survey of the lipid composition of Odontocete fats, Litchfield et al. (6) demonstrate that the composition of both blubber and head oils is correlated in several respects with the taxonomic subdivision of this suborder; they categorize the acoustic and nonacoustic fats of the entire suborder by the presence of isovaleric acid, wax esters, triacylglycerols, short chain and long chain fatty acids. An important division can be made on the basis of isovaleric acid. Species belonging to the *Delphinidae*, *Phocoenidae* and *Monodontidae* all contain high percentages of isovaleric acid, whereas the *Ziphiidae*, *Physeteridae* and *Platanistidae* do not (6). A further division was suggested (6,7): the blubber fats of several *Ziphiidae* species consist almost entirely (94%-100%) of long chain wax esters, which fact was considered to be a unique characteristic distinguishing the *Ziphiidae* from all other whales. In addition, it was shown that *Ziphiidae* melon and jaw fats contain large amounts of C_{10}-C_{12} fatty acids not found elsewhere in the *Odontoceti* (6,7,8).

On September 29th, 1977, a young male Sowerby's Whale, *Mesoplodon bidens*, (2.59 m) stranded on Ouddorp Beach, The Netherlands. As no analytical data of the acoustical fats of this species have been found and only the fats of a few *Ziphiidae* species have been studied (6,7), we have analyzed its melon, jaw and blubber fats.

EXPERIMENTAL

Samples of blubber fat of the dorsal area, of one-half of the melon, and of the total jaw fat body, freed from attached blubber fat, were extracted in a Sorvall Omnimixer with chloroform/methanol (2:1, v/v). Lipid class compositions were determined by column chromatography using 10 g samples on silicic acid deactivated with 10% water using a gradient of diethyl ether in light petroleum as eluent. The triacylglycerol and wax ester fractions of the melon and blubber were saponified with methanolic sodium hydroxide solution (0.5 mol/l), and the resulting soaps were esterified with boron trifluoride in methanol (14:100, w/v). Fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) on a Packard Becker 419 gas chromatograph equipped with a flame ionization detector (FID). A homemade, 80 m x 0.5 mm, SCOT glass column was used, precoated with fractionated Aerosil OX 50 (ex Degussa, Germany) and coated with Silar 5 CP, both by the dynamic method. Conditions: column temperature 205 C or programmed from 180 to 215 C at 2 deg/min; carrier gas He, 5 ml/min. Peaks were identified by gas chromatography-mass spectrometry (GC-MS), by comparison with the carbon numbers of known compounds and with analytical data from an EGA packed column. The GC-MS apparatus was a Kratos MS 30 fitted with the same

capillary column and temperature programming as above.

RESULTS

Lipid class analyses showed that triacylglycerols (melon 38%, jaw 46%, blubber 59%) and wax esters (melon 62%, jaw 54%, blubber 41%) were the main components of the nonpolar part of the fats. In addition, traces of other lipid classes (diacylglyceryl ethers) were found, which were not further analyzed.

The high triacylglycerol content of the blubber fat is quite unexpected, because low levels (0-6%) were considered to be a unique characteristic of the *Ziphiidae* (6,7). To exclude the possibility that these high levels of triacylglycerols are only locally present, we investigated 9 samples, taken from the upper, middle and lower layers, respectively of the dorsal and ventral part of the body behind the dorsal fin and of the jaw. However, substantial amounts of triacylglycerols were present in all samples.

The fatty acid composition of the wax esters and the triacylglycerols of the blubber and melon fats are presented in Table I and Figure 1. The fatty acids are similar to those found in most whale fats. The melon oil fatty acids ranged from 10 to 22 carbon atoms. Average chain lengths were 14.7 (triacylglycerols) and 14.8 (wax esters). The most prominent fatty acids were 12:0, 14:0, 14:1ω5 and 16:1ω7. As in other Odontoceti genera examined, the melon fatty acids had shorter chain lengths, were branched and were saturated to a higher extent than those of the blubber fat. The most prominent fatty acids of the blubber were 14:0, 16:0, 16:1ω7, 18:1ω(9+11), 20:1ω(9+11) and 22:1ω(11+13). Relatively high amounts of polyunsaturated fatty acids were present in the wax ester fraction of this fat: 20:5ω3, 6.8%; 22:5ω3, 2.7% and 22:6ω3 9.2%; this was confirmed by a high skipped-methylene signal in the NMR spectrum and by the high iodine value of 128.7 of this fraction. The other lipid samples had lower iodine values: blubber triacylglycerols 79.6; melon triacylglycerols 56.6; melon wax esters 52.9.

The average chain lengths of the fatty acids in the blubber fat were 17.1 (triacylglycerols) and 18.4 (wax esters).

DISCUSSION

It seems worthwhile to incorporate the data

TABLE I

Distribution of Fatty Acids of the Wax Esters and the Triacylglycerols (Mole %) of Sowerby's Whale *Mesoplodon bidens*

Fatty acid		Wax esters		Triacylglycerols	
		Melon	Blubber	Melon	Blubber
Iso-	C10:0	0.3		1.2	
	C10:0	0.8		3.0	0.2
Iso-	C11:0			0.5	
Anteiso-	C11:0	0.1		0.2	
	C11:0	trace		0.1	
Iso-	C12:0	2.9		5.1	0.1
	C12:0	7.6	0.4	8.7	1.2
Branched	C13:0				0.2
Iso-	C13:0	0.6		0.5	
Anteiso-	C13:0	0.2		0.3	
	C13:0	0.1		0.1	0.1
Iso-	C14:0	6.3	0.1	4.9	0.2
	C14:0	16.9	5.2	15.5	11.9
Iso-	C15:0	0.9	0.1	0.7	0.2
Anteiso-	C15:0	0.4	0.1	0.3	0.1
	C15:0	0.2	0.1	0.4	0.5
Iso-	C16:0	0.9	trace	1.8	0.2
	C16:0	2.3	2.8	7.8	12.4
Iso-	C17:0		0.1	0.1	0.1
Anteiso-	C17:0	0.1	0.1	0.1	0.1
	C17:0		0.1	0.1	0.2
Iso-	C18:0			0.1	0.1
	C18:0	0.3	0.8	0.7	1.9
	C19:0	0.1	0.1	0.1	0.1
Total saturated		41.0	10.0	52.3	29.8

TABLE I (continued)

Fatty acid	Wax esters		Triacylglycerols	
	Melon	Blubber	Melon	Blubber
C10:1	0.3		0.3	
C10:1	0.2		0.5	0.1
Iso- C12:1	4.3		3.4	
Iso- C12:1	1.5	0.2		0.2
C12:1			2.1	
C12:1	0.7	0.1	0.5	0.2
C12:1	0.2		0.1	
C14:1ω9	2.5	0.1	1.1	
C14:1ω7	2.1	0.2	2.7	0.3
C14:1ω5	11.1	6.1	5.0	4.0
C15:1			0.1	
C15:1		0.1		trace
C16:1ω11	1.2	0.2	1.1	0.3
C16:1ω9	1.1	0.2	3.8	1.1
C16:1ω7	14.6	14.3	10.8	17.6
C16:1ω5	0.3	0.2	0.3	0.4
C17:1	0.2	0.5	0.3	0.6
C17:1	0.8	0.1	0.3	
C18:1ω11 + C18:1ω9	8.3	15.3	3.0 + 6.4	18.7
C18:1ω7	0.9	1.7	0.8	1.9
C18:1ω5	0.1	0.4		0.2
C20:1ω11 + C20:1ω9	2.7	15.7	1.0 1.4	4.5 6.0
C20:1ω7		0.6		
C21:1		0.4		0.2
C22:1ω13 + C22:1ω11	0.4	5.3	0.9	7.0
C22:1ω9	0.1	0.8	0.1	0.9
Total monoene	53.6	62.5	46.0	64.2
C12:2	0.1		0.1	
C14:3	0.7		0.2	
C16:2			0.1	0.1
C16:2	0.1			
C16:2	0.2	0.4		0.3
C16:3	0.2			
C16:3	0.2			
C18:2ω6	0.6	1.8	0.4	0.9
C18:3ω3	0.4	1.8	0.2	0.6
C18:4ω3	0.8	1.8	0.2	0.4
C20:2ω6	0.1	0.4		0.1
C20:4ω6	0.1	1.1	0.1	0.2
C20:4ω3		1.5	0.1	0.4
C20:5ω3	1.1	6.8	0.3	1.2
C22:5ω3	0.2	2.7		0.4
C22:6ω3	0.4	9.2		0.1
Total polyene	5.2	27.5	1.7	5.7

of *Mesoplodon bidens* in the scheme of Litchfield et al. (6) in which they put the data of *Odontocete* whales in order. As predicted by them, no significant amounts of isovaleric acid were present, which is typical of the group of *Ziphiidae, Physeteridae* and *Platanistidae.* However. although the fatty acids of the melon are rather short and contain ca. 3% C_{10} and almost 20% C_{12}, the assignment of our speciment to the group of "C_{10}-C_{20} acids" seems more appropriate than to the group of mostly "C_{10}-C_{12} acids," to which the *Ziphiidae* species appear to belong (6). In fact, the C_{10}-C_{12} content and the average triacylglycerol carbon number of the melon of *Mesoplodon bidens* resembles that of the Sperm Whale *Physeter macrocephalus* (9), which belongs to a different family, rather than that of the more related *Ziphiidae* species *Berardius bairdii* (8,10). In the latter species, fatty acids > C_{14} were present only in minor amounts (8).

The finding of high amounts of polyun-

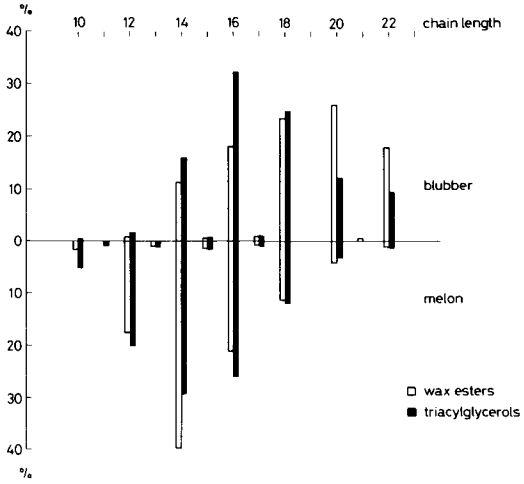

FIG. 1. Distribution of chain lengths of the fatty acids (mole %) of the blubber (above) and melon (below) of Sowerby's Whale *Mesoplodon bidens.*

saturated fatty acids in the blubber fat of our specimen compared with, for example, an almost complete lack of these acids in *Berardius bairdii* (8) is possible of limited taxonomical importance; at least for Mysticeti Whales, a relation between the iodine value of the blubber fat and the fatness of the animals exists (11).

Another unexpected result is the high triacylglycerol/wax ester ratio in the blubber fat of our specimen, which deviates substantially from that of other beaked whales; *Berardius bairdii, Hyperoodon ampullatus* and *Mesoplodon densirostris* contained 3, 6 and 0% triacylglycerols, respectively (6). Our specimen contained 59% blubber triacylglycerols. From the data of the blubber fat of a Sowerby's Whale stranded on the French coast in 1908 (12,13), it can be deduced that they indicated low levels of triacylglycerols. The saponification value of the blubber fat was 103.4; the fat contained 49% unsaponifiable material and only small amounts (0.7%) of glycerol were found. It can, therefore, be concluded that at least in *Mesoplodon bidens* the triacylglycerol/wax ester ratio in the blubber fat may be subject to substantial intraspecific variation. As our specimen differed from the previously studied *Mesoplodon bidens* and other beaked whales in that it was very young (about one year old), one may speculate that the triacylglycerol/wax ester ratio may change with age. The milk fat of *Mesoplodon bidens* is unlikely to contain wax esters as triacylglycerols were the only main lipids demonstrated in the milk of *Tursiops truncatus,* which also belongs to the Odontoceti (14). On the other hand, the food of the adult animal (squid for example) is expected to contain substantial amounts of wax esters (9). The development from infancy to independence, which is usually reached after a nursing period of about a year (roughly the estimated age of our specimen), could thus be accompanied by a change in the triacylglycerol/wax ester ratio.

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Identification of Ergosta-8,24(28)-Dien-3β,6α-Diol in A $\Delta^8 \rightarrow \Delta^7$ Sterol Isomerase-Blocked Yeast Mutant

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ABSTRACT

In addition to the monohydroxysterols found in the $\Delta^8 \rightarrow \Delta^7$ isomerase-blocked *Saccharomyces cerevisiae* mutant *erg 2*, a novel dihydroxysterol, ergosta-8,24(28)-dien-3β,6α-diol, was isolated. This sterol accumulated to the extent of 2.1% of the total sterol fraction when this mutant was treated with 23-azacholesterol, a known inhibitor of the 24-methylene-sterol-24(28)-reductase.

INTRODUCTION

Yeast produce an array of monohydroxysterols which have been extensively investigated in this and other laboratories (1). In the instances in which polyhydroxysterols, naturally occurring (2,3) or artifacts of oxidation (3), have been detected in yeast, few (2,4) have been identified. We wish to report characterization of a dihydroxysterol (0.8% of sterols) from a *Saccharomyces cerevisiae* mutant *erg 2* which is blocked at $\Delta^8 \rightarrow \Delta^7$ sterol isomerase (5). The dihydroxysterol accumulates (2.1% of sterols) when this mutant is grown aerobically in the presence of 1 μM 23-azacholesterol (5), a known 24(28)-methylene sterol reductase inhibitor (6), suggesting it possesses at least 3β-hydroxy, Δ^8 and $\Delta^{24(28)}$ structural units.

MATERIALS AND METHODS

The *erg 2* sterol mutant of *S. cerevisae* employed in this study has been described elsewhere (7,8). A 10 ml starter culture statically incubated at 30 C for 24 hr was added to 100 ml of complete liquid medium (9) containing 1 μM 23-azacholesterol, and the culture was grown at 25 C with stirring for 48 hr. The inoculum was transferred to a 4 l Virtis fermenter jar containing 1.5 l of medium to which the 23-azacholesterol had been added in ethanol (1.5 ml) to a concentration of 1 μM. For the control experiment, the azasterol was omitted from the medium. The cultures were grown for 48 hr on a Virtis fermenter at 30 C with 400 rpm stirring and 2 l/min aeration. The cells were harvested by centrifugation, washed three times with distilled water, and saponified (9).

The nonsaponifiable fraction (NSF) from *erg 2* cultured in the presence of 1 μM 23-azacholesterol was acetylated and separated by preparative thin layer chromatography (TLC) on Silica Gel GF-254, 25% $AgNO_3$ plates developed in benzene. The bottom-eluting band at R_f 0.14 was removed and found to contain one major peak by gas liquid partition chromatography (GLPC) (4,6). After purification by TLC (Silica Gel GF-254, benzene) a compound (**1**) was obtained that was 98% pure by GLPC (5,6). Analysis by GLPC of the NSFs from the azasterol-treated and control cultures revealed that **1** was 2.1% and 0.8% of the total sterols (0.028% and 0.008% of the dry cell weight), respectively.

Chemical ionization-mass spectroscopy (CI-MS) was generously performed by Professor W. Ayre at the University of Alberta, Edmonton. Electron impact-mass spectra (EI-MS) were obtained on a Perkin-Elmer Hitachi RMU-6E spectrometer using usual inlet conditions (9). Proton and carbon resonance spectroscopy was performed on a Varian XL-100-15 spectrometer equipped with TT-100 Fourier transform system. For proton spectra, samples were dissolved in $CDCl_3$ containing tetramethylsilane (TMS) while for ^{13}C spectra TMS was omitted. ^{13}C chemical shifts are reported relative to TMS ($\delta(TMS) = \delta(CDCl_3) + 76.9$ ppm).

RESULTS AND DISCUSSION

High resolution mass spectroscopy gave the molecular weight of **1** (Fig. 1) as 498.3716. This corresponds to a formula of $C_{32}H_{50}O_4$ (498.3709) which is compatible with a 28 carbon, doubly unsaturated sterol possessing two acetoxy groups and with the molecular weight (498) determined by CI-MS.

In Table I, the proton magnetic resonance (PMR) spectral data for **1** and **2** (fecosteryl acetate) are given. The two acetoxy functions of **1** absorbed as two closely spaced but resolved singlets at δ2.05 and 2.07 ppm. As chemical shifts and coupling constants of the side chain methyl groups and the C-28 protons of **1** were nearly identical to those of **2**, the presence of a $\Delta^{24(28)}$-bond on the side chain of **1** was apparent. The presence of Δ^8-double

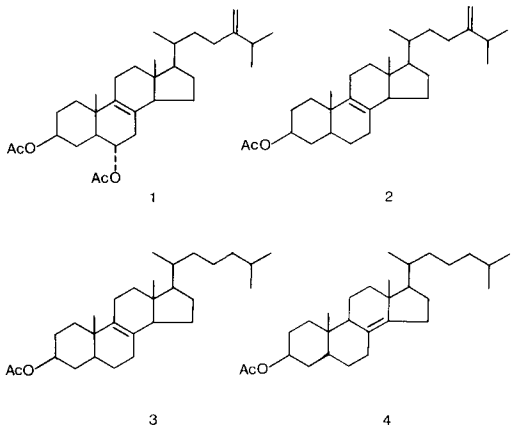

FIG. 1. Structures of 3β,6α-diacetoxyergosta-8,24(28)-diene (**1**), 3β-acetoxyergosta-8,24(28)-diene (**2**), 3β-acetoxycholest-8-ene (**3**), and 3β-acetoxycholest-8(14)-ene (**4**).

TABLE I

Proton Chemical Shifts of **1** and **2**[a]

Position	1	2
C-18	0.61	0.62
C-19	1.08	0.99
C-21	0.97 (J=7.5 Hz)	0.96 (J=8 Hz)
C-26,27	1.05 (J-7 Hz)	1.04 (J=6.5 Hz)
$COOCH_3$	2.05	2.03
	2.07	
C-3α	ca. 4.65	ca. 4.65
C-6β	ca. 4.90	
C-28	4.65 (J=5 Hz)	4.65 (J=5 Hz)

[a]In ppm downfield from TMS.

TABLE II

Carbon-13 Chemical Shifts of 3β,6α-Diacetoxyergosta-8,24(28)-Diene (**1**), 3β-Acetoxyergosta-8,24(28)-Diene (**2**), 3β-Acetoxycholest-8-ene (**3**), and 3β-Acetoxycholest-8(14)-Ene (**4**)[a]

Carbon No.	1	2	3[b]	4[b]
1	34.8	34.7	34.8	36.2
2	27.0	27.4	27.5	27.5
3	72.7	73.4	73.3	73.4
4	28.1	34.0	34.1	34.0
5	44.3	40.4	40.5	44.0
6	70.2	25.2	25.3	28.7
7	33.9	27.2	27.0	29.4
8	125.9	128.2	128.1	125.8
9	134.5	134.6	134.4	49.1
10	37.4	35.5	35.6	36.6
11	22.4	22.6	22.7	19.8
12	36.4	36.7	36.8	37.1
13	41.8	42.0	42.0	42.6
14	51.2	51.7	51.7	142.5
15	23.4	23.6	23.9	25.7
16	28.4	28.6	28.7	26.9
17	54.4	54.6	54.8	56.8
18	11.0	11.1	11.2	18.1
19	18.4	17.6	17.6	12.6
20	35.9	36.1	36.2	34.3
21	18.4	18.6	18.7	19.0
22	34.4	34.5	36.1	35.9
23	30.8	30.9	23.7	23.7
24	156.5	156.5	39.4	39.4
25	33.5	33.6	27.9	27.9
26	21.6	21.7	22.5	22.4
27	21.6	21.7	22.7	22.7
28	105.3	105.8		
$COOCH_3$	20.9	21.2	21.3	21.2
	21.0			
$COOCH_3$	170.2	170.3	170.1	170.1
	170.6			

[a]In ppm downfield from TMS.
[b]Data from Ref. 12.

bond in **1** was suggested by chemical shift of the C-13 methyl group and lack of additional olefinic absorptions downfield from those of the *exo* methylene. Additional support for this observation was given by the calculated chemical shifts (10) for the C-10 and C-13 methyl groups of **4** (Fig. 1) possessing any additional ring acetoxy substitutent. These were all higher than that measured for **1** and **2**.

The C-10 methyl group of **1** was shifted ca. 0.1 ppm downfield, and this indicated that the second acetoxy group was probably attached to ring A or B. Examination of the calculated shifts (10) for C-10 methyl group of **3** (Fig. 1) for all ring A and B positions of the second acetoxy group revealed 1α (1.03 ppm), 2α (1.06 ppm), 2β (1.12 ppm), 6α (1.05 ppm), 6β (1.15 ppm) and 7α (1.05 ppm) to be compatible (± 0.1 ppm) with that observed. While these calculations established the attachment of the second acetoxy group to ring A or B, no decision could be made as to its location or configuration.

The ^{13}C magnetic resonance (CMR) spectrum of **1** was more definitive than its PMR spectrum, and not only established the presence of the Δ^{8}- and $\Delta^{24(28)}$-bonds but also permitted assignment of the second acetoxy group of **1** to the C-6 position. In Table II are listed the ^{13}C chemical shifts for **1** and **2**. Schroepfer and coworkers have most recently reported the ^{13}C chemical shifts for a variety of Δ^{8}- and $\Delta^{8(14)}$-sterols and their derivatives (11,12). Of these, 3β-acetoxycholest-8-ene (**3**) is the key reference compound which greatly facilitated the assignment of the ^{13}C chemical shifts of **1** and **2**. The ^{13}C chemical shifts for **3** and its double bond isomer **4** (3β-

acetoxycholest-8(14)-ene) are also given in Table II.

As seen in Table II, the CMR spectrum of **1** readily confirmed that it possessed a $\Delta^{24(28)}$-bond and two acetoxy groups as were indicated by mass and PMR spectral data. The assignment of the chemical shifts for the $\Delta^{24(28)}$-double bond were made by comparison to those given for limonene (13). Although the ^{13}C chemical shift of C-8 in **1** was the same as in **4** and slightly unfield from that in **2** and **3**, no peak near 142 ppm was present in the CMR spectrum of **1**. Hence, the second double bond in **1** was Δ^8 and not $\Delta^{8(14)}$. The upfield shift of the C-8 carbon was probably due to a γ shift induced by the second acetoxy at C-6, 11 or 15. As the two latter sites of attachment of the acetoxy group have been ruled out by PMR data (see above), only C-6 remained for consideration.

The C-6 carbon of **2** and **3** absorbed at δ25.2 and 25.3 ppm, respectively, but **1** lacked an absorption in the 24-26 ppm region of the spectrum and exhibited a prominent peak at 70.2 ppm. These data established the position of the second acetoxy group of **1** at C-6.

The configuration of the C-6 acetoxy substituent and the chemical shift assignments for C-4, 5 and 7 are based upon the substituent effects of a ring acetoxy group on ^{13}C shifts of steroids (14). From these data, the β shifts for C-5 and C-7 and γ shift for C-4 were calculated for the two possible configurations of the C-6 acetoxy group. For a C-6α acetoxy group, the values are -5.9, 3.6 and 6.7 ppm for C-4, 5 and 7, respectively, whereas for a C-6β acetoxy, the values are -3.7, 1.1 and 4.7 ppm, respectively. When the former set of substituent effects are added to the chemical shifts of the appropriate carbons of **2**, the shifts for C-4, 5 and 7 are predicted to be 28.1, 44.2 and 33.9 ppm, respectively. Indeed, carbon resonances in the spectrum of **1** were found at these calculated positions. For the case of a C-6β acetoxy group of **1**, the shifts calculated for C-4, 5 and 7 are 30.9 and 41.8 and 31.9 ppm, respectively, and while there is coincidence with two of these values, the third resonance (31.9 ppm) is absent in the spectrum of **1**. The remaining datum supporting the α configuration of the C-6 acetoxy of **1** is the shift of the C-19 carbon. The calculated shift (12,14) of C-19 due to a C-6β acetoxy is ca. 20.5 ppm while that due to this group of the α configuration is 18.7 ppm. The latter value is more consistent with the observed shift of 18.4 ppm.

Additional proof of structure of **1** is given by its relative retention time (RRT) of 2.37 (cholestanyl acetate = 1.00) on an OV-101 glass capillary column at 245 C (5) which was identical to the RRT calculated for **1** (15). Finally, the EI-MS of **1** was similar to that of peniocerol diacetate (3β,6α-diacetoxycholest-8-ene) whose spectrum shows major ions at m/e 426 (M^+ - HOAc), 366 (M^+ - 2HOAc), 351 (M^+ - 2HOAc - CH_3) and 143 (16). The mass spectrum of **1** exhibited major ions at m/e 438, 378, 363 and 143, the first three of these being 12 mass units larger due to the *exo* methylene on the side chain.

Since the *S. cerevisiae* mutant from which this sterol is derived is blocked at $\Delta^8 \rightarrow \Delta^7$ isomerase, this metabolite may be involved in a Δ^5-unsaturation. If this is indeed the case, Δ^5-unsaturation may proceed via hydroxylation at C-5 and/or C-6 since 5α-hydroxyergosterol derivatives have previously been encountered in wild type strains of this yeast and have been shown to proceed in vivo to Δ^5-metabolites (4). This sterol resembles in hydroxylation pattern hydroxysterols which inhibit cholesterol biosyntheses in mammals (17) and is structurally similar to peniocerol, which was isolated from the catus *P. fosterianus* (18). Whether the diol of **1** is a regulator of yeast sterol biosynthesis has yet to be determined.

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Inhibition of Hepatic Lipogenesis by 2-Tetradecylglycidic Acid

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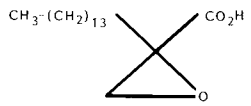
ABSTRACT

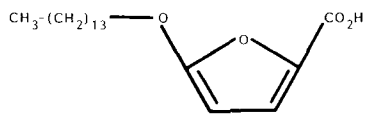
2-Tetradecylglycidic acid (TDGA), a hypoglycemic agent, has been found to be a very effective inhibitor of de novo fatty acid synthesis by isolated hepatocytes. A comparison was made between the effectiveness of TDGA and 5-(tetradecyloxy)-2-furoic acid (TOFA), a hypolipidemic agent, on the metabolic processes of isolated hepatocytes. These compounds are structurally related and both inhibit fatty acid synthesis; however, they have opposite effects from each other on the oxidation and esterification of fatty acids. TDGA inhibits whereas TOFA stimulates fatty acid oxidation. TDGA stimulates whereas TOFA inhibits fatty acid esterification.

INTRODUCTION

Previous studies from this and other laboratories have established that 5-(tetradecyloxy)-2-furoic acid (TOFA) is a potent inhibitor of fatty acid synthesis by the liver (1-5). TOFA is converted to the CoA ester which is an inhibitor of acetyl-CoA carboxylase (5). Tutwiler et al. (6,7) recently demonstrated that 2-tetradecylglycidic acid (TDGA), a compound structurally related to TOFA, is a hypoglycemic agent and a powerful inhibitor of fatty acid oxidation. The structures of the compounds are given below:



2 - Tetradecylglycidic acid



5 (Tetradecyloxy)-2-furoic acid

We became interested in comparing the effects of these compounds on hepatic fatty acid synthesis because, in contrast to TDGA, TOFA is an activator of fatty acid oxidation (1,5,8). It is shown in this study that TOFA and TDGA have almost identical effects on de nova fatty acid synthesis, but do indeed have opposite effects upon fatty acid oxidation and esterification.

MATERIALS AND METHODS

The hepatocytes were prepared from meal-fed female Wistar rats (220-280 g) by the method of Berry and Friend (9) with the modifications described previously (10). The cells were suspended (80 mg wet wt) in 2 ml Krebs-Henseleit buffer supplemented with 2.5% bovine serum albumin (Fraction V, Sigma Co., St. Louis, MO, charcoal-treated and dialyzed) under an atmosphere of 95% O_2, 5% CO_2 in stoppered 25 ml Erlenmeyer flasks. Incubations were conducted in a shaking water bath at 37 C and terminated by adding 0.25 ml of 50% $HClO_4$. Metabolite assays were conducted on KOH-neutralized $HClO_4$ extracts spectrophotometrically by enzymatic methods according to the methods of Hohorst et al. (11) for pyruvate and lactate and Williamson et al. (12) for acetoacetate and β-hydroxybutyrate. Oxidation of fatty acids was assayed by the accumulation of acid-soluble, radioactive products and $^{14}CO_2$ from [1-^{14}C]oleate (13). The rate of fatty acid synthesis, expressed as μmoles of acetate equivalents/min/g wet weight of hepatocytes, was determined by the incorporation of ^3H_2O into total lipid fatty acids as described earlier (10). To determine the extent of [1-^{14}C]oleate esterification, the precipitate from the $HClO_4$ extracts was extracted for total lipids with chloroform-methanol by the method of Kates (14). Lipid classes were separated by thin layer chromatography on Silica Gel G plates with the solvent system of petroleum ether/ether/acetic acid (70:30:1, v/v). The various lipid classes (phospholipids, mono-, di-, and triacylglycerol, cholesterol, free fatty acids, and cholesteryl esters) were located by staining with iodine vapor, scraped from the plates into scintillation vials, and counted for radioactivity with Aquasol 2 (New England Nuclear, Boston, MA) as the scintillation fluid. Results are reported in terms of the sum of the total μmoles of [1-^{14}C]oleate incorporated into mono-, di-, and triacylglycerol plus phospholipids.

TOFA (RMI-14514) was a gift from Dr. Alfred Richardson, Jr. of Merrill-National Laboratories, Cincinnati, OH. TDGA (McN-3802) was a gift from Dr. Gene F. Tutwiler of McNeil Laboratories, Washington, PA. The

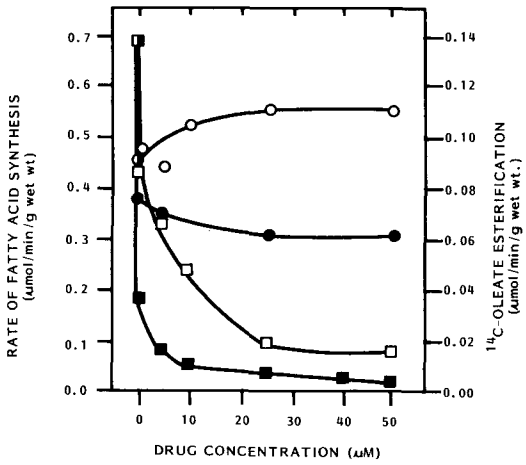


FIG. 1. Effect of TOFA and TDGA upon de novo fatty acid synthesis and [1-^{14}C] oleate esterification by isolated hepatocytes. Squares refer to fatty acid synthesis (measured by $^{3}H_2O$ incorporation between 30 and 60 min of incubation) and circles to esterification (measured by [1-^{14}C] oleate incorporation into esterified lipid fractions between 0 and 45 min of incubation); open symbols to TDGA; closed to TOFA; and half closed to control values without either drug. [1-^{14}C] Oleate at 0.5 mM initial concentration was present for the determination of esterification but was absent when $^{3}H_2O$ was present to measure fatty acid synthesis. Experimental data are reported for one hepatocyte preparation. However, the results have been reproduced with essentially the same results with another hepatocyte preparation.

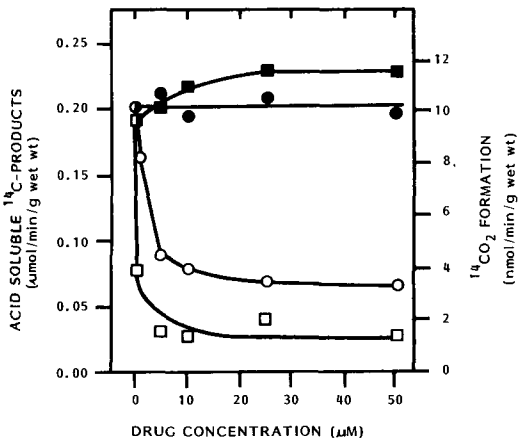


FIG. 2. Effect of TOFA and TDGA upon the oxidation of [1-^{14}C] oleate to acid-soluble, radioactive products and $^{14}CO_2$. Squares refer to the accumulation of acid-soluble, radioactive products and circles to $^{14}CO_2$; open symbols to TDGA; closed to TOFA; and half closed to control values without either drug. [1-^{14}C] Oleate was present in all flasks at an initial concentration of 0.5 mM. Incubations were for 45 min. As with Figure 1, the results of this experiment have been reproduced with another hepatocyte preparation.

compounds were added to the incubation flasks by the procedure described by Panek et al. (1).

RESULTS AND DISCUSSION

Both TOFA and TDGA are potent inhibitors of fatty acid synthesis by isolated hepatocytes (Fig. 1). TOFA is much more effective than TDGA. However, compared to other known inhibitors of fatty acid synthesis, TDGA is still one of the most effective synthetic compounds ever found to inhibit fatty acid synthesis. For example, TDGA is effective at lower concentrations than (-)hydroxycitrate or β-cyano-4-hydroxycinnamic acid (3). In studies reported previously from this laboratory (4,5), TOFA was demonstrated to cause an inhibition of net glucose utilization as well as lactate plus pyruvate accumulation, indicating an inhibition of glycolysis. In results not shown, TDGA did not have this effect upon glycolytic activity.

TOFA and TDGA have similar effects on fatty acid synthesis by isolated hepatocytes but have opposite effects upon the esterification of [1-^{14}C] oleate (Fig. 1). Although neither effect is as dramatic as that on de novo fatty acid synthesis, TOFA inhibits whereas TDGA stimulates esterification. Benito and Williamson (15) have also shown a slight inhibition of esterification of 1 mM oleate in the presence of TOFA.

TOFA and TDGA also have opposite effects upon the conversion of [1-^{14}C] oleate to acid-soluble, radioactive products (mainly ketone bodies) and $^{14}CO_2$ (Fig. 2). As shown in studies conducted previously in this laboratory (4,5), TOFA causes a significant stimulation of [1-^{14}C] oleate oxidation to acid-soluble, radioactive products but has no effect upon $^{14}CO_2$ formation. As expected from the studies of Tutwiler et al. (6), TDGA is a very potent inhibitor of [1-^{14}C] oleate oxidation to both acid-soluble, radioactive products and $^{14}CO_2$. In studies not shown but carried out under the same conditions described in Figure 2, TOFA was found to cause a significant increase in the formation of acetoacetate and β-hydroxybutyrate, whereas TDGA was a very effective inhibitor of the accumulation of ketone bodies. Thus, TOFA is a stimulator of fatty acid oxidation and an inhibitor of fatty acid synthesis and esterification, whereas TDGA is an inhibitor of fatty acid oxidation and synthesis and a stimulator of fatty acid esterification.

It is of considerable interest that these two compounds, although structurally related, have opposite effects upon fatty acid oxidation and

esterification but the same effect upon fatty acid synthesis. Elucidation of the mechanisms responsible for the effects of these compounds may be of some importance to our understanding of the regulation of fatty acid metabolism. Presumably, multiple enzymatic steps with differing inhibitor constants for these compounds or derivatives of these compounds are involved. It is known that TOFA is converted to its CoA ester (4,5) and that inhibition of acetyl-CoA carboxylase by TOFyl-CoA is probably responsible for inhibition of fatty acid synthesis by TOFA (5). Experiments are in progress to determine whether TDGA can be converted enzymatically to the CoA ester and whether TDGA and its CoA ester are inhibitors of acetyl-CoA carboxylase. Another possibility under investigation is that inhibition of fatty acid oxidation by TDGA causes the accumulation of long chain acyl CoA esters which inhibit acetyl-CoA carboxylase. Although several possibilities must be explored, it seems most likely that both compounds bring about an inhibition at the level of acetyl-CoA carboxylase but only TDGA or one of its metabolites inhibits a step of the fatty acid oxidation pathway. Since TDGA inhibits both fatty acid oxidation and fatty acid synthesis, it may, in contrast to TOFA, turn out to be both a hypolipidemic as well as a hypoglycemic agent.

ACKNOWLEDGMENTS

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Effect of Serum Lipoproteins of Bile Obstructed Rats on 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Perfused Rat Liver

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ABSTRACT

Total lipoproteins as well as fractionated VLDL + LDL and HDL from fasted control rats and bile-ligated rats were tested in liver perfusion for their effect on 3-hydroxy-3-methylglutaryl CoA reductase activity in normal rat livers. The total lipoproteins of bile-obstructed rats had 3 times greater capacity to increase 3-hydroxy-3-methylglutaryl CoA reductase activity than that of the control total lipoproteins. When the fractionated lipoproteins were tested from fasted control rats, it was found that the major stimulating activity was in the HDL fraction with minor activity in the VLDL + LDL fraction. When these plasma components isolated from fasted bile-ligated rats were tested, it was found that the major activity had shifted to the VLDL + LDL fraction with the HDL having only a minor stimulatory role. The possible mechanism of action of the abnormal lipoproteins associated with bile obstruction in regulating 3-hydroxy-3-methylglutaryl CoA reductase activity is discussed.

INTRODUCTION

It has been known for a long time that the ingestion of cholesterol results in a reduction of hepatic cholesterol synthesis. This feedback mechanism is believed to function by controlling the amount of the rate-limiting enzyme in cholesterol synthesis, 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase. Although it has recently been demonstrated that chylomicron remnants have the capacity to effect feedback inhibition (1-4) of hepatic cholesterol synthesis, it is quite possible that the liver is also governed by alternative regulatory mechanisms (5).

It has been known for many years that obstruction of the biliary tract is accompanied by hypercholesteremia (6-8). In 1954 Frederickson et al. (9) demonstrated that, following bile ligation in rats, cholesterol synthesis in the liver was elevated despite high plasma cholesterol levels. Studies from this laboratory (10) have shown that blood from bile-obstructed rats had the ability to stimulate cholesterogenesis in normal perfused rat livers. Since it has been demonstrated both in animals and humans (11-14) that abnormal serum lipoproteins are produced following bile ligation, the present study was undertaken to determine whether the potentiator of cholesterol synthesis of the blood of bile-obstructed animals was associated with the lipoprotein fraction and in which individual group of lipoproteins it was contained.

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MATERIALS AND METHODS

Liver Perfusion

The experimental model used in these studies was the isolated perfused liver technique previously described by our laboratory (10). All animals used as liver donors in the study were nonfasted, male, Sprague-Dawley albino rats weighing ca. 250 g. The animals were housed under controlled lighting conditions (light 7 AM to 6 PM; dark 6 PM to 7 AM) for at least a week prior to the perfusion experiments which commenced at 9 AM. Under light ether anesthesia prior to removing the liver from the animal, the portal vein was cannulated, the inferior vena cava severed and the liver perfused with oxygenated Krebs-Ringers phosphate buffer, pH 7.4. This step shortened the anoxic period of the liver during the preparative procedures. The caudate lobe was removed, iced, and used to obtain preperfusion levels of HMG CoA reductase activity. Microsomal HMG CoA reductase was determined according to the method of Goodwin and Margolis (15). Following a 3 hr perfusion period, HMG CoA reductase activity was measured again in the left lateral lobe of the liver. Microsomal protein was measured by the method of Lowry et al. (16).

Bile Duct Ligation

The animals were anesthetized with pentobarbital and bile duct ligation conducted under sterile surgical conditions. The bile duct was tied off in two positions with silk ligatures as close to the liver as possible and the duct

severed between the ligatures. Control rats were sham operated, but the bile ducts were left intact. Since it was found early in the study that bile-obstructed rats would not eat for at least 48 hr following surgery, both the control and experimental animals were only given water after surgery. After 48 hr, the animals were anesthetized with ether and exsanguinated.

Preparation of Serum Lipoproteins

When total lipoproteins were to be studied, the sera from both bile duct ligated and control rats were adjusted to 1.20 g/ml and total lipoproteins isolated by ultracentrifugation according to Havel et al. (17). The infranate (lipoprotein-free serum) from the lipoprotein isolation was saved and used for baseline perfusion experiments.

Since in fasted, normal rats there is little low density lipoprotein available for harvesting, the very low and low density lipoproteins could not be studied separately. Therefore, when fractionated lipoproteins were to be studied, VLDL + LDL ($d < 1.063$), HDL ($d < 1.20$) and lipoprotein-free infranate ($d > 1.20$) were separated from the same serum sample according to the method of Havel et al. (17).

Following the isolation of the total lipoproteins and the various lipoprotein fractions, all samples were dialyzed exhaustively against Krebs-Ringer phosphate buffer (pH 7.4) containing 0.01% EDTA. Total cholesterol was determined by the method of Zak (18).

Perfusate Preparation

The sera of the control rats had a total cholesterol concentration of ca. 100 mg/dl. Sera from bile-obstructed rats contained ca. 3 times as much total cholesterol. In the perfusion experiments, the perfusates had a volume of 100 ml but were fabricated to contain the various serum components as listed below. These perfusates contained 25% fresh, washed beef red blood cells and were used to perfuse livers from nonfasted, male 250 g rats.

Total lipoprotein perfusates. To 25 ml of washed red blood cells was added the total lipoprotein ($d = 1.20$ g/ml) isolated from 50 ml of plasma of either control or bile-obstructed rats. This mixture was diluted to 100 ml with a 3% solution of bovine serum albumin (Cohn's Fraction V). The perfusates containing lipoproteins from control rats contained ca. 50 mg cholesterol per 100 ml and those from bile-obstructed rats contained ca. 150 mg cholesterol per 100 ml.

VLDL+LDL perfusates. To 25 ml of washed red blood cells was added VLDL + LDL ($d <$ 1.063 g/ml) containing 16.5 mg of total cholesterol from either control or bile-obstructed rats. This amount of VLDL + LDL was found to be the amount in 50 ml of normal rat serum and was comparable to that reported by Havel et al. (17). This mixture was diluted to 100 ml with a 3% solution of bovine serum albumin (Cohn's Fraction V).

HDL (High Density Lipoprotein) Perfusates. To 25 ml of washed red blood cells was added high density lipoproteins ($d < 1.20$ g/ml) containing 33.5 mg of total cholesterol from either control or bile-obstructed rats. This amount of high density lipoprotein was determined to be that amount found in 50 ml of normal rat serum and was comparable to that reported by Havel et al. (17). This mixture was diluted to 100 ml with a 3% solution of bovine serum albumin (Cohn's Fraction V).

Infranate (lipoprotein-free serum) perfusates. To 25 ml of washed red blood cells was added 75 ml of infranate from either total lipoprotein or lipoprotein fraction isolation. This type of perfusate was used in the baseline experiments since it was found by Breslow et al. (19) to neither alter significantly hepatocyte HMG CoA reductase activity nor to effect the regulation of cholesterol synthesis.

Pre- and postperfusion HMG CoA reductase activity was expressed as nmoles of mevalonate formation/min/mg microsomal protein. The percent increase in activity from preperfusion to postperfusion time was calculated according to the formula:

$$\frac{\text{Postperfusion Activity} - \text{Preperfusion Activity}}{\text{Preperfusion Activity}} \times 100.$$

RESULTS

Table I demonstrates that the fasting lipoprotein-free sera (infranates) from either control animals or bile-obstructed animals did not significantly stimulate HMG CoA reductase activity. However, the total lipoprotein fraction of 50 ml of serum from control rats induced HMG CoA reductase activity. The total lipoprotein fraction from bile-obstructed animals increased HMG CoA reductase activity by 3-fold more than did the total lipoproteins from control animals despite the presence of 3 times the amount of cholesterol than was present in the control lipoproteins.

Since in the above experiments it was shown that the total lipoproteins of bile-obstructed animals had an increased capacity to increase HMG CoA reductase activity, experiments were conducted to determine which lipoprotein fraction contained the capacity for enhancing HMG CoA reductase activity.

TABLE I

Influence of Total Lipoproteins of Control and Bile-Obstructed Rats on 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Perfused Rat Liver[a]

Perfusate	HMG CoA Reductase (μmole/mg per min.)			
	Preperfusion	Postperfusion	Pre vs. Post[b]	Mean % increase
Control lipoprotein-free serum (n = 5)	0.19 ± 0.03	0.21 ± 0.03	NS	4.06 ± 1.83
Control total lipoprotein (n = 4)	0.18 ± 0.03	0.25 ± 0.05	$p < 0.05$	34.5 ± 11.8
Bile-obstructed lipoprotein-free serum (n = 3)	0.21 ± 0.02	0.25 ± 0.07	NS	17.5 ± 10.4
Bile-obstructed total lipoprotein (n = 3)	0.21 ± 0.03	0.64 ± 0.14	$p < 0.02$	197.0 ± 37.4

[a]Mean ± SEM.

[b]Paired t test; NS, Not Significant.

TABLE II

Effect of Low Density and High Density Lipoproteins of Control and Bile-Obstructed Rats on Hepatic 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase[a]

Perfusate	HMG CoA Reductase (μmole/mg per min.)			
	Preperfusion	Postperfusion	Pre vs. Post[b]	Mean % increase
Control infranate (n = 5)	0.19 ± 0.03	0.21 ± 0.03	NS	4.06 ± 1.83
Control VLDL + LDL (n = 3)	0.22 ± 0.01	0.41 ± 0.06	$p < 0.05$	82.6 ± 23.8
Control HDL (n = 6)	0.27 ± 0.02	0.98 ± 0.15	$p < 0.001$	259.3 ± 60.7
Bile-obstructed infranate (n = 3)	0.21 ± 0.02	0.25 ± 0.07	NS	17.6 ± 10.4
Bile-obstructed VLDL + LDL (n = 7)	0.17 ± 0.02	0.67 ± 0.07	$p < 0.001$	352.0 ± 65.9
Bile-obstructed HDL (n = 3)	0.25 ± 0.01	0.40 ± 0.01	$p < 0.001$	59.6 ± 8.0

[a]Mean ± SEM.

[b]Paired t test: NS, Not Significant.

As shown in Table II, control VLDL + LDL containing 16.5 mg cholesterol (representing 33% of normal total lipoprotein cholesterol) significantly stimulated HMG CoA reductase. However, the control high density lipoproteins which contained 33.5 mg cholesterol (representing 67% of normal total cholesterol) had a stimulatory capacity 2.5 times greater than that of the VLDL + LDL fraction.

Following bile obstruction, the majority of plasma cholesterol was found in the VLDL + LDL fraction of the rat. However, when isolated lipoproteins from bile-obstructed rats were added to the perfusate on the basis of the same amount of cholesterol used in the control experiments, an interesting observation was made. The VLDL + LDL fraction of bile obstruction stimulated HMG CoA reductase activity 4-fold over that obtained by control VLDL + LDL. High density lipoproteins of bile obstruction showed only one-fourth the stimulatory activity when compared to control HDL.

DISCUSSION

Cholestasis is known to produce elevated levels of plasma cholesterol in humans (6,7). Chanutin and Ludewig (8) first demonstrated in animals that bile ligation produced elevated blood cholesterol levels. Although several mechanisms have been proposed to explain the increase in plasma cholesterol following bile obstruction (20), enhanced hepatic cholesterol synthesis appears to be the major mechanism (9).

To date no one has identified the determinant in bile obstruction responsible for the stimulation of cholesterol synthesis. Ferris et al. (10) have shown that blood obtained from bile-obstructed rats stimulated cholesterol synthesis in normal perfused rat liver.

Several recent studies have suggested that circulating plasma lipoproteins may be important components in the regulation of cholesterogenesis. In the human fibroblast system (21), it has been shown that VLDL and LDL from human plasma inhibit HMG CoA reductase activity, while HDL has no effect. In liver tissue, however, the situation is entirely different. Edwards (22) has reported that rat lipoprotein fraction VLDL + LDL or HDL increased HMG CoA reductase activity in rat hepatocytes in a 3 hr period. Breslow et al. (19) demonstrated that human and rat serum VLDL and HDL fractions stimulated activity of HMG CoA reductase in rat liver cell cultures while the LDL fraction was ineffective. Jakoi and Quarfordt (5) have shown that infusion of low and high density lipoproteins from a human source influenced cholesterol synthesis and HMG CoA reductase activity in intact rats.

The data in Table I show that total lipoproteins from bile-ligated animals stimulated HMG CoA reductase more than 4 times that obtained with total lipoproteins from control animals. This increased enzyme activity occurred despite 3 times as much cholesterol in the added lipoprotein fraction. Since perfusion with lipoprotein-free serum from these animals did not increase HMG CoA reductase activity, it suggests that the potentiator of cholesterol synthesis resides in the total lipoprotein fraction. These data are consistent with the observations of Ferris et al. (10) who described increased cholesterol synthesis in normal livers by perfusing with whole blood from bile duct ligated rats.

The data in Table II demonstrate that the cholesterol synthesis stimulating ability of the plasma from bile-obstructed rats resides within the VLDL + LDL fraction. This is a complete reversal of the results obtained with control lipoproteins. The control data is consistent with that shown by Edwards (22) and Breslow et al. (19) in hepatocytes, in which the HDL was the fraction holding the major stimulatory ability with the VLDL + LDL fraction containing only one-third of this activity.

Comparison of the data in Tables I and II discloses that fractionated lipoproteins from control and bile duct-ligated animals increase HMG CoA reductase activity more than do total lipoproteins in either instance. A partial explanation for these results may be found in the work of Mahley and Innerarity (23) who demonstrated a competition between low and high density lipoproteins for surface receptor sites on the cell (fibroblasts).

The relative ratios of cholesterol to phospholipid in lipoproteins and apoprotein moieties are also believed to be important in regulating cholesterol synthesis in cells (5,24). These factors may also account for the differences seen in HMG CoA reductase stimulating activity between total lipoproteins and fractionated lipoproteins since as shown by Havel et al. (17), the quantities of apoproteins and the ratios of cholesterol to phospholipid between these entities undoubtedly varied widely.

Following bile obstruction, several abnormal lipoproteins appear which differ chemically from their normal counterparts. For example, Lipoprotein X, the major abnormal lipoprotein to emerge in humans and rats in cholestasis (25,26) contains different amounts of free cholesterol, cholesteryl esters, phospholipid and an entirely different protein than normal LDL along with which it is isolated by ultracentrifugal floatation techniques (27). It is a distinct possibility that the increased hepatic HMG CoA reductase activity and cholesterol synthesis seen following bile ligation may be a direct result of these molecular changes.

These results demonstrate that under normal conditions the fasting plasma lipoproteins may play a role in regulating HMG CoA reductase activity in the liver. Following bile obstruction, the abnormal lipoproteins enhance the activity of HMG CoA reductase possibly by disturbing the normal regulatory balance at the level of the receptor sites.

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Serum Lipids in Suckling and Post-Weanling Iron-Deficient Rats

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ABSTRACT

Serum lipids were studied in iron-deficient and control rats during suckling and after weaning at 21, 30, and 60 days of age. Diets providing 5 or 307 ppm iron were fed to dams and their offspring during gestation, lactation, and after weaning. Rats on the deficient diet throughout the experimental period developed a hyperlipidemia characterized by elevated triglycerides, cholesterol, and phospholipids which was present at 21, 30, and 60 days. Control pups weaned to the deficient diet developed anemia at 30 days of age and hypertriglyceridemia at 60 days of age. Repletion of deficient rats with iron after weaning caused a rapid decline in serum lipid levels after only 9 days on the control diet. The hyperlipidemia of iron deficiency thus appears to be reversible with iron supplementation. The time required to develop hypertriglyceridemia in iron deficiency is longer postweaning than during suckling.

INTRODUCTION

Elevated concentrations of serum lipids have been associated with dietary iron deficiency in rats and chicks (1-4). Previous reports from this investigator have found abnormal lipid metabolism in 18-day-old offspring of rats fed iron-deficient diets during pregnancy and lactation (1,2). Hyperlipidemia characterized by significant increases in the triglyceride, cholesterol, and phospholipid fractions of the serum was reported in pups of dams fed the deficient diet containing 5 ppm iron throughout gestation and lactation (1). Sera from pups of dams fed the control diet (307 ppm iron) during either gestation and/or lactation and dams fed both diets had normal lipid concentrations. The elevated serum lipids were observed only in pups whose dams were fed the lowest concentration of iron (5 ppm). Although feeding 29 ppm iron resulted in anemia, it was not associated with hyperlipidemia (2).

Subsequent experimentation has investigated the mechanisms which may be involved in the etiology of this hyperlipidemia. Since increases in serum lipids occurred in the offspring and not in the maternal organism, lipids were measured in milk of control and deficient dams to determine if iron-deficient pups had greater dietary fat levels. No differences were found in this exogenous source of lipids. Lipoprotein lipase was determined in the post-heptarin plasma of pups, and no significant differences were found between control and deficient pups. This indicated that decreased clearance of dietary lipids from the blood was not responsible for the hyperlipidemia. Production of triglycerides in vitro from [U-^{14}C]glucose was found to be significantly higher in liver slices of iron-deficient pups than in controls. Apparently, during iron deficiency, this increased endogenous lipid pool may contribute to the increased serum triglyceride concentrations (2). Lipogenesis has also been studied in iron-deficient adult rats which were not hyperlipidemic (5). Triglyceride synthesis from [$^{3}H_2O$] and [U-^{14}C]glucose in adipose tissue was two to three times greater in iron-deficient rats than in controls. Amine et al. found that iron-deficient rats had increased incorporation of ^{14}C-glucose into intestinal lipids than did controls and reported that the lipid synthesis increased as the level of dietary fats increased (6). The present study was initiated to investigate the effects of iron restriction during the prenatal and suckling and/or postweanling periods on serum lipid levels later in life. Pups of iron-deficient dams were repleted with iron after weaning and control pups were depleted in iron after weaning to compare the effects of iron deficiency on serum lipid levels during these two stages in the life cycle.

MATERIALS AND METHODS

Experimental Design

Nulliparous Sprague-Dawley CD rats (Charles River, Wilmington, MA) were obtained at a weight of 180 g and maintained on a cereal-based stock diet (Purina Rat Chow) until they weighed ca. 270 g. They were then bred and fed ad libitum either the iron-deficient diet (5 ppm iron) or the control diet (307 ppm iron) (Table I) (n=16 per group) and glass-distilled water from the first day of pregnancy until the pups were weaned. On the day following parturition, litters were adjusted to contain six female pups. Food was placed in the cage to limit access to the dams and the iron-free bedding in the solid bottom "maternity" cages was changed frequently. On day 21 the pups were weaned to either the same or opposite diet as that fed to their dams and

TABLE I

Composition of Diets

	307 ppm iron[1] %	5 ppm iron %
Casein[2]	22.00	22.00
Sucrose	29.70	29.76
Cornstarch	29.70	29.76
Iron-free salt mix[3]	5.48	5.48
Vitamin mix[4]	1.00	1.00
Corn oil[5]	10.00	10.00
Cellulose[6]	2.00	2.00

[1]Iron levels determined by atomic absorption.

[2]Vitamin-free casein, Teklad, Chagrin Falls, OH.

[3]The levels of minerals used is at least 12.5% more than NRC recommendations for lactation (7). Composition of salt mixture (mg/100 g diet): $CaCO_3$, 1,680.000; $CoCl_2 \cdot 6\ H_2O$, 0.100; $CuSO_4 \cdot 5\ H_2O$, 9.800; $MgSO_4 \cdot 7\ H_2O$, 278.500; $MnSO_4 \cdot H_2O$, 19.000; KI, 0.022; NaCl, 1,000.000; K_2HPO_4, 2,483.600; $ZnCl_2$, 3.000. $FeSO_4 \cdot 7\ H_2O$ was added to the supplemented diet in place of sucrose.

[4]Teklad, Chagrin Falls, OH, supplies in g/kg of diet; *p*-aminobenzoic acid, 0.110132; ascorbic acid, 0.9912; biotin, 0.000441; vitamin B_{12}, 0.0000297; calcium pantothenate, 0.066079; choline, 1.4337; folic acid, 0.001982; inositol, 0.110132; menadione (vitamin K_3), 0.049559; niacin, 0.099119; pyridoxine HCl, 0.022026; riboflavin, 0.022026; thiamin HCl, 0.022026; supplies in units per kg of diet: dry retinyl palmitate, 19,824; dry ergocalcifrol 2.202.5; dry tocopheryl acetate, 121.15; corn starch, 4.666878 g.

[5]Mazola corn oil, Best Foods, Englewood Cliffs, NJ.

[6]Alphacel nonnutritive cellulose, Teklad, with iron extracted by the method of Houk et al. (8).

placed in individual stainless steel screen bottom cages. There were four different experimental treatments depending on the dam's diet during pregnancy and lactation and the diet fed to weanlings after day 21. Control-Control (CC) rats were born to control dams and weaned to the control diet, CD (Control-Deficient) rats were born to control dams and weaned to the iron-deficient diet, DC (Deficient-Control) rats were repleted with iron after weaning, DD (Deficient-Deficient) rats were born to iron-deficient dams and weaned to iron-deficient diets (n=8-14 litters per treatment). Diets and glass-distilled water were offered ad libitum and weekly food intakes and body weight records were kept.

Littermates were fasted for 4 hr on days 21, 30 and 60 and tail blood samples were taken for hemoglobin and hematocrit determinations (9). After brief exposure to chloroform anesthesia, blood was collected by cardiac puncture, serum removed and frozen for analysis of lipids.

Lipids

Serum triglycerides, cholesterol, cholesteryl ester and phospholipids were determined. Lipids were extracted from serum samples by the method of Folch et al. (10) and separated on thin layer chromatography plates developed in a solvent system of petroleum ether/ethyl ether/acetic acid (70:30:1, v/v/v). Following exposure to iodine vapors, lipid fractions were identified by comparison with triolein, cholesterol, cholesteryl linoleate, and lecithin standards which were run simultaneously. The spots were recovered and the following methods were used to quantitate the lipid fractions: triglyceride concentration was determined by the method of Stern and Shapiro (11) nonesterified cholesterol and cholesteryl esters were measured by the method of Searcy and Bergquist (12) and phospholipid concentration was determined by measuring phosphorus in the lipids remaining at the origin using the method of Chen et al. (13).

Statistical Methods

Differences between the two groups at 21 days of age were determined with the Student's t test. Analysis of variance was used to determine differences among group means when four experimental groups were compared (days 30 and 60). Duncan's multiple range test was used to find the means which differed significantly (14).

RESULTS

Twenty-one-day-old pups of iron-deficient dams had lower body weights, hemoglobin and hematocrit levels (Table II) and higher levels of serum cholesteryl ester, cholesterol, triglycerides, and phospholipids (Table III) than did pups of control dams. The elevations in cholesteryl ester and cholesterol were approximately 2-fold, triglycerides were 4-fold and phospholipids were elevated 3-fold. The iron content of the weaning diet had dramatic effects on body weight, hematological values, and serum lipid levels as early as 30 days of age. Weaning deficient pups to the control diet (DC) increased body weight, hemoglobin, and hematocrit to control levels after only 9 days on the diet. Serum cholesteryl ester, triglycerides, and phospholipids returned to control levels at this time also. At 30 days old, serum cholesterol remained somewhat higher in DC rats than in CC rats. Pups weaned to the deficient diet from the control diet (CD) had lower levels of hemoglobin and hematocrits than CC rats but not as low as DD rats which had been exposed to the iron-deficient diet throughout the

TABLE II

Body Weight, Hemoglobin Levels, and Hematocrits of Rats 21, 30 and 60 Days Old

	21 Days			30 Days					60 Days				
	C (n=15)	D[1] (n=10)	Significance	CC (n=13)	CD (n=12)	DC (n=8)	DD (n=9)	Significance	CC (n=14)	CD (n=13)	DC (n=8)	DD (n=9)	Significance
Body weight (g)	52.3[2a] ± 1.9	40.1[b] ± 3.3	p<0.005	92.6[a] ± 5.3	94.0[a] ± 4.8	78.8[a] ± 7.1	58.9[b] ± 3.9	p<0.001	206.1[a] ± 10.7	189.5[a] ± 11.7	206.3[a] ± 10.0	107.1[b] ± 7.4	p<0.001
Hemoglobin (g/dl)	11.1[a] ± 0.5	4.1[b] ± 0.5	p<0.001	12.9[a] ± 0.3	9.1[b] ± 0.6	11.7[a] ± 0.3	3.4[c] ± 0.2	p<0.001	16.5[a] ± 0.4	6.1[b] ± 0.5	16.4[a] ± 0.3	3.0[c] ± 0.7	p<0.001
Hematocrit (%)	39.7[a] ± 1.4	15.7[b] ± 1.9	p<0.001	45.2[a] ± 0.6	31.5[b] ± 1.5	43.5[a] ± 0.7	17.5[c] ± 1.5	p<0.001	49.4[a] ± 0.6	26.1[b] ± 1.3	50.0[a] ± 0.7	12.3[c] ± 0.8	p<0.001

[1]C=control; D=deficient; CC=control during gestation, lactation, and post-weaning; CD=control during gestation/lactation, deficient after weaning; DC=deficient during gestation/lactation, control after weaning; DD=deficient during gestation/lactation and after weaning. Number in parentheses equals number of rats in group.

[2]Mean ± SE within each age group, means followed by different superscripts are significantly different as determined by Student's t test (21 day) or analysis of variance (30 and 60 day).

TABLE III

Serum Lipid Concentration (mg/dl)

	21 Days			30 Days					60 Days				
	C (n=9)	D[1] (n=9)	Significance	CC (n=7)	CD (n=9)	DC (n=6)	DD (n=6)	Significance	CC (n=10)	CD (n=10)	DC (n=8)	DD (n=7)	Significance
Cholesteryl ester	113.1[a2] ± 23.7	215.2[b] ± 22.4	p<0.01[2]	116.8 ± 21.7	83.9 ± 11.1	87.0 ± 16.1	95.4 ± 6.6	NS	93.2 ± 12.6	60.7 ± 9.0	84.4 ± 8.0	69.3 ± 6.3	NS
Cholesterol	54.0[a] ± 14.2	125.9[b] ± 23.3	p<0.02	29.9[a] ± 3.1	48.1[ab] ± 7.8	46.7[ab] ± 15.0	66.3[b] ± 6.3	p<0.001	33.8[a] ± 4.3	34.9[a] ± 5.0	34.6[a] ± 3.0	77.7[b] ± 13.7	p<0.001
Triglyceride	89.9[a] ± 22.8	405.3[b] ± 99.5	p<0.01	63.5[a] ± 12.1	28.9[a] ± 4.9	41.5[a] ± 9.7	328.5[b] ± 80.4	p<0.01	48.9[a] ± 8.9	163.0[b] ± 48.3	60.3[a] ± 15.1	309.0[b] ± 78.6	p<0.01
Phospholipids	29.9[a] ± 6.7	101.4[b] ± 11.4	p<0.001	32.0[a] ± 2.8	29.0[a] ± 3.8	28.0[a] ± 3.7	51.8[b] ± 5.6	p<0.002	37.8[a] ± 5.6	48.6[a] ± 5.4	31.1[a] ± 6.4	68.0[b] ± 8.1	p<0.002

[1]C=control; D=deficient; CC=control during gestation, lactation, and postweaning; CD=control during gestation-lactation, deficient after weaning; DC=deficient during gestation-lactation, control after weaning; DD=deficient during gestation-lactation and after weaning. Number in parentheses equals number of rats in group.

[2]Mean ± SE within each age group; means followed by different superscripts are significantly different as determined by Student's t test (21 day) or analysis of variance (30 and 60 day).

experiment. No significant elevations in serum lipids were seen in the CD rats at 30 days of age, although cholesterol concentrations were slightly elevated relative to the CC rats. The DD rats continued to have elevated serum cholesterol, triglycerides, and phospholipids after weaning.

At 60 days of age, body weights were similar in CC, CD, and DC rats, and significantly lower in DD rats (Table II). Hematological values (Table II) in CC and DC rats were the same, CD rats had hemoglobin levels and hematocrits approximately one-half of the control rats. Hemoglobin levels and hematocrits in DD rats were significanlty lower than those of all of the other groups. Serum cholesterol, triglycerides and phospholipids continued to be highest in the DD group at 60 days (Table III). Sixty-day-old DC and CC rats had serum lipid concentrations similar to those found in 30-day-old littermates. Rats restricted in iron after weaning (CD) developed a hypertriglyceridemia at 60 days of age. However, serum cholesterol and phospholipids were not significantly elevated in the CD rats at that time.

Initial plans to continue the study longer than 60 days were changed when DD rats had high mortality rates after 60 days of age.

DISCUSSION

The anemia and stunted growth of the suckling iron-deficient rat observed here is similar to and confirms previously reported findings (1,2). This results from iron restriction in the maternal animal during gestation and lactation and from the low concentrations of iron in milk secreted by the iron-deficient dam (2). The hyperlipidemia in the suckling iron-deficient pups may be related to increased endogenous synthesis of lipid in liver (2) and/or to the decreased zinc/copper ratio in the livers of the pups (15).

Rats weaned to the control diet (DC) were repleted with iron rapidly. This was evident after only 9 days on the diet when hemoglobin levels and hematocrit values increased to control values. This was probably due to the increased efficiency of iron absorption in iron-deficient rats (16) and the high level of iron found in the control diet. As the animals were repleted with iron, serum lipids decreased to normal concentrations and remained at normal levels for the duration of the study. Cholesteryl ester, cholesterol, and phospholipid in the serum decreased to approximately one-half of the preweaning levels. At 30 days of age, serum triglyceride concentrations were one-tenth of the levels found in 21-day-old littermates. Apparently the hyperlipidemia of iron-deficiency is reversible when dietary iron is increased and body stores of iron are repleted. No indications of prolonged ill effects of the hyperlipidemia during suckling were observed in DC rats during this experiment. Histological studies of heart, lung, and kidney using light microscopy did not reveal any unusual pathologies in the DC rats.

Although at 30 days of age the rats weaned to the deficient diet from the control diet (CD) were anemic, they were not sufficiently depleted in iron for an elevation in serum lipids to be manifested or for a depression in growth to occur. The hyperlipidemia seen in the 30-day-old DD rats was somewhat different in character than the hyperlipidemia observed during the suckling period. Cholesteryl ester was not elevated, and the levels of cholesterol and phospholipid were lower than in the sera of suckling pups. Serum triglycerides continued to be the lipid fraction most affected in the postweaning DD rats, with concentrations approximately five times greater than those found in control sera. The extent to which serum triglyceride concentrations remained elevated in the DD rats was fairly constant; concentrations at 21, 30, and 60 days did not differ significantly from each other. After 39 days on the iron-deficient diet, the 60-day-old CD rats were anemic and hypertriglyceridemic. Thus, the depression in iron status as reflected by the hematological values precedes the elevation in serum triglycerides by one month.

The results of this study suggest that elevations in serum lipids during iron deficiency occur primarily in the more severely deficient rats. The age of the animal during the deficient period influences both the extent of anemia and the characteristics of the lipemia. The anemia in pups exposed to iron deficiency in utero and during suckling is quite severe as indicated by the blood hemoglobin concentrations and hematocrit values less than half of control levels and is associated with elevations in triglycerides, cholesterol, and phospholipids. When these rats are weaned to the deficient diet (DD), hemoglobin and hematocrit levels remain low and serum lipids remain high. However, serum phospholipid and cholesterol levels are not as high after weaning as during suckling. The lipemia of rats fed the deficient diet after weaning (CD) is limited to the triglyceride fraction and is not as severe as in the DD rats, and is related to a milder anemia than that found in the rats deprived of iron for a longer time period (DD). Amine and Hegsted (3) reported elevated triglycerides and phospholipids in Charles River rats fed low-iron diets for

8 weeks after weaning. The lipemia they reported was observed when the diet contained 15% coconut oil and 1% safflower oil. In the present study, a hypertriglyceridemia was produced in the control-deficient animals fed 10% corn oil and no saturated fat for 5½ weeks after weaning. The relationship between saturation of dietary fat, iron, and serum lipids may be related to differences in genetic strain. In a second study, Amine et al. (6) found that serum triglycerides were affected only by fat in Charles River rats, whereas, in Buffalo rats iron, fat, and an iron-fat interaction affected triglycerides. In the present study using Sprague Dawley rats, hypertriglyceridemia was observed in rats fed iron-deficient diets after weaning. However, when the deficiency was induced in utero cholesterol, triglycerides, and phospholipids were all elevated above control levels.

The hypertriglyceridemia seen in the 60-day-old iron-deficient (DD) and (CD) rats may be due to increased lipogenesis in adipose and liver tissue which has been reported previously in adult iron-deficient rats (5). In a previous study, the increased incorporation of [^3H_2O] and [U-^{14}C]glucose into triglycerides in adipose tissue and increased incorporation of glucose into polar lipids in liver was not accompanied by a hypertriglyceridemia as was found in the 60-day-old deficient rats in the present study. This may be directly related to the more severe anemia seen in the present study (hemoglobin levels of 6.1 ± 0.5 g/dl in CD rats and 3.0 ± 0.7 g/dl in DD rats vs. 7.1 ± 0.4 in deficient rats of the previous study [5]). The postulated sequence of events may be such that anemia is associated with increased lipogenesis in adipose and liver which precedes the appearance of increased concentrations of triglycerides in serum. When the iron deficiency anemia becomes more severe and increased lipogenesis continues, the resultant serum has a higher concentration of triglycerides. The hypertriglyceridemia in iron-deficient adult rats may also be related to a decreased activity in tissue lipoprotein lipase activity as reported by Lewis and Iammarino (4).

The exact mechanism by which iron regulates or functions in lipid metabolism has not yet been established. Conflicting reports of serum lipid levels in anemic human subjects appear in the literature since early in this century. Bloor and MacPherson (17) and Erickson et al. (18) reported elevated lipids in anemic subjects. Others have found hypolipidemia in patients with anemias of various origins (19-22) or no significant relationships between anemia and serum lipids (23). Since hyperlipidemia is recognized as a risk factor in the development of atherosclerotic heart disease, all nutritional influences on serum lipid concentrations assume considerable importance and warrant further study to enable us to more clearly understand the etiology of this disease.

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End Product Specificity of Triacylglycerol Lipases from Intestine, Fat Body, Muscle and Haemolymph of the American Cockroach, *Periplaneta americana* L.

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ABSTRACT

The triacylglycerol-hydrolyzing capacity of tissue homogenates has been investigated for midgut, fat body, thoracic musculature and haemolymph of the American cockroach, *Periplaneta americana*. The greatest lipolytic activity was demonstrated in midgut homogenates with decreasing levels of activity present in fat body, muscle and haemolymph. Comparison of the lipolytic products resulting from triacylglycerol hydrolysis indicates that midgut homogenates effect the production of *sn*-2-monoacylglycerols and free fatty acids, whereas the other tissues that were examined favor the accumulation of diacylglycerols. Stereospecific analysis of the diacylglycerol products of triacylglycerol hydrolysis demonstrated that the lipolytic activities of midgut and muscle homogenates result in the production of a racemic mixture of the *sn*-1,2- and *sn*-2,3-enantiomers, but the fat body and haemolymph show a preference for the accumulation of the *sn*-1,2-isomer.

INTRODUCTION

A capacity to hydrolyze long chain acylglycerols has been reported for a number of insect tissues but few data are available to describe the substrate and end product specificity of the various lipases (1). The preferential cleavage of fatty acids located at the *sn*-1 and *sn*-3 positions of triacyl-*sn*-glycerols by the midgut lipase of *Periplaneta americana* has been reported (2) but, as the substrate employed in this study was 1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol, no distinction could be made between positional and fatty acid specificity. The midgut lipase of *Locusta migratoria* hydrolyzes trioleoylglycerol more readily than tripalmitoylglycerol (3), a property that parallels the preferential cleavage of unsaturated fatty acids that has been reported for mammalian pancreatic lipase (4). Stevenson (5,6) reported low rates of hydrolysis of triacylglycerol and diacylglycerol relative to monoacylglycerol by thoracic musculature of *Prodenia eridania*, and this trend was confirmed by Crabtree and Newsholme (7) who demonstrated in a number of insect species that the rate of acylglycerol hydrolysis by muscle lipases increases in the order triacylglycerol: diacylglycerol: monoacylglycerol. By contrast, the fat body lipase of *P. eridania* displays homogeneity with regard to acylglycerol specificity. Two lipases have been described in the fat body of *L. migratoria*, one of which (alkaline lipase) preferentially hydrolyzes monoacylglycerol, whereas the other (acid lipase) is also strongly active against triacylglycerol and diacylglycerol (8).

The present study was undertaken to determine the triacylglycerol-hydrolyzing activities of four tissues of *P. americana* and thus contribute further to our understanding of lipid metabolism in insects. The report demonstrates that lipases in the haemolymph, fat body, and thoracic musculature favor the accumulation of diacylglycerol from triacylglycerol, whereas the midgut lipase effects a more complete hydrolysis of the substrate. A preliminary report has been published (9).

MATERIALS AND METHODS

Animals

Adult male American cockroaches were taken at between 2 and 3 months after the final adult ecdysis from a colony maintained in this laboratory under standard conditions (10).

Preparation of Homogenates

Experimental tissues were dissected from 16 fed insects following decapitation. Midgut, fat body and thoracic musculature were removed under ice cold Ringer's solution, rinsed thoroughly, pooled and placed in 5.0 ml ice cold buffer for homogenization (midgut, 0.1 M Tris-Maleate, pH 5.0; muscle, fat body, 0.1 M Tris-HCl, pH 7.0). Care was taken to remove adhering fat body and Malpighian tubular tissue from the intestinal tracts. Haemolymph was collected from 32 insects by the centrifugal method of Sternburg and Corrigan (11) and,

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following a second centrifugation at 1700 x g for 10 min to remove haemocytes, was mixed with 0.1 M Tris-HCl buffer, pH 7.0. Tissues were homogenized by hand in a ground glass pestle and mortar, and centrifuged at 1700 x g for 10 min. The supernatant (or infranatant in the case of the fat body) was placed in fresh buffer solution, and final protein concentrations in μg per ml of homogenate buffer were determined by the method of Lowry et al. (12) and found to be: midgut, 877; fat body, 419; muscle, 550; haemolymph, 4499.

Triacylglycerol Emulsions

For assay of midgut lipase activity, a triacylglycerol emulsion was prepared containing 5 ml 0.1 M Tris-Maleate buffer (pH 5.0), 5 mg triolein, 20 μCi [2-(n)^{3}H]-glycerol triolein and 3 mg *sn*-1-oleoyl-3-phosphatidylcholine. For assay of lipolytic activities in muscle, fat body and haemolymph tissues, the emulsion contained 5 ml 0.1 M Tris-HCl buffer (pH 7.0), 20 mg triolein, 80 μCi [2-(n)^{3}H]-glycerol triolein and 12 mg *sn*-1-oleoyl-3-phosphatidylcholine. The ratio of triacylglycerol to lysolecithin was 1:0.6 (wt/wt) as suggested by Morley and Kuksis (13). The emulsions were generated by sonication for 60 seconds in a 30 ml conical centrifuge tube, with a Biosonik III microprobe (Bronwill Scientific Company, Rochester, NY) and used immediately.

Assay of Triacylglycerol Lipase Activity

Prior to the enzyme assays, aliquots of triacylglycerol emulsions (100 or 300 μl) were mixed with (600 μl of) appropriate buffers containing 5% bovine serum albumin (BSA), fatty acid-free. In the case of the midgut tissue, this buffer (400 μl) also contained 1 mM $CaCl_2$. These solutions were vortexed and kept on ice. To commence the reaction, aliquots of haemolymph (100 μl) or tissue homogenate (300 μl) were pipetted into each tube, the tubes capped, their contents mixed rapidly and the samples then placed at 30 C in a Buchler Evapo-mix vortex shaking bath (Buchler Instruments, Fort Lee, NJ) and incubated at medium shaking speed. Reactions were halted by removing the tubes at various times, placing them in ice, and adding 15 ml chloroform/methanol (2:1, v/v).

Analytical Methods

Following extraction of lipids into chloroform/methanol, the nonlipid contaminants were removed by washing with 3 ml 0.9% NaCl. The lower phase was removed, dried over sodium sulphate, transferred to 7-ml vials, and the solvent dried down at 40 C under nitrogen. Lipids were dissolved in 100 μl of chloroform/methanol (2:1, v/v) and the samples immediately applied to thin layer plates coated with a 500 micron layer of Silica Gel G (E. Merck, Darmstadt, Germany) impregnated with 12.5% (by weight) boric acid. The lipid classes were resolved by development in chloroform/acetone (95:5, v/v) according to Thomas et al. (14), visualized by spraying with 2,4-dichlorofluorescein followed by exposure to short wave ultraviolet light. Lipids were identified by their R_f values compared with those of authentic cochromatographed standards and areas corresponding to 1-(3)-monoacylglycerols, 2-monoacylglycerols, 1,2(2,3)-diacylglycerols, 1,3-diacylglycerols, and triacylglycerols scraped into glass scintillation vials. Ten ml Aquasol (New England Nuclear, Boston, MA) cocktail was added, the vials capped and vortexed well, cooled to 4 C and the radioactivity determined by an Isocap 300 liquid scintillation counter (Searle Instrumentation, Don Mills, Ont. Canada). The radioactivity of the labeled glycerol remaining in the aqueous phase was determined by placing 1 ml of this solution in a scintillation vial with 10 ml Aquasol. All cpm data were converted to dpm by the use of the external standard ratio method of quench correction. The results presented represent the mean ± range/2 for two experimental determinations, each analyzed in duplicate.

Materials

[2-(n)^{3}H]-Glycerol triolein (SA: 458 μCi/μ Mol) was purchased from Amersham/Searle Corp. (Don Mills, Ontario). Trioleoylglycerol, dioleoylglycerol, monooleoylglycerol, bovine serum albumin (fatty acid-free), and Tris-Maleate buffer (mono-tris(hydroxymethyl) aminomethane maleate) were obtained from Sigma Chemical Co. (St. Louis, MO). The purity of labeled and unlabeled acyglycerols was checked and found to be in excess of 99.9%. Tris-HCl buffer was prepared with reagent grade hydrochloric acid and Tham (tris(hydroxymethyl)aminomethane). These and all other reagents and solvents were of Fisher Certified Reagent grade, and were used without further purification.

Stereospecific Analysis of Diacylglycerols

Stereospecific analyses of the *sn*-1,2-(2,3)-diacylglycerols were performed by a modification of the method of Brockerhoff (15) as described by Morley and Kuksis (13). Radioactive diacylglycerols purified by borate thin layer chromatography (TLC) were combined with 50 μg of unlabeled *sn*-1-palmitoyl-2-linoleoylglycerol and 200 μg of rac-1,2-(2,3)-

dipentadecanoyl glycerol, prior to their conversion to the phosphatidylphenol derivatives. Further purification of these compounds and removal of any remaining *sn*-1,3-diacylglycerols was accomplished by TLC on Silica Gel H plates in a solvent system of chloroform/methanol/3.5N NH_4OH (80:17.5:2.5, v/v/v) as described by Akesson (16). The purified phenolphosphatides were extracted from the gel, dried down inside 7-ml vials, and 0.2 ml anhydrous diethyl ether along with 2.0 ml of 0.1 M triethylamine buffer (pH 7.5 containing 7 μMol $CaCl_2$ and 2 mg *Crotalus atrox* venom (Sigma Scientific Co., St. Louis, MO) added. The vials were tightly capped, and the samples incubated for 4 hr at 37 C in a Buchler Vortex Evapo-Mix shaker at moderate speed. The digested lipids were extracted by the method of Arvidson (17), the solvent removed and spotted on Silica Gel H thin layer plates. The plates were developed twice: first in a neutral lipid system (heptane/isopropyl ether/acetic acid (60:40:4, v/v/v) to separate the fatty acids; the gel was then scored transversely across the plate just below the fatty acids; then the plates were re-run in a polar lipid system (15) to separate the lyso- and residual phenolphosphatides. Lyso- and residual phenolphosphatides were recovered and their radioactivity measured. The products from the rac-1,2(2,3)-dipentadecanoyl internal standard were quantified by gas liquid chromatography (GLC), and the results used to confirm the thoroughness of the digestion. When necessary, the sample ratios of *sn*-1,2:*sn* 2,3-enantiomers were normalized with respect to the 50:50 ratio for the internal standard.

RESULTS

The relative lipolytic activities of midgut, fat body, muscle and haemolymph were determined by measuring the linear rate of lipolytic product formation during the first few minutes of incubation. The rate at which the triacylglycerol substrate is hydrolyzed by each of the tissue homogenates is demonstrated in Table I. It is evident that the most powerful triacylglycerol lipase(s) occurs in the midgut, whereas the haemolymph and muscle are at least 10 times less active against triacylglycerols.

Rapid hydrolysis of triacylglycerol by midgut lipase(s) is further demonstrated in Figure 1 which illustrates the formation of lipolytic products during a 30-min incubation. Although monoacylglycerol is the primary product during the first few minutes of the incubation, appreciable amounts of glycerol are detected after 5 min, thus indicating that hydrolysis is proceeding to completion. Table II identifies the nature of the end products produced by midgut lipase(s) after 30-min of incubation and shows that at this time 78.17%

TABLE I

Rate of Triacylglycerol Hydrolysis in Midgut, Fat Body, Muscle and Haemolymph of *Periplaneta americana* L.

Tissue	Lipolytic products at T^{1a}		Rate of triacylglycerol hydrolysis (dpm/μg protein/minute)
	Lipid class	Radioactivity (dpm)	
Midgut	Glycerol	26,178 ± 3,798	
	Monoacylglycerol	174,886 ± 29,599	
	Diacylglycerol	117,403± 2,566	
	Total	318,467	605
Fat body	Glycerol	1,826 ± 371	
	Monoacylglycerol	4,923 ± 1,111	
	Diacylglycerol	28,868 ± 3,486	
	Total	35,617	141
Muscle	Glycerol	2,663 ± 874	
	Monoacylglycerol	3,518 ± 1,382	
	Diacylglycerol	12,194 ± 604	
	Total	18,375	56
Haemolymph	Glycerol	842 ± 303	
	Monoacylglycerol	9,947 ± 196	
	Diacylglycerol	36,284 ± 756	
	Total	47,073	52

[a]T^1 = 2 min for midgut, 5 min for haemolymph, fat body, muscle.

of the original triacylglycerol substrate has undergone hydrolysis. The formation of 1,2-(2,3)-diacylglycerols is greater than that of 1,3-diacylglycerol (Table II), and *sn*-2-monoacylglycerol predominates over the 1(3)-isomer.

Diacylglycerol is the primary end-product following incubation of the triacylglycerol substrate with fat body homogenate, with lesser amounts of free glycerol and monoacylglycerol produced during the course of the 60-min incubation. The principle diacylglycerol products are the 1,2(2,3)-isomers, and *sn*-2-monoacylglycerol is the dominant monoacylglycerol (Table II).

The lowest levels of lipolytic activity among the tissues that were examined were found in the muscle and haemolymph, and the lipolytic end-products in incubations containing homogenates of these tissues are indicated in Table II. The major lipolytic product detected in incubations containing haemolymph or muscle homogenates was diacylglycerol which accounted for 69% and 74%, respectively, of the total end-products. Both tissues favor the

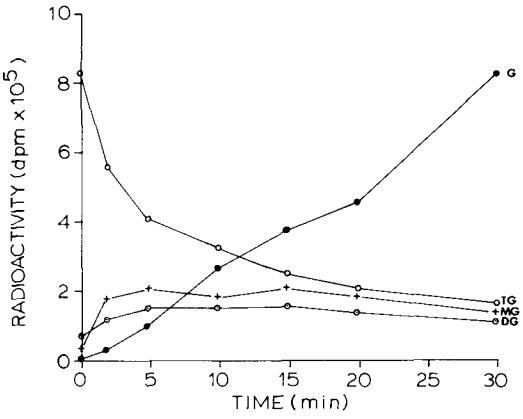

FIG. 1. Hydrolysis of [^{3}H]glycerol triolein by midgut homogenate of the American cockroach, *Periplaneta americana*. Each sample contained 300 µl tissue homogenate, 400 µl Tris-maleate buffer (0.1 mM, pH 5.0) containing 1 mM $CaCl_2$ and 5% BSA, and 300 µl triacylglycerol emulsion containing 1.2 x 10^6 dpm [^{3}H]glycerol triolein. G - glycerol; MG - monoacylglycerols; DG - diacylglycerols; TG - triacylglycerols.

TABLE II

Nature of Lipolytic Products Resulting from Hydrolysis of Triacylglycerol by Midgut, Fat Body, Muscle and Haemolymph of *Periplaneta americana* L.

Tissue	Radioactivity of triacylglycerol at T°[a] (dpm)	Lipolytic products at T[1a]		% of Triacylglycerol hydrolyzed
		Lipid class	Radioactivity (dpm)	
Midgut		1-MG	53,482 ± 3,891	4.44
		2-MG	91,034 ± 14,789	7.56
		1,3-DG	6,633 ± 231	0.55
		1,2-DG	110,712 ± 7,586	9.20
		glycerol	679,356 ± 146,533	56.42
	1204024	Total	948,803	78.17
Fat body		1-MG	2,533 ± 51	0.08
		2-MG	6,186 ± 1,754	0.19
		1,3-DG	3,686 ± 60	0.12
		1,2-DG	108,186 ± 5,374	3.40
		glycerol	39,866 ± 7,920	1.25
	3180456	Total	160,457	5.04
Muscle		1-MG	2,367 ± 371	0.07
		2-MG	3,380 ± 359	0.11
		1,3-DG	3,505 ± 1,177	0.11
		1,2-DG	52,063 ± 2,214	1.64
		glycerol	13,901 ± 1,400	0.44
	3180456	Total	75,217	2.37
Haemolymph		1-MG	26,615 ± 4,920	0.84
		2-MG	42,371 ± 15,909	1.33
		1,3-DG	4,532 ± 359	0.14
		1,2-DG	195,639 ± 1,638	6.15
		glycerol	20,973 ± 666	0.66
	3180456	Total	289,947	9.12

[a]T° Indicates zero time and T[1] 30 min (midgut) or 60 min (other tissues) after commencement of incubation. Abbreviations: 1-MG, 1(3)-monoacylglycerols; 2-MG, *sn*-2-monoacylglycerol; 1,3-DG, 1,3-diacylglycerols; 1,2-DG, 1,2(2,3)-diacylglycerols.

production of 1,2(2,3)-diacylglycerols over that of 1,3-diacylglycerol, and of *sn*-2-monoacylglycerol over the 1(3)-isomer (Table II).

The results of a stereospecific analysis of the x-1,2(2,3)-diacylglycerols produced during the incubations are presented in Table III. Samples from the midgut incubation were taken at 30 min, whereas those from the haemolymph, fat body and muscle incubations were collected after 15 min or 60 min. The midgut homogenate produces a racemic mixture of *sn*-1,2 and *sn*-2,3-diacylglycerols, and the thoracic muscle enzyme(s) also favors the formation of a racemic mixture of the two enantiomers. However, the haemolymph and fat body lipases appear to produce more of the *sn*-1,2-isomer than the *sn*-2,3-isomer.

DISCUSSION

Diacylglycerols comprise the major form in which neutral lipids are transported in most insects (1). Tissue lipases may be expected to contribute to this unusual mode of lipid transport, and an appreciation of enzyme specificities is essential to our understanding of the various mechanisms of acylglycerol release and uptake by the tissues. The present study provides preliminary information on the capacity of several tissues to hydrolyze triacylglycerol and identifies several areas for future investigation. In particular it should be recognized that the lipolytic activities of tissue homogenates do not necessarily reflect the activities of single enzymes, and purification of the individual enzymes is required before any definitive conclusions can be reached concerning stereospecificity or acylspecificity. Furthermore, the demonstration that lipolytic activity is greater in lyzed fat body tissue than in intact tissue (6) indicates that tissues have the capacity to regulate lipolytic activity; therefore, results obtained in vitro may not reflect in vivo conditions.

Midgut

The hydrolysis of dietary long chain triacylglycerols in the alimentary tract of the American cockroach is effected through a lipase which originates in the epithelial cells of the midgut and associated caecae (1). The midgut enzyme shows several similarities to mammalian pancreatic lipase, including an enhancement of activity in the presence of calcium ions (18) and the preferential cleavage of fatty acids located in the primary positions of the triacylglycerol molecule (2). The substrate employed in the latter study was *sn*-1,3-dipalmitoyl-2-oleoyl glycerol; thus the investigation did not accommodate the possible influence of fatty acyl specificity on the nature of triacylglycerol hydrolysis.

Studies on *L. migratoria* show that the midgut enzyme hydrolyzes trioleoylglycerol more readily than tripalmitoylglycerol (3), thereby indicating a fatty acyl specificity that is analogous to that of mammalian pancreatic lipase which preferentially cleaves unsaturated fatty acids from the 1- and 3-positions of the triacylglycerol molecule (4). The similarity between midgut lipase and mammalian pancreatic lipase is strengthened by the results of the present study. Table II demonstrates that the primary monoacylglycerol resulting from

TABLE III

Stereospecific Analysis of Diacylglycerol Products Resulting from the Hydrolysis of [H^3]-Glycerol Triolein by Homogenates of Midgut, Fat Body, Muscle and Haemolymph of *Periplaneta americana* L.[a]

	Radioactivity of phosphatidylphenols (dpm)				
		After digestion with phospholipase A_2		Percentage of diacylglycerols (DG)	
Tissue	Predigestion	lyso-phosphatidyl phenol	residual phosphatidylphenol	*sn*-1,2-DG	*sn*-2,3-DG
Midgut	54,384[b]	17,420	17,288	50.19	49.81
Fat body	A. 3,395	1,596	1,166	57.75	42.26
	B. 69,438	25,444	17,194	59.67	40.33
Muscle	32,568	9,591	9,231	50.96	49.04
Haemolymph	A. 99,913	39,210	30,863	55.82	44.18
	B.124,528	48,653	39,229	55.36	44.64

[a]Incubation times were: midgut, 30 min; muscle, 60 min; fat body and haemolymph, 60 min (A) or 15 min (B) (2 separate sets of experiments).

[b]Values represent the mean of duplicate experiments.

the hydrolysis of trioleoylglycerol is the *sn*-2-isomer, and Table III indicates that the diacylglycerol products of midgut lipolytic activity comprise a racemic mixture of *sn*-1,2-and *sn*-2,3-enantiomers. The role of the midgut lipase appears to be the formation of *sn*-2-monoacylglycerol and free fatty acids for absorption across the intestinal wall. These observations, together with recent reports of monoacylglycerol acyltransferase activity in the midgut (19) and the uptake of diacylglycerol from midgut by the haemolymph (20), suggest that the uptake of dietary triacylglycerols in the cockroach requires prior hydrolysis to monoacylglycerols and free fatty acids in the gut lumen with subsequent resynthesis of diacylglycerols occurring in the intestinal mucosa.

Fat body

Insect fat body contains large reserves of triacylglycerol which may be released into the haemolymph for distribution to other tissues in the form of diacylglycerol (1). It has been suggested, on the basis of observed differences in the fatty acid composition of haemolymph diacylglycerol and fat body triacylglycerol of the locust, that fat body triacylglycerols are hydrolyzed to monoacylglycerols and free fatty acids prior to their being resynthesized to diacylglycerols for release to the haemolymph (21). This suggestion is supported by the demonstration of monoacylglycerol acyltransferase activity in the fat body (19,22) and the report that an acid lipase from locust fat body yields equimolar amounts of monoacylglycerol and free fatty acids following incubation with a micellar dispersion of tri-, di- and monoacylglycerols (8). However, other studies indicate that monoacyl cleavage of the stored triacylglycerol is the primary route of diacylglycerol production in the fat body of another locust, *Schistocerca gregaria* (23), and the results presented herein suggest that this situation may prevail also in the cockroach fat body. The present study does not distinguish between several possible mechanisms of diacylglycerol production. Thus, the results reported herein may result from the action of a triacylglycerol lipase that preferentially yields *sn*-1,2-diacylglycerol, preferential degradation of *sn*-2,3-diacylglycerols by a stereospecific diacylglycerol lipase, or resynthesis of *sn*-1,2-diacylglycerols from monoacylglycerol precursors. Further studies on purified enzymes are required in order to resolve these possibilities.

Muscle

The primary source of energy for thoracic muscle metabolism in the cockroach is carbohydrate (24,25) with no lipid being employed for this purpose under normal physiological conditions (26). The predominant role of carbohydrate in satisfying the energy requirements of cockroach thoracic musculature is reflected in the relatively low levels of triacylglycerol hydrolysis that are observed in muscle homogenates (Table I). Comparative studies on the lipolytic activity of flight muscle from several insect species indicate that the capacity for hydrolysis is related to the importance of lipid as a substrate for muscle metabolism (7). Species which employ lipid as the primary substrate for muscle metabolism tend to show a specificity for acylglycerol hydrolysis which increases in the order tri: di: monoacylglycerol. A similar trend is suggested in the cockroach, although the absolute levels of lipase activity are lower (7). The results presented indicate that diacylglycerol is the primary product of triacylglycerol hydrolysis by muscle homogenates of the cockroach. The demonstration of lipolytic activity is influenced markedly by the form in which the substrate is presented to the enzyme (27) and, in the absence of a uniform emulsification procedure, direct comparison of the disparate reports is inappropriate. However, in interpreting comparative data of this nature, it is important to ensure that the differing solubilities of the various substrates are recognized and that equimolar concentrations of the substrates are included in the incubation medium. The possibility that muscle samples may be contaminated with haemolymph is negated by the apparent differing stereospecific ratios of diacylglycerol products resulting from lipolysis of triacylglycerol by the fat body and the haemolymph (Table III). Thus, the fat body homogenate produces a racemic mixture of *sn*-1,2- and *sn*-2,3-isomers, whereas the haemolymph favors the production of the *sn*-1,2-configuration (Table III).

Haemolymph

Lipolytic activity has been reported in the haemolymph of several insect species (28-32), but neither the source nor the role of the enzyme is known. Demonstration of lipolytic activity in haemolymph requires precisely defined experimental conditions; incubation of freshly collected haemolymph with emulsified triacylglycerol failed to reveal any capacity for hydrolysis and the presentation of diacylglycerol bound to the naturally occurring diacylglycerol-carrying lipoprotein similarly failed to demonstrate any lipolytic capacity in haemolymph (H. Chino and R.G.H. Downer, unpublished obser-

vations). The presence, in haemolymph, of high concentrations of diacylglycerol argues against the continual presence of an active lipase in this tissue, and it is necessary to propose a mechnism of enzyme activation that results in lipolytic activity being expressed under particular physiological conditions. It may be assumed that the incubation conditions employed in the present investigation serve to activate the haemolymph enzyme, and it is interesting to note, in this regard, that mammalian lipoprotein lipase is activated by phosphoacylglycerols (4). However, the demonstrated inhibition of cockroach haemolymph lipase by fluoride ions (30) indicates that the insect enzyme is dissimilar to that of the mammal. The present study demonstrates that the haemolymph lipase produces diacylglycerol as the principal product of triacylglycerol hydrolysis and stereospecific analysis of the resulting diacylglycerols reveals an accumulation of *sn*-1,2-enantiomers over the *sn*-2,3-isomer (Table III). This indicates a further difference from the mammalian lipoprotein lipase for which a preponderance of the *sn*-2,3-diacylglycerol has been reported (4).

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Reaction between Peroxidized Phospholipid and Protein: II. Molecular Weight and Phosphorus Content of Albumin after Reaction with Peroxidized Cardiolipin

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ABSTRACT

Peroxidized cardiolipin (diphosphatidylglycerol) reacts covalently with albumin. Incubation of albumin with increasing amounts of peroxidized cardiolipin produces a gradual increase in molecular size. Incubation with a small amount of peroxidized cardiolipin (molar ratio of cardiolipin/albumin 21) produces a mixture of complexes that differs considerably with respect to the number of cardiolipin molecules bound per molecule of albumin. With larger amounts of peroxidized cardiolipin (molar ratios of cardiolipin/albumin 54 and 114), the complexes formed seem to be of a more uniform type since the numbers of cardiolipin molecules bound per molecule of albumin are similar. No polymerization occurs for reactions in which up to at least 15 moles of cardiolipin have become bound per mole of albumin, and 20-25 moles may be bound with only very little polymerization. Only when the ratio of peroxidized cardiolipin to albumin was increased to a high value of 314 did polymerization occur. The present findings show that extensive covalent binding of peroxidized cardiolipin to albumin can occur without intermolecular crosslinking of the protein.

INTRODUCTION

Peroxidized lipids cause important polymerization of proteins (1-3). Observation of Roubal and Tappel (1) shows that co-oxidation of cytochrome C and ethyl linolenate converts almost all of the cytochrome C to dimer, tetramer and higher polymers. In a similar experiment, they observed that ribonuclease is also converted to polymers with the dimer constituting the major part. Furthermore, co-oxidation of α-chymotrypsin and ethyl arachidonate produces polymers of the protein (1). The polymers are considered to be protein-protein crosslinked polymers arising by free radical chain polymerization. This mechanism is supported by more recent investigations (4,5). Menzel (2) has observed that both peroxidizing linolenic acid and malondialdehyde cause dimerization of ribonuclease and has suggested that formation of malondialdehyde during oxidation of polyunsaturated fatty acids accounts for some of the effects of oxidizing lipids on protein. Chio and Tappel (3) have shown that polymerization of ribonuclease A by peroxidizing ethyl arachidonate and by malondialdehyde produces qualitatively similar mixtures of polymers and apparently the same fluorophor which was tentatively identified as N,N′-di-substituted 1-amino-3-iminopropene. It has been suggested that crosslinking of ribonuclease A by peroxidizing ethyl arachidonate is caused by malondialdehyde arising during peroxidation. Recently, we have observed that peroxidized cardiolipin binds covalently to albumin (6). The present work was undertaken to examine this reaction and we now report on molecular weight and phosphorus content of albumin after reaction with peroxidized cardiolipin.

MATERIALS AND METHODS

Chemicals

Albumin (human) was obtained from Kabi, Sweden; ovalbumin from Miles, Seravac, England; ribonuclease-A (from bovine pancreas) and γ-globulins (Bovine Cohn Fraction II) from Sigma; myoglobin (whale sperm) from Koch and Light; and pepsin from Boehringer. Albumin disulfide dimer (in a mixture with albumin monomer) was prepared by addition of Hg^{++} to a solution of albumin (7) followed by oxidation of the resulting mercury dimer with iodine (8). Ox heart cardiolipin, Na salt form, was prepared as previously described (6) and stored dry under N_2 (-25 C) in brown glass ampoules, solvent being removed by blowing with N_2 prior to sealing. Under these conditions, cardiolipin was stable over at least 1 year since no change in absorption at 232 nm or chromatographic behavior was detected. The same batch of cardiolipin was used throughout the work. Other chemicals used were of analytical grade. Cardiolipin suspension was prepared as previously described (6).

Peroxidation of the cardiolipin suspension and its subsequent incubation with albumin was previously described (6) except that UV irradiation was stopped at a 10% oxygen uptake.

Phosphorus Analysis

Phosphorus was determined as previously

described (6) by a modified micro-procedure (9) of Bartlett's method (10).

Protein Analysis

Protein was determined according to Lowry (11), and by total N analysis involving wet combustion with perchloric acid (12) and colorimetric quantitation by the Nessler reaction.

Gel Filtration

Gel filtration was performed on a Sephadex G-200 column (bed dimension ca. 2.7 x 60 cm) in 10 mM sodium deoxycholate, 0.1 M Na_2SO_4 and 0.05 M sodium borate (pH 8.5) at a flow rate of 17-18 ml/hr. Samples were applied on the column in 4 ml buffer containing 60 mg sodium deoxycholate, and fractions of 6.3-6.7 ml were collected. V_o and V_t were determined from elution volumes of Blue dextran and LiCl, respectively, the latter being analyzed by flame emission spectrophotometry.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-Electrophoresis)

SDS-electrophoresis (Baker, Bremel, and Sobieszek, personal communication) was performed in the ORTEC 4200 electrophoresis system. The tank buffer was composed of 0.020 M sodium acetate, 0.18 mM Na_2 EDTA, 0.040 M Tris/acetic acid (Tris concentration), pH 7.4, and 2% (w/v) sodium dodecyl sulfate. A 7.5% running gel was prepared from 8% (w/v) acrylamide and 0.22% (w/v) bis-acrylamide (N,N′-methylene-bis-acrylamide) in 30 ml tank buffer with further addition of 5 μl TEMED (N,N,N′,N′-tetramethylenediamine) and 2 ml ammonium persulfate (2.5%, w/v). Wells were cast from a mixture of the same composition except that ammonium persulfate concentration was doubled and five-fold more TEMED was used. Samples were made into a solution of buffer containing 3% SDS and heated for 1½ min on a boiling water bath. Mercaptoethanol was intentionally omitted because of the possibility that it might disrupt bonds involved in an eventual polymerization of the protein. After cooling, bromophenol blue (0.1% in 50% aqueous glycerol) was added to a concentration of 0.015%, and 25-50 μg protein was subjected to electrophoresis at room temperature at 300 V and 75 pps (pulses per second) for the initial 10 min to give a current intensity of 38 mA. Pulse rate was then increased to 150 pps. Electrophoresis was stopped after ca. 2 hr, at which time the tracking dye had reached bottom edge of the gel. Gels were stained either with 0.25% Coomassie blue in methanol/water/acetic acid (50:40:10, v/v) according to Weber and Osborn (13), or with 0.085% Coomassie blue in 20% (w/v) trichloroacetic acid (14). The latter method stained far more effectively the complexes of albumin and peroxidized cardiolipin than the former.

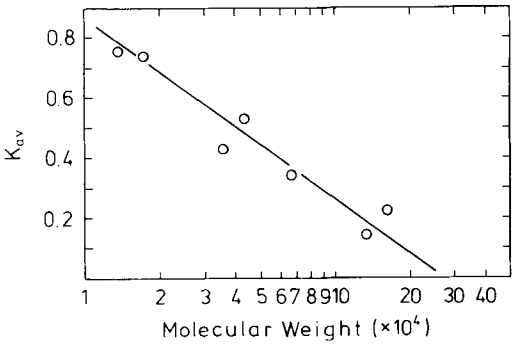

FIG. 1. Calibration curve for gel filtration on Sephadex G-200. Molecular weight standards in order of increasing molecular weight are ribonuclease (13700), myoglobin (17000), pepsin (35500), ovalbumin (43000), albumin (66200), albumin dimer (132500) and γ-globulin (160000).

RESULTS

Gel Filtration on G-200 in Presence of the Detergent Sodium Deoxycholate

Figure 1 shows calibration curve for the Sephadex G-200 column used in the study. The K_{av} values for albumin monomer and dimer, 0.34 and 0.15, respectively, differ sufficiently to assure their separation.

Figure 2 shows gel filtration of albumin after incubation with increasing amounts of peroxidized cardiolipin (B through E) as compared to albumin that has been incubated with non-peroxidized cardiolipin (A). It is apparent that when the ratio of peroxidized cardiolipin to albumin in the incubation mixture is increased there is a gradual decrease in K_{av} value for albumin indicating an increase in molecular size. Only in the experiment where the incubation was performed at the higher ratio of peroxidized cardiolipin to albumin (E) does the elution profile show a secondary peak (K_{av} = 0.067).

Figure 3 and 4 which refer to experiment B and C, respectively, of Figure 2 show the elution profiles for protein and covalently bound cardiolipin, and the ratio of moles cardiolipin bound per mole of albumin (right ordinate). The results of a typical experiment in which low amounts of peroxidized cardiolipin have been incubated with albumin are depicted in Figure 3. It is observed that covalently

bound P is eluted earlier than the protein, and the number of moles of cardiolipin bound covalently per mole of albumin varies widely through the eluted protein peak. When albumin is incubated with a considerably higher amount of peroxidized cardiolipin, the number of cardiolipin molecules bound per molecule of albumin is much increased and elution for protein and phosphorus tends to become coincident (Fig. 4).

When experiments D and E of Figure 2 were analyzed in the same way, elution profile for protein and covalently bound P appeared to be coinciding. However, the number of cardiolipin molecules bound per molecule of albumin did vary slightly throughout the protein peaks. For experiment D, the number varied from 25 (for the first part of the protein peak) to 20 (for the last part); for experiment E, the number varied from 29 to 23.

Determination of protein both according to Lowry (11) and by total N analysis (12) gave similar values; thus, covalent binding of cardiolipin to albumin did not interfere with Lowry's method.

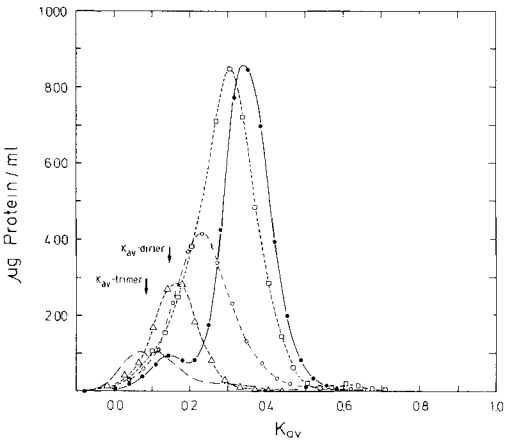

FIG. 2. Gel filtration of albumin after reaction with increasing amounts of peroxidized cardiolipin (A through E). Position of elution for albumin dimer and trimer is indicated by arrows at respective K_{av} values. K_{av} value for albumin trimer was arrived at by interpolation on the calibration curve. Composition of incubation mixtures were, A (●-●): 0.124 mM albumin + 4.63 mM nonperoxidized cardiolipin; B (□—□): 0.165 mM albumin + 3.39 mM peroxidized cardiolipin (2.4 moles O_2/mole cardiolipin); C (○---○): 0.127 mM albumin + 6.80 mM peroxidized cardiolipin (2.6 moles O_2/mole cardiolipin); D (△--△): 0.058 mM albumin + 6.62 mM peroxidized cardiolipin (2.7 moles O_2/mole cardiolipin); and E (···): 0.039 mM albumin + 12.3 mM peroxidized cardiolipin (2.6 moles O_2/mole cardiolipin). This corresponds to the following increasing molar ratios of peroxidized cardiolipin to albumin in the incubation mixture for B through E: 21, 54, 114 and 314.

SDS-electrophoresis

Figure 5 shows electrophoresis of different segments of the protein eluted in Figure 3 together with appropriate controls. Controls were obtained from gel filtration of an incubation mixture of albumin and nonperoxidized

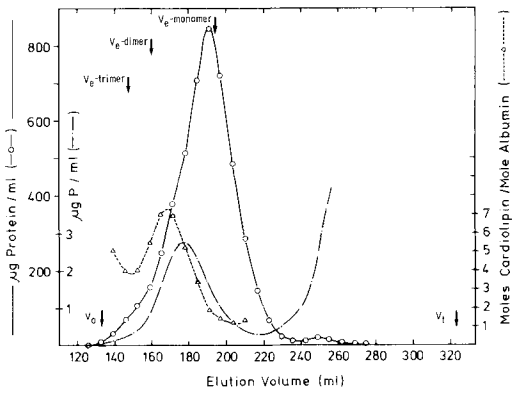

FIG. 3. Gel filtration on Sephadex G-200 of albumin reacted with peroxidized cardiolipin (2.4 moles O_2/mole cardiolipin). Peroxidized cardiolipin (3.39 mM) and albumin (0.165 mM) were incubated under N_2 at 30 C for 24 hr with continuous shaking whereafter 3 ml was subjected to gel filtration. From protein concentration in eluate (-o-) determined according to Lowry (11) and P concentration (---), moles of covalently bound cardiolipin per mole of albumin (--△--) were calculated (right ordinate). Void volume (V_o), total volume (V_t), and elution volumes for albumin monomer, dimer and trimer are marked with arrows.

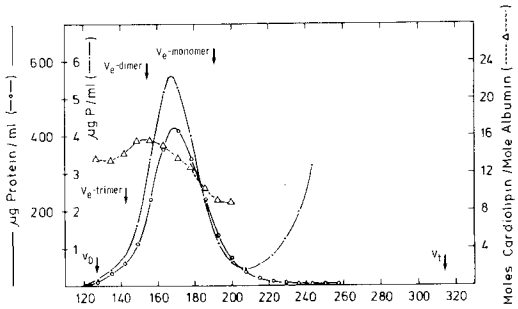

FIG. 4. Gel filtration on Sephadex G-200 of albumin after reaction with peroxidized cardiolipin (2.6 moles O_2/mole cardiolipin). Peroxidized cardiolipin (6.80 mM) and albumin (0.127 mM) were incubated under N_2 at 30 C for 22 hr with continuous shaking whereafter 2 ml of the reaction mixture was subjected to gel filtration. From protein concentration in eluate (-o-) determined according to Lowry (11) and P concentration (---), moles of covalently bound cardiolipin per mole of albumin (--△--) were calculated (right ordinate). Void volume (V_o), total volume (V_t), and elution volumes for albumin monomer, dimer and trimer are marked with arrows.

cardiolipin and subsequent pooling of corresponding segments of eluted protein. Suffice to mention that the nonperoxidized cardiolipin had been preincubated under the same conditions (including UV irradiation at 5 ppm Cu^{++}) used for peroxidation of cardiolipin, except that oxygen had been replaced by nitrogen. Pools corresponding to application 1, 2 and 3 constituted 8, 38 and 54%, respectively, of the total eluted protein. Pools corresponding to application 1C, 2C and 3C constituted 10, 34 and 56%, respectively, of the total eluted protein. For none of the segments is any difference apparent between sample and control neither in their electrophoretic mobility nor in their content of dimeric albumin. The front segments (1 and 1C) consist mainly of albumin dimer which is known to be present in native albumin (15). The succeeding segments (2, 2C and 3, 3C) consist of albumin monomer.

The protein eluted in Figure 4 was pooled for SDS-electrophoresis (Fig. 6). The first 5% and the last 3% of the eluted protein were not included because of lack of material. Pools corresponding to samples 1 and 2 constituted 54% and 38%, respectively, of the eluted protein. 1C and 2C are appropriate controls. Since there is no difference between samples and controls with respect to content of dimer and higher polymers of albumin, at least 15 molecules of peroxidized cardiolipin may be bound per molecule of albumin without intermolecular crosslinking. There is a slight decrease in electrophoretic mobility and some broadening of the bands of samples. The gel was stained with Coomassie blue in methanol/water/acetic acid, and it is apparent that the samples stain less well than their controls. Incubation at still higher ratios of peroxidized cardiolipin to albumin (D and E of Fig. 2) produced complexes that could not be stained by this procedure. However, in these cases staining could be done with Coomassie blue in 20% trichloroacetic acid (Fig. 7).

The protein of D in Figure 2 was made into two pools corresponding to the first and the second half of the eluted protein. The first half contained one major broad band with a mobility slower than that of the control monomer but higher than that of albumin dimer (Fig. 7). This fraction is claimed to be monomers of albumin-peroxidized cardiolipin complexes. In addition, a minor band was present at a position corresponding to the 3 mm mark of the inserted ruler. This band is claimed to be polymers – probably dimer – of the monomeric albumin-peroxidized cardiolipin complexes; it clearly exceeds the amount of polymeric material in its control (compare 1 with 1C of Fig. 7; polymeric material of control is not visible on photograph). The second half of the eluted protein contained exclusively the asserted monomeric albumin-peroxidized cardiolipin complexes (Fig. 7, lane 2). Thus, 20-25 moles of peroxidized cardiolipin may be bound covalently per mole of albumin with only very little polymerization. The considerable decrease in electrophoretic mobility of the asserted monomeric and dimeric complexes relative to albumin monomer and dimer might be anticipated because the reaction with

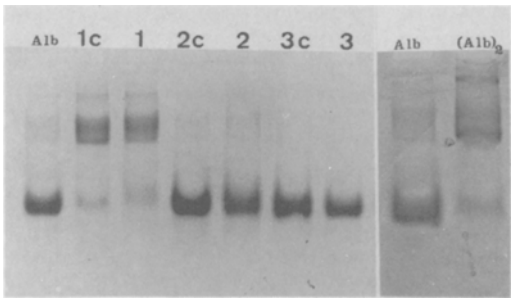

FIG. 5. SDS-electrophoresis of three albumin preparations with varying amounts of covalently bound peroxidized cardiolipin (1, 2 and 3). The preparations were obtained by pooling eluate from gel filtration of Figure 3 as indicated. 1: Protein eluted from 122-160 ml; 2: Protein eluted from 161-187 ml; 3: Protein eluted from 188-232 ml; 1C, 2C and 3C controls for 1, 2, and 3, respectively, as explained under *SDS-electrophoresis*. The far left application (Alb) is native albumin. The far right two lanes show electrohporesis of native albumin (Alb) and albumin dimer ($(Alb)_2$) in another electrophoretic run performed under the same conditions. Each application contained 29 μg protein.

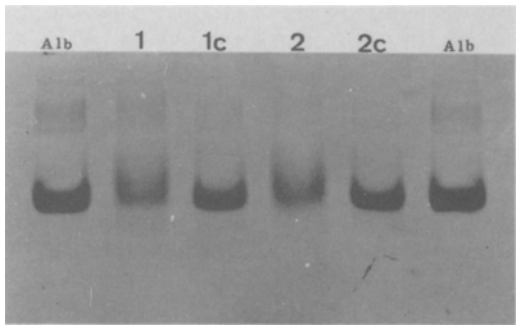

FIG. 6. SDS-electrophoresis of two albumin preparations, 1 and 2, containing 14-16 moles and 9-13 moles, respectively, of covalently bound cardiolipin per mole of albumin. The preparations were obtained by pooling eluate from gel filtration of Figure 4 as indicated below. 1: Protein eluted from 145-174 ml; 2: Protein eluted from 175-203 ml. 1C and 2C are appropriate controls. Far left and far right application is native albumin (Alb). Each application contained 30 μg protein.

peroxidized cardiolipin will increase the molecular size of the protein.

The protein of E of Figure 2 was similarly made into two pools corresponding to the first half and the second half of the eluted protein. Electrophoresis demonstrated the presence of two major bands with similar electrophoretic mobilities as the asserted monomeric and dimeric complexes of D and therefore probably of the same type. The assumed dimeric complex was the major component of the first half of the eluted protein, whereas the assumed monomeric complex constituted the major component of the second half of the eluted protein. Hence, polymerization appears to be quantitatively important in E.

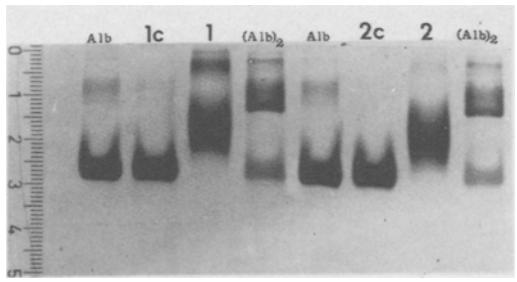

FIG. 7. SDS-electrophoresis of the first (1) and second half (2) of the eluted protein of D of Figure 2 containing 23-25 moles and 20-23 moles, respectively, of covalently bound cardiolipin per mole of albumin. 1C and 2C are appropriate controls. Alb is native albumin, and $(Alb)_2$ is a preparation of albumin dimer. Each application contained 45 μg protein.

DISCUSSION

The linear dependence of elution volume on the logarithm of the molecular weight in gel filtration of globular proteins in dilute salt solutions is well established (16). The deviations from linearity seen in Figure 1 are probably caused by the detergent of the buffer producing different degrees of unfolding of the various proteins. Such unfoldings will be tantamount to increased molecular size. However, to detect polymerization of albumin, the gel filtration procedure is valid since the K_{av} value for a protein is reproducible and the difference between K_{av} values for albumin monomer and dimer assures separation of the two compounds. Thus, three determinations of K_{av} for albumin monomer gave values of 0.336; 0.344 and 0.340, and two determinations of K_{av} for albumin dimer gave values of 0.147 and 0.148.

Figure 2 shows that when albumin is incubated with increasing amounts of peroxidized cardiolipin, its maximum of elution is gradually shifted towards smaller K_{av} value, and only for the incubation with the highest ratio of peroxidized cardiolipin to albumin does a secondary peak appear (E). The increase in molecular size of albumin is probably the result of space-filling by the bound cardiolipin. In addition, there may be unfolding of the albumin due to altered intramolecular forces. This would contribute to increased molecular size. An increase in molecular size produced in these ways will probably be gradual with increasing amounts of reacting lipid as seen for B, C and D in Figure 2. However, reactions causing intermolecular cross-linking of the albumin may also occur. This need not result in the appearance of additional peaks because a range of monomeric complexes of albumin-peroxidized cardiolipin and their dimers may give rise to an elution profile with a broader base rather than definite peaks. Consequently, the fact that only one peak is present in B, C and D does not necessarily exclude such polymerization. To account for the appearance of a secondary peak in E, it is tentatively assumed that quantitatively important cross-linking has occurred for monomeric albumin-peroxidized cardiolipin complexes of a relatively narrow molecular weight range. These assumptions are supported by the results of SDS-electrophoresis and by the relative small variation in the number of moles cardiolipin bound per mole of albumin when relatively high amounts of peroxidized cardiolipin is incubated with albumin (C, D and E).

The weight ratio of lipid to protein in the incubation mixtures ranged from ca. 0.5-7. For most biological membranes, the ratio is within the range of 0.25-4 (17); the ratios used in A through D are well within this range.

SDS-electrophoresis of albumin of experiments A through E in Figure 2 indicates that no polymerization has occurred in B and C, and only very little in D (Figs. 5, 6 and 7, respectively). In E, however, quantitatively important polymerization did occur. In E the ratio of lipid to protein in incubation mixture was increased 3-fold over that in D. It may be that a cross-linking product of lipid peroxidation thereby reaches a concentration that makes substantial polymerization possible. This may be the essential difference between D and E since the number of moles cardiolipin bound per mole of albumin are rather similar.

The elution of covalently bound peroxidized cardiolipin and protein in experiments B-E of Figure 2 was studied as shown for B and C in Figures 3 and 4, respectively. Generally, if only one species of complex is produced in the reaction between peroxidized cardiolipin and

albumin (no albumin being left unreacted), elution profiles for covalently bound P and protein would coincide. The ratio of moles cardiolipin/mole albumin calculated on basis of the concentration of P and protein in eluate would be constant and express the actual number of cardiolipin molecules bound per albumin molecule. When more species of complexes differing with respect to the number of moles of cardiolipin bound per mole of albumin are formed in the reaction, elution profiles for P and protein would still coincide if no fractionation of the complexes according to their P content occurs during gel filtration. The ratio of moles cardiolipin/mole albumin would be constant to give an average value. If, however, fractionation occurs, the elution profile for P will be displaced relative to the protein elution profile. The analytical determined ratio of moles cardiolipin/mole albumin will vary for the eluate and express average values in so far as the elution of different complexes overlap each other.

In Figure 3, the elution profile for P is considerably displaced relative to the elution profile for protein. This indicates that more than one molecular species is present and fractionation has occurred on basis of difference in P content. The ratio of moles cardiolipin/mole albumin which varies from 1-7 is a minimum range because the ratios are average values, and complexes containing far more than 7 cardiolipin molecules per albumin molecule may be present. It is, however, not possible to decide the kinds and amounts of the individual complexes actually present.

In Figure 4, the elution profiles for P and protein are almost coincident, except for a very small displacement of the P elution profile towards smaller elution volume, and the analytical determined ratio of moles cardiolipin/mole albumin varies from about 9 to 15. The real composition of the complexes may cover a somewhat broader range corresponding to the presence of complexes with a higher and lower number of molecules of covalently bound cardiolipin per molecule of albumin. The species containing about 14 moles of cardiolipin per mole of albumin apparently may be the most abundant, lesser amounts of complexes containing more than 14 and less than 14 moles of cardiolipin per mole of albumin being present.

Molecular weights of monomeric albumin-peroxidized cardiolipin complexes based on K_{av} values (Fig. 2) and the calibration curve are considerably higher than molecular weights calculated from the number of cardiolipin molecules bound per albumin molecule; e.g., K_{av} for D of Figure 2 is 0.16. A molecular weight of about 150000 is read from the calibration curve. However, since 20-25 moles of cardiolipin are bound per mole of albumin, a molecular weight of only 95000-100000 should be expected (assuming a molecular weight of 1400 for cardiolipin). Presumably, the bound cardiolipin molecules are relatively loosely packed, and also unfolding of the albumin may have occurred. Both phenomena would cause a greater increase in molecular size than could be expected from its actual increase in molecular weight.

The present reaction between peroxidized cardiolipin and albumin differs from previous views of the reactions between peroxidized lipid and protein by extensive covalent binding of peroxidized lipid to protein and absence of substantial intermolecular crosslinking of the protein. As discussed previously (6), the present model system differs in several respects from systems used by other authors, and this probably accounts for the different findings. Since albumin and cardiolipin have also been used as model compounds in our earlier study (6), it is not known if the reaction occurs in general between peroxidized phospholipids and proteins. However, the fact that peroxidized cardiolipin binds covalently to the same extent to albumin and γ-globulin (18) may indicate that the reaction applies to proteins in general. We are currently investigating if peroxidized phospholipids, in general, bind covalently to proteins.

ACKNOWLEDGMENT

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Inhibition of Cholesterol Synthesis in Mammary Tissue, Lung, and Kidney Following Cholesterol Feeding in the Lactating Rat

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ABSTRACT

Pregnant rats were randomly allocated to one of 3 experimental dietary groups: Group 1 – 15.5% butter, 2% cholesterol, 0.78% sodium cholate purified diet; Group 2 – standard rat diet with the addition of 10% lard and 2% cholesterol, and Group 3 -- standard rat diet. Plasma and milk cholesterol at 10 days postpartum were significantly elevated in dams fed exogenous cholesterol. The rat of incorporation of [1-^{14}C] acetate into digitonin-precipitable sterols of mammary tissue slices from dams in Group 1 and Group 2 was eight-fold and two-fold, respectively, less than controls. Mammary tissue cholesterol was greater in dams fed dietary cholesterol. Thus, our data, for the first time, demonstrate that cholesterol synthesis in lactating rat mammary tissue is suppressed following cholesterol feeding. In a second experiment, the rate of incorporation of [1-^{14}C] acetate into digitonin-precipitable sterols in kidney and lung tissue of Group 1 rats was suppressed; however, this response was not as marked as that observed in lactating mammary tissue. The concentration of cholesterol in kidney and lung was greater than controls. These results suggest that extrahepatic inhibition of cholesterol synthesis exists in the rat with a concomitant increase in tissue cholesterol.

INTRODUCTION

While there have been extensive investigations about the rate and regulation of cholesterol synthesis in many mammalian tissues (1,2), there is no information about the control of cholesterogenesis in lactating mammary tissue. It is recognized that lactating mammary tissue is metabolically active, synthesizing fatty acids at an exceptionally high rate (3). As early as 1950, it was demonstrated that lactating mammary tissue is capable of de novo synthesis of cholesterol from radio-labeled acetate (4-6). These findings prompted us to examine lactating mammary tissue for the suppression of cholesterol biosynthesis subsequent to cholesterol feeding.

In view of recent investigations which have demonstrated extrahepatic suppression of cholesterol synthesis in response to dietary cholesterol in rabbits (7), mice (8), and guinea pigs (9-11), investigations were conducted to determine whether the rate of cholesterol synthesis in the extrahepatic tissues of the rat is sensitive to exogenous cholesterol. Swann and coworkers (10) concluded that the sensitivity of cholesterol synthesis in extrahepatic tissues of the guinea pig is due to the accumulation of exogenous cholesterol in these tissues. The second purpose of this report was to examine whether this sensitivity existed in the rat.

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PROCEDURES

Animal Preparations

At 14 days' gestation, Holtzman rats (Holtzman Co., Madison, WI) were allocated to one of three experimental dietary groups: Group 1 was fed a purified diet (Table I) containing 15.5% butter, 2% cholesterol and 0.78% sodium cholate; Group 2 was fed a standard rat diet (Purina Rat Chow, Ralston Purina, St. Louis, MO) with the addition of 10% lard and 2% cholesterol; and Group 3, which served as a reference group, was fed a standard rat diet. Rats were housed in individual cages and maintained on a reverse lighting schedule (2000-0800 light). Five days postpartum, litters were adjusted to six pups. Milk and plasma samples were collected at 10 days postpartum. Dams were separated from their litters 5 hr prior to the collection of milk and plasma. Milk was collected while dams were under light anesthesia 10 min after a 0.2 ml intraperitoneal injection of oxytocin (Pitocin, 10 units per ml, Parke Davis and Co., Detroit, MI). Approximately 2 ml of milk was collected. Blood samples were taken from the tail in EDTA tubes. Mammary tissue samples were excised from the right proximal inguinal gland while dams were under light ether anesthesia at 10 days postpartum between 0800 and 1200. In a second experiment, lung and kidney tissue was collected both from dams at 10 days postpartum and female rats which were not lactating. These rats had been maintained on a high fat, high cholesterol purified diet (Table I) or

TABLE I

Composition of High Fat, High Cholesterol Purified Diet

	g/100 g Diet
Cholesterol[a]	1.94
Butter (sodium chloride free)	15.51
Casein[b]	31.03
Sucrose	31.12
Sodium cholate[b]	0.78
Choline chloride[b]	1.55
Cellulose flour[b]	7.76
Salt mix W[b]	6.21
Vitamin diet fortification mixture[b]	3.10

[a]USP grade, Sigma Chemical Co., St. Louis, MO.
[b]ICN Pharmaceuticals, Inc., Cleveland, OH.

standard rat diet for 17 days. Mammary tissue from 2 dams which had no access to food for 24 hr, but were allowed free access to water, was also excised under the aforementioned experimental conditions.

Tissue Preparation Incubation

Immediately following tissue excision, lung, kidney, and mammary tissue were immersed in 4 C Krebs Ringer phosphate solution (pH 7.2) and rinsed well. Tissue slices, 0.5 mm in thickness, were made with a Stadie-Riggs Microtome (A.H. Thomas Co., Philadelphia, PA). Approximately 200 mg of tissue was transferred to a 25 ml erlenmeyer flask which contained 3 ml of Krebs-Ringer phosphate buffer and 5 μ Ci of [1-^{14}C]acetate (0.45 μ Ci/μmole). The flasks were gassed with 95% O_2-5% CO_2, capped and incubated in a Dubnoff Metabolic Incubator (Precision Scientific Co., Chicago, IL) with 120 oscillations/min at 37 C for 2 hr, at which time 0.3 ml of 3N H_2SO_4 was added through the stopper to terminate the incubation (12).

Isolation of ^{14}C-Labeled Sterols

This method is discussed in detail elsewhere (12). Briefly, tissue slices were rinsed with an isotonic saline solution and transferred to screw cap culture tubes with 2 ml H_2O. The tissues were digested with 2 ml of 30% KOH while 7 ml ethanol was added to make a 50% ethanol solution. The tubes were heated for 5 hr at 70 C for saponification at which time the unsaponifiable material was extracted 3 times with 10 ml of petroleum ether. The extracts were evaporated to dryness and dissolved in 6 ml of a mixture of acetone/ethanol (1:1), 3 ml of 1% digitonin in a 50% ethanol solution, heated for 30 seconds in boiling water, and left to sit overnight at 4 C.

The precipitate, containing the β-hydroxy sterols, was washed 3 times with acetone in centrifuge tubes, and dried under a slow stream of N_2. Two ml methanol was added to dissolve the digitonides and 0.5 ml was added to 10 ml DPO-POPOP scintillation fluid containing 0.5% diphenyloxazole and 0.05% p-bis-phenylorazolyl-benzene in toluene. All samples were counted in an Ansitron liquid scintillation counter, II 1300 (Picker Nuclear, New Haven, CT). Corrections for quenching were made. Results are expressed as the percent incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols per 100 mg tissue per 2 hr incubation period.

Isolation of ^{14}C-Labeled Fatty Acids

Masoro and coworkers (13) described a method for isolating fatty acids in detail. In short, to the saponifiable solution, two drops of brom cresol green (as a color indicator) and enough 6 N HCl to acidify the solution (yellow end point) were added. Fatty acids were extracted three times with 8 ml hexane, washed once with 8 ml H_2O and centrifuged (2500 RPM for 5 min). One-tenth (2.4 ml) of the hexane-fatty acid aliquot was evaporated to dryness in a scintillation vial to which the aforementioned scintillation fluid was added.

Determination of Tissue, Plasma and Milk Cholesterol

Tissue cholesterol was determined by an adaptation of the Sperry and Webb method (14) from the cholesterol digitonide. Milk cholesterol was determined by a method developed by Morin and Elms (15) utilizing a Packard Gas Chromatograph 837 (Packard Instrument Co., Inc., Downers Grove, IL). Plasma cholesterol was determined by the Technicon Autoanalyzer II method (16).

Statistical Analyses

A Student's t test was used to analyze all data (17).

RESULTS

Milk and Plasma Cholesterol

Results indicate a significant effect of dietary cholesterol (Group 1 and 2) on plasma and milk cholesterol (Table II) in the lactating rat at 10 days postpartum. These findings are in agreement with previous reports from our laboratory (18). In the two groups of rats fed cholesterol (Group 1 and 2), milk cholesterol was not significantly different between Group 1 and 2 while plasma cholesterol was, (397 ± 40 and 128 ± 7 mg%, respectively, ($p<0.0005$).

Incorporation of [1-^{14}C] Acetate into Digitonin-Precipitable Sterols and Fatty Acids in Mammary Tissue

Cholesterol feeding and fasting inhibited the incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols (Table III). There was an eight-fold decrease in cholesterol synthesis in lactating mammary tissue from dams fed the high cholesterol purified diet (Group 1). To examine the exclusive effect of cholesterol feeding, and not the effect of other purified dietary components on cholesterol synthesis in mammary tissue, dams were fed a standard rat diet with the addition of 2% cholesterol and 10% lard (Group 2). The percent incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols was suppressed ca. 2.5 times. Therefore, the addition of exogenous cholesterol to the diet of a lactating rat suppressed cholesterol synthesis in lactating mammary tissue.

TABLE II

Plasma and Milk Cholesterol of Dams at 10 Days Lactation

Treatment	Plasma cholesterol mg%	Milk cholesterol mg%
Group 1[a]		
1	428	
2	291	
3	438	
4	364	
5	534	
6	500	
7	467	60
8	215	44
9	203	50
10	333	84
11	458	57
12	224	50
13	708	63
	(397 ± 40)	(58 ± 5)[b]
Group 2[a]		
14	117	71
15	125	45
16	144	52
17	112	60
18	145	45
	(128 ± 7)[c]	(55 ± 5)[d]
Group 3		
19	69	
20	73	
21	91	
22	69	
23	75	30
24	68	41
25	63	37
26		25
27		41
28		34
29		37
30		38
31		50
32		43
	(73 ± 4)	(38 ± 2)

Values in parentheses are means and standard errors.

[a]Denotes significant difference when comparisons are made between respective treatment and Group 3.

[b]$P<0.01$.

[c]$P<0.005$.

[d]$P<0.025$.

Group 1 – High fat, high cholesterol diet.
Group 2 – 10% Lard, 2% cholesterol, standard rat diet.
Group 3 – Standard rat diet.

The percent incorporation of [1-^{14}C]-acetate into fatty acids was examined as an assurance that dams were eating the experimental diet in view of our findings, and those of others (1,2) that fasting has an inhibitory effect on cholesterol synthesis. Although there was a significant difference in the percent incorporation of [1-^{14}C]acetate into fatty acids in dams in Group 2, a closer examination of each value reveals that every value falls within the range of normal values. We have indications that dams in Group 2 consumed the experimental diet. Serum cholesterol was elevated, milk was produced and from past preliminary experiments, we have found that rats fed this diet gain weight at a faster rate than rats fed a standard stock diet. Furthermore, mammary tissue from dams in Group 2 (and Group 1) had a significantly higher concentration of cholesterol than mammary tissue from dams fed standard rat diets. Accordingly, the inhibition of cholesterol synthesis in mammary tissue of dams fed a 2% cholesterol, 10% lard, and standard rat diet was the result of cholesterol feeding even though fatty acid synthesis was moderately depressed.

Finally, it appears that cholesterol feeding increased the concentration of digitonin-precipitable sterols in mammary tissue of lactating rats.

Incorporation of [1-^{14}C] Acetate into Digitonin-Precipitable Sterols and Fatty Acids in Lung and Kidney. Lung and Kidney Cholesterol

The incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols was significantly inhibited in lung tissue of lactating rats fed a high cholesterol purified diet (Table IV). Cholesterol synthesis was also suppressed in kidney tissue of dams fed a high cholesterol purified diet, although these results were not statistically significant. Both lung and kidney tissue from rats in Group 1 contained more cholesterol than controls.

DISCUSSION

While the liver is the primary site of an inhibition of cholesterol synthesis following cholesterol feeding in the rat, data presented in this paper suggest that other tissues have a participatory role as well. Under comparable experimental conditions, there was also inhibition of cholesterol synthesis in mammary tissue of the lactating rat. Of importance, also, is that cholesterol synthesis may be suppressed in lung

TABLE III

Incorporation of [1-^{14}C] Acetate into Digitonin-Precipitable Sterols and Fatty Acids in Mammary Tissue, and Mammary Tissue Cholesterol

Treatment	^{14}C Incorporation into digitonin-precipitable sterols/100 mg tissue	^{14}C Incorporation into fatty acids/ 100 mg tissue	Digitonin-precipitable sterols/100 mg tissue
Group 1[a]	%	%	mg
1	0.11	0.62	0.109
2	0.15	3.37	0.158
3	0.04	1.83	0.167
4	0.10	0.80	0.145
5	0.02	4.12	0.153
6	0.07	2.31	0.178
7	0.03	2.31	0.240
8	0.06	1.28	0.115
9	0.06	1.56	0.134
10	0.02	3.06	0.173
11	0.09	1.15	0.123
12	0.04	0.91	0.149
13	0.04	1.31	0.148
	(0.06 ± 0.01)[b]	(1.89 ± 0.30)	(0.153 ± 0.009)[c]
Group 2[a]			
14	0.03	1.31	0.162
15	0.19	0.52	0.128
16	0.25	0.75	0.124
17	0.40	1.35	0.140
18	0.04	0.99	0.178
	(0.18 ± 0.07)[d]	(0.98 ± 0.16)[d]	(0.140 ± 0.10)[c]
Group 3			
19	0.28	3.41	0.107
20	0.85	3.56	0.107
21	0.45	1.69	0.111
22	0.62	2.58	0.102
23	0.19	1.56	0.107
24	0.53	1.48	0.113
25	0.30	0.42	0.009
	(0.46 ± 0.09)	(2.10 ± 0.43)	(0.107 ± 0.002)
Fasted dams			
33	0.01	0.42	
34	0.004	0.10	
	(0.007 ± 0.003)[d]	(0.26 ± 0.16)[d]	

Values represent the average of duplicate samples.
Values in parentheses are means and standard errors.

[a]Denotes significant difference when comparisons are made between respective treatment and Group 3.

[b]$P<0.005$.

[c]$P<0.01$.

[d]$P<0.05$.

Group 1 – High fat, high cholesterol diet.
Group 2 – 10% Lard, 2% cholesterol, standard rat diet.
Group 3 – Standard rat diet.

TABLE IV

Incorporation of [1-^{14}C] Acetate into Digitonin-Precipitable Sterols and Fatty Acids in Lung and Kidney. Lung and Kidney Cholesterol

Treatment	^{14}C Incorporation into digitonin-precipitable sterols/100 mg tissue %		^{14}C Incorporation into fatty acids/100 mg tissue %		Digitonin-precipitable sterols/100 mg tissue mg	
Group 1[a]	Lung	Kidney	Lung	Kidney	Lung	Kidney
1	0.006	0.0060	0.320	0.080	0.170	0.189
2	0.010	0.0185	0.410	0.090	0.181	0.159
3	0.004	0.0155	0.370	0.040	0.224	0.209
4	0.004	0.0040	0.490	0.100	0.171	0.163
5	0.010	0.0060	0.660	0.120	0.143	0.163
	(0.007 ± 0.002)[2]	(0.010 ± 0.003)	(0.450 ± 0.059)	(0.086 ± 0.013)	(0.177 ± 0.013)[3]	(0.179 ± 0.009)[4]
Group 3 (lactating)						
6	0.009	0.0320	0.240	0.070	0.120	0.168
7	0.010	0.0095	0.270	0.040	0.118	0.116
(nonlactating)						
8	0.010	0.0095	0.370	0.060	0.124	0.123
9	0.015	0.0215	0.700	0.150	0.134	0.133
	(0.011 ± 0.002)	(0.019 ± 0.005)	(0.395 ± 0.105)	(0.080 ± 0.24)	(0.124 ± 0.004)	(0.135 ± 0.012)

Group 1 – High fat, high cholesterol diet
Group 3 – Standard rat diet

Values represent the average of duplicate samples.
Values in parentheses are means and standard errors.
[a]1 Denotes significant difference when comparisons are made between respective treatment and Group 3. 2 $P<0.05$. 3 $P<0.005$. 4 $P<0.025$.

and kidney tissue in the rat under severe experimental conditions.

Dietschy and McGarry (19) demonstrated the limitation of acetate as a substrate for measuring cholesterol synthesis. These investigators compared the rates of cholesterol synthesis in rat liver slices using [^{14}C]acetate and [^{14}C]octanoate as precursors reasoning that isotopic acetate would generate isotopic acetyl-CoA in the cytosol while labeled octanoate would generate labeled acetyl-CoA in the mitochondrion. By simultaneously incubating liver slices with both precursors, Dietschy and McGarry were able to assess the degree of intracellular dilution and compartmentalization of the acetyl-CoA pools. They found that the rate of cholesterol synthesis was 47% of the maximum when acetate was used as a precursor. These investigators cautioned against using acetate to precisely measure cholesterol synthesis because of changes in the concentration of the acetate pool.

Our investigations did not attempt to quantify the cholesterol synthetic rate in tissue slices incubated with labeled acetate. While the caveat of Dietschy and McGarry (19) cannot be ignored, it is of importance to note that in one experiment we found an 8- to 10-fold suppression in the percent incorporation of acetate into cholesterol. Unless there were vast changes in the acetate pool size induced by cholesterol feeding, such a marked inhibition of cholesterol synthesis could be attributed only to an actual suppression in cholesterol synthesis. To date, vast changes in the acetate pool sizes, leading to a 10-fold inhibition in cholesterol synthesis have not been demonstrated. Therefore, we believe that the significant reduction in the percent incorporation of labeled acetate into cholesterol is the result of an actual suppression in the rate of cholesterol synthesis rather than a major dilution in the acetate pool size. The percent incorporation of labeled acetate into cholesterol in lung and kidney tissue, however, warrants further investigation.

We attribute the suppression of cholesterol synthesis in kidney, lung and mammary tissue in the rat to the accumulation of cholesterol in these tissues. Frantz and associates (20) first suggested that hepatic cholesterol content was the prime determinant of the rate of hepatic cholesterol synthesis. Gould and associates (21) later suggested that as well. This was subsequently demonstrated in the isolated perfused rat liver (22). Our data offer further evidence to support this phenomenon in the extrahepatic tissues of the rat.

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Isolation and Characterization of Phospholipase D from Fababeans

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ABSTRACT

An enzyme activity in crude extract of fababeans hydrolyzed phosphatidylcholine-U-^{14}C to produce choline and phosphatidic acid. This enzyme, phospholipase D, was stable at 50 C in the presence of 5 mM DTT but was inactivated at 55 C. The enzyme was precipitated with cold acetone, concentrated between 30% saturation to 40% saturation with ammonium sulphate, absorbed on calcium phosphate gel and eluted with 0.2 M phosphate buffer. This procedure resulted in a 20-fold increase in specific activity. The activity of fababean phospholipase D was much higher when assayed at 38 C than that at room temperature. There was an obligatory requirement for calcium, and for maximal activity 40 mM calcium was required. A narrow pH optimum of about pH 5.7 was observed. The enzyme activity was extremely dependent on substrate dispersion. When 5 mM phosphatidylcholine (PC) was sonicated with increasing levels of sodium dodecyl sulphate (1 mM to 4 mM), the enzyme activity kept increasing. By using equimolar concentrations of PC and sodium dodecyl sulphate (1 mM to 5 mM), the Michaelis constant (Km) was estimated to be 1.74 mM. Addition of choline and serine at 10 mM concentration reduced phospholipase D activity by 31% and 22%, respectively.

INTRODUCTION

Processed fababeans become rancid after mechanical disruption of the beans, presenting storage and acceptability problems. This rapid development of rancidity has been attributed in part to the enzymic degradation of lipids. Eskin and Henderson (1,2) purified lipoxygenase (linoleate: O_2 oxidoreductase, EC 1.13.11.12) from fababeans which, in the presence of molecular oxygen, catalyzed the oxidation of *cis,cis*-1,4-pentadiene systems in unsaturated fatty acids. Subsequent work by Dundas et al. (3) demonstrated the presence of lipase (triacylglycerol hydrolase, EC 3.1.1.3) in fababeans, which hydrolyzed triglycerides to fatty acids and glycerol. Since approximately two-thirds of the lipids in fababeans are present as phospholipids (4), this study attempted to elucidate the first step in the hydrolysis of phospholipids.

EXPERIMENTAL

Materials

Fababean protein concentrate (FBPC), prepared by air classification of fababean flour (*Vicia faba* L. var. minor CV. Diana), was provided by the NRC Prairie Regional Laboratory, Saskatoon. This material was subsequently stored at -40 C. Phosphatidylcholine-U-^{14}C, phosphatidylcholine-methyl-^{14}C and Aquasol-2 were obtained from New England Nuclear, Boston, MA. Protein binding dye was purchased from BioRad Laboratories, Mississauga, Ontario. Other reagents were of A/R quality and purchased from Sigma Chemical Co., St. Louis, MO or Fisher Scientific Company, Fair Lawn, NJ.

Enzyme Preparation

The enzyme was extracted by homogenizing FBPC with 10 parts (w/v) of 50 mM Tris acetate buffer, pH 5.7, containing 5 mM DTT and 1 mM EDTA, in a Waring blender for 2-3 min. Any undissolved material was removed by centrifugation at 40,000 g for 30 min. The enzyme was partially purified according to the scheme shown in Table I. All procedures were carried out at 5 C and at every step the precipitate was collected by centrifugation at 40,000 g for 20 min.

Enzyme Assay

Substrate. Adequate amount of labeled phosphatidylcholine (PC), 250 μ moles of carrier PC and a trace amount of butylated hydroxytoluene (2,6-di-tert-butyl-p-cresol) were added into a test tube and the mixture dried under a stream of nitrogen, at room temperature. The residue was suspended in 10 ml of 10 mM sodium dodecyl sulphate (SDS) and sonicated at 5 C for 15 min (bursts of 15 sec, about 15 sec apart) with Model 1000 Insinator (Ultra Sonic System, Inc., Farmingal, NY). The sonicated substrate was stored at -20 C under nitrogen.

Procedure. The assay system of Heller et al. (5) with some modifications was adapted and all assays carried out in duplicates. The standard assay mixture (1 ml) contained 50 mM Tris acetate buffer, pH 5.7; 2 mM $Na_2S_2O_5$; 40 mM $CaCl_2$; 2 mM SDS and 5 mM phosphatidylcholine-methyl-^{14}C (ca. 2,400 dpm/μ mole).

TABLE I

Partial Purification[a] of Fababean Phospholipase D

Procedure step	Total[b] activity (Units)	Protein (mg/ml)	Specific activity (Units/mg protein)
1. Crude extract	1630	16.2	1.01
2. Heat treated at 50 C	1600	11.2	1.43
3. Precipitation with acetone at -20 C	1550	7.84	1.98
4. Amm. sulph. fraction (30%-40% saturation)	750	6.52	5.75
5. Elution[c] at pH 6.5			
(a) 0.2 M phosphate	216	1.54	6.36
(b) 0.2 M phosphate	218	0.55	19.82
(c) 0.5 M phosphate	96	0.48	10.00

[a]One unit = 1 μ mole of choline/min.

[b]Crude extract (100 ml) was prepared from 10 g of Fababean protein concentrate.

[c]Enzyme protein from step 4 was adsorbed on calcium phosphate gel (16.3 mg/mg protein), washed with 0.1 M phosphate buffer pH 6.5, and then eluted twice with 0.2 M and once with 0.5 M phosphate buffer.

The reaction was carried out in 15 ml centrifuge tubes with screw caps at 38 C in a shaking water bath. The reaction was started by adding enzyme and terminated by adding 4 ml of chloroform/methanol (2:1) mixture, vortexing and cooling to -20 C. The tubes were vortexed again for 1 min, centrifuged, and aliquots of aqueous layer containing free choline were mixed with 9 ml of aquasol and counted for radioactivity in a Mark III, model 6880, Liquid Scintillation System (Searle Canada Ltd.), using a channel ratio method.

Identification of products. Uniformly labeled PC (ca. 100,000 dpm/μ mole) was incubated with crude extract of fababean protein concentrate at room temperature for 1 or 2 hr. The enzyme was inactivated by adding 3.0 ml of 2% perchloric acid. The assay mixture was extracted repeatedly with 4 ml lots of ethyl ether. After 8-10 extractions, aliquots from the aqueous phase were counted for radioactivity in water soluble products. The water soluble products were identified by thin layer chromatography (6).

The first three ethyl ether extracts were pooled, dried under a stream of nitrogen gas; the resulting residue was hydrolyzed under very mild alkaline conditions (7). Free fatty acids were removed by partitioning with 1 vol of water and 2 vol of chloroform/isobutanol (2:1). The phosphorus-containing products in aqueous phase were separated by descending paper chromatography with the solvent system containing phenol saturated with H_2O/ acetic acid/ethanol (100:10:12) and detected with a molybdenum spray reagent (8). Areas of filter paper, corresponding to molybdenum reactive spots were cut into small pieces and counted for radioactivity by the scintillation procedure.

Determination of protein. The protein contents of various enzyme preparation were assayed by a dye binding method of Bradford (9), using bovine serum albumin as a standard. The presence of DTT does not interfere in this method.

RESULTS AND DISCUSSION

Characterization of Phospholipase D

Incubation of 1.6 μ moles of phosphatidylcholine-U-^{14}C with 1.6 mg and 8.0 mg protein of crude enzyme extract produced 1.39 ± 0.06 and 1.54 ± 0.01 μ moles of choline or phosphorylcholine within 2 hr. When substrate concentration was doubled to 3.2 μ moles of PC, 8.0 mg of enzyme protein produced 1.92 ± 0.03 μ moles of product in 1 hr (Table II). Thin layer chromatography (TLC) of products extracted in the aqueous phase showed that 91% of the radioactivity was in free choline. Examination of ethyl ether extract by TLC using Silica Gel G plates developed with petroleum ether/ethyl ether/acetic acid (85:25:2) failed to show any radioactive spot corresponding to free fatty acids or diglyceride standards. Thus, it was apparent that most likely fababeans contained phospholipase D. However, a sequential action of phospholiase C and some phosphatase acting on phosphoryl-

TABLE II

Hydrolysis of PC-U-^{14}C

Expt. No.	Enzyme (mg)	Assay time (min)	Substrate (μ moles)	Product[a] (μ moles)	Hydrolysis (%)
1	1.6	120	1.6	1.39 ± 0.06	88
1	8.0	120	1.6	1.54 ± 0.01	96
2	8.0	60	3.2	1.92 ± 0.04	60

[a]Choline and phosphatidic acid were confirmed as the end products.

choline could also yield free choline.

The identity of the products in ethyl ether extract was established by mild alkaline hydrolysis to remove fatty acids and paper partition chromatography of phosphorous-containing derivatives. Control runs gave major spots (74.7% and 78.9% of radioactivity) corresponding to glyceryl phosphorylcholine, indicating that PC was the parent phospholipid. Incubations with the enzyme gave major spots (58.4% and 66.6% of radioactivity) corresponding to glyceryl phosphate, indicating that phosphatidic acid (PA) was the parent phospholipid.

Thus, it was confirmed that crude extracts of fababeans contained enzyme activity hydrolyzing PC to PA and choline, i.e., phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4). Similar enzyme activities have been reported in other plant materials including cabbage leaves (10,11); sugar beets, spinach and carrots (12); cottonseed (13); peanuts (5); and red alga (14).

Properties of Phospholipase D from Fababeans

The enzyme in the crude extract was sensitive to heat. Short time (5 min) exposure at 50 C, 55 C, 60 C and 70 C reduced the enzyme activity by 19, 91, 98 and 100%, respectively (Fig. 1). Addition of 5 mM DTT protected the enzyme activity almost completely at 50 C but reduced the extent of inactivation from 81% to 61% at 55 C. Thus, the inactivation of phospholipase D around 50-55 C might be related to sulphydryl groups. Thermal inactivation at ca. 60-70 C, of phospholipase D extracted from other plant materials have been reported (11-13).

The specific activity of enzyme preparation increased from 1.01 units (μ moles/mg protein/min) in crude extract to 19.82 units in the second elution with 0.2 M phosphate buffer pH 6.5, from calcium phosphate gel (Table I). After precipitation with acetone, the enzyme became cold labile and all buffers, subsequently used, contained 20 mM DTT. This seemed to protect the enzyme against low temperature.

The enzyme activity was much higher when assayed at 38 C than that at room temperature (Fig. 2). There was a linear relationship between enzyme concentration and choline produced in 10 min assays (Fig. 2), as well as between assay time and choline production (Fig. 3) up to ca. 2 μ moles of product accumulation.

A narrow pH optimum of ca. pH 5.7 (Fig. 4) was in the range reported for phospholipase D extracted from other plant sources (5,10,15).

The requirement for calcium was obligatory; i.e., without added calcium, the enzyme

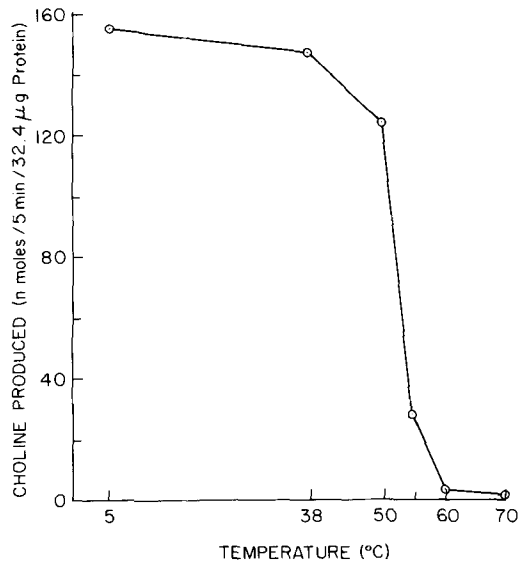

FIG. 1. Effect of heat treatment (Crude enzyme extract in 10 ml lots was heated in a water bath to the desired temperature within 2-3 min, held at that temperature for 5 min and then cooled quickly to 5 C within 2-3 min; inactivated protein was removed by centrifugation), on phospholipase D activity (Equal volumes–equivalent to 32.4 μg protein before heating–were used).

activity was negligible. The calcium concentration of 40 mM required for maximal activity (Fig. 5) was similar to that reported for cabbage enzyme (11) and peanut enzyme (5).

The enzyme activity was extremely dependent on the extent of substrate dispersion. Dawson and Hemington (11) reported that ultrasonication of lecithin increased the initial rate of reaction of phospholipase D purified from Savoy cabbage. In the present study, when 5 mM PC was sonicated with increasing levels of SDS (1 mM to 4 mM), the enzyme activity kept increasing (Table III). Also a combination of 10 mM PC and 2 mM SDS resulted in enzyme activity similar to that with 5 mM PC and 4 mM SDS. Other workers have reported different combinations of phospholipids and SDS for maximal activity of phospholipase D from various sources (5,11).

A preliminary estimation of Km by using equimolar concentrations of PC and SDS (1 mM to 5 mM) gave a value of 1.74 mM for PC. This was of the same order as reported for soluble phospholipase D from peanut seeds (15).

The nitrogenous bases, the usual end products of phospholipase D action on phospholipids, were found to be potential inhibitors of fababean enzyme. At 10 mM concentration, the choline reduced the enzyme activity by

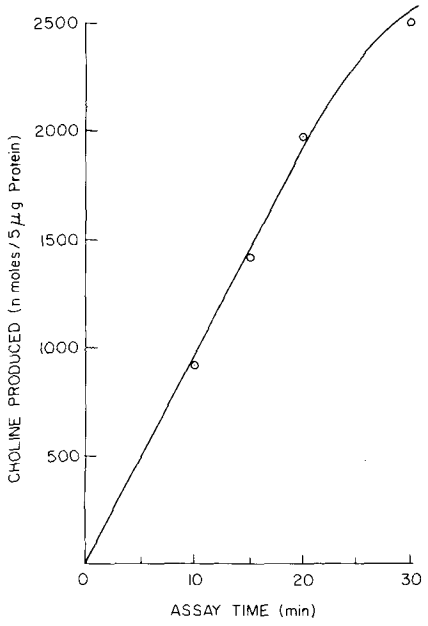

FIG. 3. Effect of assay time on phospholipase D activity at 38 C.

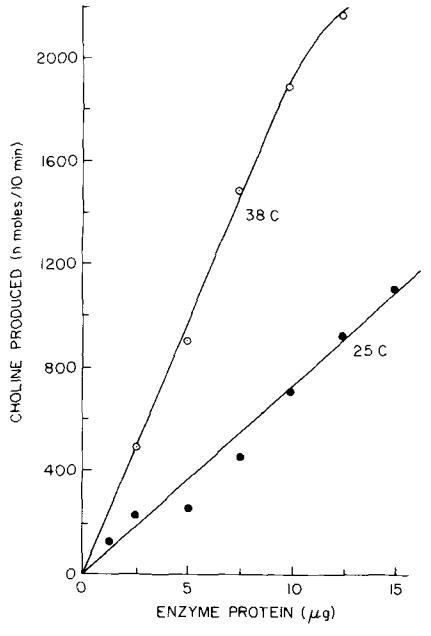

FIG. 2. Effect of concentration of phospholipase D on the hydrolysis of phosphatidylcholine at 25 C and 38 C.

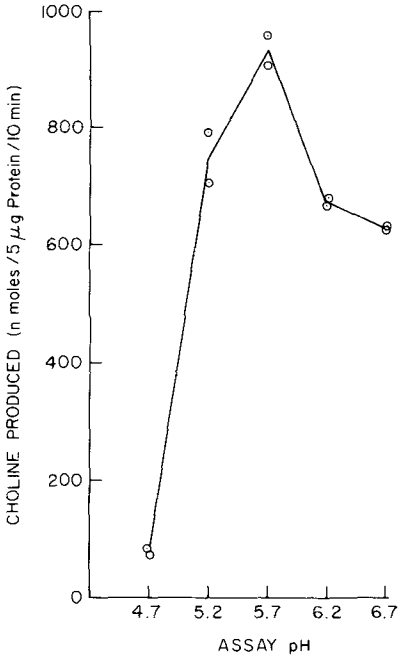

FIG. 4. Effect of pH of assay medium on phospholipase D activity at 38 C.

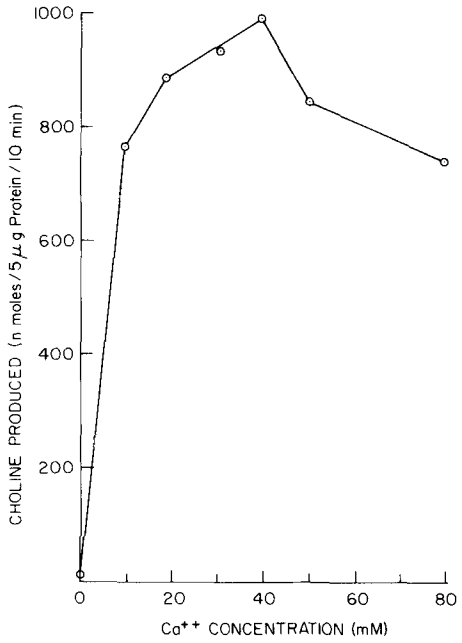

FIG. 5. Effect of calcium concentration on phospholipase D activity at 38 C.

31% and serine by 22% (Table IV). Thus, it seems that phospholipase D of fababeans might also hydrolyze phosphatidylserine and perhaps other phospholipids as well.

TABLE III

Effect of SDS[a] Concentration on Phospholipase D

PC[b] (mM)	SDS (mM)	Choline produced Exp. 1 (n moles/5 μg protein/10 min)	Choline produced Exp. 2 (n moles/5 μg protein/10 min)
5	1	77	67
5	2	962	912
5	3	1008	1195
5	4	1180	1267
10	2	1122	1253

[a]Sodium dodecyl sulphate.
[b]Phosphatidylcholine.

TABLE IV

Effect of Bases on Phospholipase D[a]

Addition	Choline produced (n moles)	Relative activity (%)
---	953	100
10 mM choline	661	69
10 mM serine	743	78

[a]5 μg of enzyme protein was incubated with 5 mM PC for 10 min at 38 C.

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On the Membrane Phospholipids and Their Acyl Group Profiles of Adrenal Gland

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ABSTRACT

The phospholipid composition and their acyl group profiles from subcellular fractions of guinea pig adrenal gland and the same fractions from the cortex and medulla of the bovine gland were compared. The phospholipids of guinea pig adrenal were enriched in diacyl-glycerophosphocholines (GPC) which comprised over 50% of the total phospholipids, but the proportions of ethanolamine and choline plasmalogens, sphingomyelin and diacyl-glycerophosphoserine (GPS) were lower in guinea pig adrenals as compared to the bovine adrenals. In the bovine adrenal, sphingomyelin and diacyl-GPS were enriched in the medulla, whereas diacyl-glycerophosphoinositol (GPI) were enriched in the cortex. Although lysolecithin was present (up to 4.5%) in the bovine adrenal, only trace amounts of this lipid were detected in the guinea pig adrenal. Characteristic acyl group profiles were found associated with each type of the phosphoglycerides in adrenal membranes. However, acyl group profiles of the phosphoglycerides were not greatly different either between the bovine and guinea pig adrenal or with respect to the type of subcellular membranes isolated. Diacyl-GPC were enriched in 16:0 and 18:1, but also contained considerable amounts of 18:0, 18:2 and 20:4. Diacyl-GPE were enriched in 18:0. 18:1 and 20:4, while diacyl-GPI, diacyl-GPS, as well as alkenylacyl-GPE, were enriched in 20:4. The lysolecithin from bovine adrenal membranes contained mainly 16:0, 18:0 and 18:1 with only trace amounts of the polyunsaturated fatty acids. Other polyunsaturated fatty acids, such as 22:4 and 22:6, are apparently not prominent in the phosphoglycerides from either the bovine or the guinea pig adrenal gland.

INTRODUCTION

The adrenal gland is an organ important in regulating body growth and other metabolic functions. It is made up of two physiologically and morphologically distinct compartments: the cortex, containing cells active in synthesis of corticosteroid hormones, and the medulla which is of neural origin and contains chromaffin cells and catecholamine storage granules.

Subcellular membranes can be isolated from homogenates of the adrenal by differential and sucrose gradient centrifugation techniques (1). An earlier study of the lipids of microsomes, mitochondria and chromaffin granules of the bovine adrenal medulla indicated the presence of large amounts of lysolecithin in the chromaffin granule fractions, comprising up to 17% of the total lipid phosphorus (2). Since this characteristic feature was not found in other types of membranes in the adrenal, the enrichment in lysolecithin in these catecholamine storage granules was implicated in the coupled stimulus-secretion activity of the chromaffin cells, especially during the process of exocytosis which involves membrane fusion (3). The role of lysolecithin in membrane fusion has been suggested because of its detergent property (4). In addition to lysolecithin, other types of lyso-compounds, such as lyso-glycerophosphoethanolamines (GPE), were also found to be present in the granule membranes (5).

Apparent differences in membrane lipid composition were shown in comparing the subcellular membranes from adrenal medulla and cortex. A plasma membrane fraction isolated from the postmitochondrial supernatant of bovine adrenal medulla indicated an enrichment in sphingomyelin (6), but the plasma membranes isolated from the bovine adrenal cortex showed a low level of sphingomyelin instead (7). Apparently, differences in lipid composition may also exist among membranes of the subcellular fractions of the two morphologically distinct regions of the gland. In spite of the importance in adrenal membranes in relation to their functions, little is known about the acyl group composition of the membrane phosphoglycerides. The present study compares the phospholipid and acyl group composition of subcellular membranes isolated from guinea pig adrenals and bovine adrenal medulla and cortex. Information from this study would be useful in aiding future studies regarding the structure and function of membranes isolated from the adrenal gland.

MATERIALS AND METHODS

Guinea pigs were obtained from Camm Research Laboratory (Wayne, NJ), and bovine adrenals were obtained from the University slaughter house. Adrenals from both species were excised immediately after sacrifice, and bovine adrenals were transported to the laboratory in ice within 30 min of dissection. The

fatty material surrounding the adrenal was trimmed off carefully while maintaining the adrenal in ice-cold condition. The medullary portion of the guinea pig adrenal was dark brown in color and contained loosely packed cellular material which was not clearly separated from the cortical region. Therefore, no further attempt was made to separate the medulla from the cortex, but tissue was briefly sliced. Normally, adrenals from two guinea pigs were homogenized in 20 vol (by wt.) of ice-cold 0.32 M sucrose with 1 mM EDTA, 1 mM Mg^{++} and 50 mM Tris-HCl (pH 7.4) using a Teflon-pestle Tri-R tissue homogenizer. The homogenate was pooled prior to subcellular fractionation procedure. The medullar portion of the bovine adrenal was dissected and separated from the cortical portion, and the tissues were homogenized individually in sucrose medium. The tissue homogenates were subjected to differential and sucrose gradient centrifugation to obtain the microsomes, mitochondria and chromaffin granules according to the procedure described by Smith and Winkler (1). The chromaffin granules isolated from the guinea pig adrenal formed a dark brown pellet, whereas the same fraction isolated from the bovine adrenal medullae had a pinkish-beige color. Subcellular fractions were verified by taking aliquots of the samples for electron microscopic examination. Practically no intact mitochondria was found in the microsomal fraction.

The membrane pellets were suspended in H_2O and a 4 vol of chloroform/methanol (2:1, v/v) was added to each membrane suspension for extraction of the lipids. After phase separation, the lower organic layer was taken to dryness and the lipid residue was redissolved in chloroform and stored at -10 C until further analysis.

The phospholipids of adrenal membranes were separated by two dimensional thin layer chromatography (TLC) using Silica Gel G plates (8). Briefly, lipids were first separated by TLC using a solvent system containing chloroform/methanol/15N NH_4OH (130:65:10, v/v). After development in the first dimension, the plates were exposed briefly to HCl fumes in order to cleave the alkenyl side chain of phosphoglycerides. After HCl reaction, the plates were inverted and further developed in a solvent system containing chloroform/methanol/acetone/acetic acid/0.1M ammonium acetate (130:50:55:3.5:10, v/v). Lipid spots were visualized either by exposing the TLC plates to iodine vapors when lipid phosphorus was determined (9), or by spraying with 2′7′-dichlorofluorescein when the acyl groups of phosphoglycerides were to be converted to methyl esters by alkaline methanolysis (10). The fatty acid methyl esters were separated by gas liquid chromatography (GLC) using a Hewlett-Packard GLC model 3850A equipped with dual flame detectors and automatic peak integration device. The conditions for separation of fatty acid methyl esters by columns packed with EGSS-X on Gas Chrom P (Applied Science Laboratories, State College, PA) were essentially the same as described previously (11).

RESULTS

Substantial differences were found regarding the phospholipid content of guinea pig and bovine adrenals. In general, the phospholipids of guinea pig adrenal were enriched in diacyl-GPC and therefore contained lower levels of sphingomyelin, choline and ethanolamine plasmalogens and diacyl-GPS as compared to the phospholipids in the bovine adrenal membranes (Table I). On the other hand, the bovine adrenal seemed to be especially enriched in ethanolamine plasmalogens. Although the presence of lysolecithin was prominent in the bovine adrenal membranes, only trace amounts of this compound could be detected in the guinea pig adrenal.

A typical TLC separation of the phospholipids in microsomes and mitochondria of guinea pig adrenal is shown in Figure 1. Obvious differences are found when comparing the phospholipids of mitochondria and microsomes. For example, the phospholipids in mitochondria were enriched in diacyl-GPC, whereas alkenylacyl-GPE was more prominent in the microsomal fraction instead. In this separation, diacyl-GPS is present in the microsomes but not mitochondria, and phosphatidate and cardiolipin are found mainly in the mitochondria but not microsomes. Most of these differences correlated well with the data indicated in Table I. The chromaffin granules isolated from guinea pig adrenal indicated a phospholipid profile similar to both the mitochondrial and microsomal fractions, except that there was less alkenylacyl-GPC and more alkenylacyl-GPE present in the granule membranes than in other fractions.

When the phospholipids of microsomes and mitochondria from the bovine adrenal cortex and medulla were compared, the microsomes in both cortex and medulla indicated a higher proportion of alkenylacyl-GPE than the mitochondria. On the other hand, the mitochondria was specifically enriched in cardiolipin. The chromaffin granules isolated from the bovine medulla gave a phospholipid profile resembling that of the mitochondria with the exception

TABLE I

Phospholipid Composition of Adrenal Membranes[a]

	Guinea pig			Bovine				
	Adrenal			Cortex		Medulla		
Phospholipids	Microsomes	Mitochondria	Chromaffin granules	Microsomes	Mitochondria	Microsomes	Mitochondria	Chromaffin granules
				mole, %				
PA	---	2.1	---	0.7	2.3	1.3	0.4	1.0
GPS	1.6	3.3	1.4	6.2	5.7	8.6	4.6	4.0
GPI	9.5	7.8	7.6	10.1	12.2	2.9	2.8	2.0
Sph	3.3	3.4	2.9	8.0	8.8	11.2	11.6	8.9
aGPE	4.0	3.0	10.5	23.9	10.5	21.1	18.3	29.7
dGPE	25.5	16.2	25.8	19.3	16.8	12.8	22.2	22.0
aGPC	2.4	3.2	---	6.0	5.2	3.2	4.9	1.7
dGPC	52.2	54.9	46.7	21.6	24.7	34.3	25.0	26.1
CL	1.1	6.2	5.1	3.3	10.1	0.8	8.1	0.2
LPC	tr	tr	tr	1.1	3.7	4.1	2.3	4.4

[a]Lipid extracts were separated by two dimensional TLC procedure as described in text. The lipid spots were taken for lipid phosphorus determination (9). Abbreviations used: PA, phosphatidic acids; GPS, diacyl-glycerophosphoserines; GPI, diacyl-glycerophosphoinositols; Sph, sphingomyelin; aGPE, alkenylacyl-glycerophosphoethanolamines; dGPE, diacyl-glycerophosphoethanolamines; aGPC, alkenylacyl-glycerophosphocholines; dGPC, diacyl-glycerophosphocholines; CL, cardiolipins; LPC, lyso-glycerophosphocholines; tr, trace.

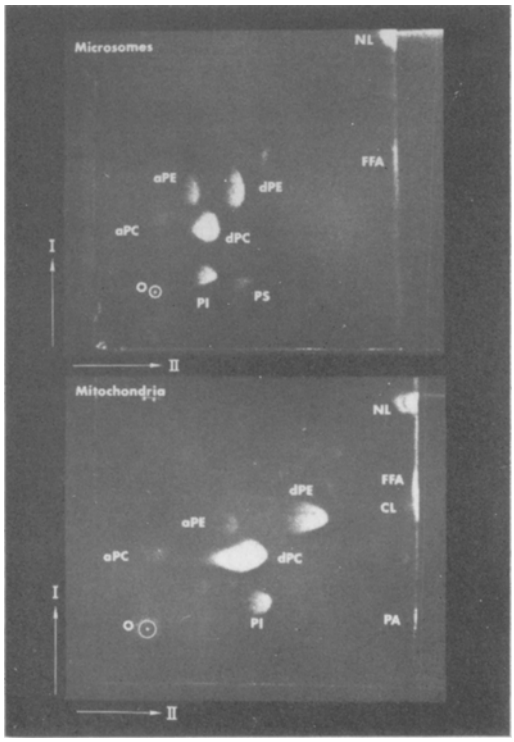

FIG. 1. A typical two dimensional TLC separation of lipids from guinea pig adrenals. Refer to Table I for abbreviations of phospholipids. O, origin. FFA, free fatty acids; NL, neutral lipids.

that more alkenylacyl-GPE and less cardiolipin were present. It is interesting to find that membrane phospholipids in the bovine cortex contained less diacyl-GPS than diacyl-GPI, whereas those in the medulla contained more diacyl-GPS than diacyl-GPI.

The acyl groups of phosphoglycerides in the guinea pig and bovine adrenals are comprised mainly of 16:0, 18:0, 18:1 and 20:4 (Tables II and III). Some minor components were also present, such as 14:0, 18:2, 20:3, 22:4 and 22:5. However, the level of 22:4 and 22:5 was, in general, higher in the bovine adrenal than in the guinea pig adrenal. The differences in acyl group profiles of phosphoglycerides among the subcellular membranes are small with the exception that the proportion of 20:4 in diacyl-GPC and diacyl-GPE in the mitochondrial membranes is normally higher than the microsomal membranes. The chromaffin granules from bovine medullae indicated a relatively small amount of 20:4 in the phosphoglycerides, but this feature was not as prevailing in the chromaffin granules isolated from the guinea pig adrenals.

TABLE II

The Acyl Group Composition of Guinea Pig Adrenals[a]

Acyl groups	diacyl-GPC			diacyl-GPI			diacyl-GP		diacyl-GPE			alkenylacyl-GPE		cardiolipin
	Micro	Mito	Gran	Micro	Mito	Gran	Micro	Mito	Micro	Mito	Gran	Micro	Mito	Mito
14:0	0.6	0.6	0.6	---	---	---	---	---						
15:0	0.5	0.5	0.5	---	---	---	---	---						
16:0	30.0	31.2	31.4	3.1	3.2	3.8	4.5	---	5.6	5.0	8.4	2.6	---	2.2
18:0	10.4	9.2	9.3	45.6	46.6	46.9	43.9	43.9	26.8	29.9	26.4	4.4	9.7	5.6
18:1	41.1	37.4	40.1	10.1	9.6	10.6	27.1	28.7	30.7	21.5	26.5	25.8	14.4	41.0
18:2	7.1	7.2	7.2	3.0	2.7	3.1	3.8	6.0	5.5	3.5	5.2	9.4	4.7	45.9
18:3	0.7	0.7	0.6	---	---	---	---	---	0.8	0.3	---	1.8	---	3.2[b]
20:3	0.4	0.8	---	---	---	---	---	---	---	---	---	---	---	---
20:4	9.3	11.7	10.4	38.1	37.9	35.7	20.8	21.5	29.8	35.5	33.6	50.1	56.8	6.6
22:4									0.7	1.0	---	2.1	5.7	---
22:5[b]									---	---	---	3.9	8.8	---

[a]Acyl groups from individual phosphoglycerides were converted to their methylesters by base methaolysis. Results are expressed as % weight of the total.
[b]Fatty acids chain length and unsaturation tentatively identified.

TABLE III

Acyl Group Composition of Phosphoglycerides from Bovine Adrenals

Acyl groups	Cortex		Medulla		
	Micro	Mito	Micro	Mito	Gran
		diacyl-GPC			
16:0	30.7	27.1	33.2	30.2	35.8
18:0	18.3	16.6	16.0	14.6	15.0
18:1	31.0	27.4	32.0	29.4	32.4
18:2	8.6	8.8	10.0	10.2	10.3
20:4	10.1	17.4	8.7	15.1	6.6
22:4	0.8	1.1	0.2	0.5	---
		diacyl-GPE			
16:0	6.9	2.0	11.2	4.6	6.8
18:0	46.2	33.6	40.8	45.2	44.4
18:1	19.5	14.7	16.8	14.8	20.7
18:2	4.5	5.9	2.1	5.0	4.9
20:4	18.0	35.5	24.9	27.7	21.1
22:4	4.0	7.4	4.2	2.8	2.1
22:5[a]	0.8	0.9	---	---	---
		alkenylacyl-GPE			
16:0	4.1	3.2	2.6	1.1	5.5
18:0	2.5	7.2	2.4	5.2	3.2
18:1	9.2	8.9	7.7	5.3	8.6
18:2	2.6	3.8	1.3	1.4	2.3
20:4	62.5	64.0	70.6	71.1	70.7
22:4	13.4	9.6	13.0	11.8	9.7
22:5[a]	5.7	3.3	2.4	4.1	---
		diacyl-GPI			
16:0	3.8	5.9	3.4	---	1.8
18:0/18:1[b]	69.3	57.2	60.8	54.5	72.9
18:2	2.2	2.0	0.9	---	---
20:4	23.5	32.4	34.9	43.8	25.3
22:4	1.2	2.5	---	1.7	---
		diacyl-GPS			
16:0	2.7	1.1	1.0	2.5	3.2
18:0	52.0	51.3	47.9	48.3	52.4
18:1	40.3	40.6	40.3	34.1	30.1
18:2	4.6	4.1	4.7	5.2	4.1
20:4	2.8	2.0	4.0	5.5	6.7
22:4	1.8	2.0	1.8	4.0	3.5
		lysolecithin			
16:0	20.1	---	23.9	9.3	29.8
18:0	55.0	---	63.3	67.9	56.8
18:1	13.4	---	---	13.5	10.8
18:2	---	---	---	---	---
20:4	11.5	---	12.8	9.3	2.6

[a]Fatty acids' chain length and unsaturation tentatively identified.

[b]18:1 appeared as a shoulder and was not separated from 18:0.

Characteristic acyl group profile is found associated with individual types of the phosphoglycerides in the adrenal gland. The diacyl-GPC were enriched in 16:0, 18:1 with 18:0 and 20:4 in almost equal proportions. The diacyl-GPE were enriched in 18:0, 18:1 and 20:4, whereas the acyl groups of alkenylacyl-GPE yielded over 50% of 20:4 and only small amounts of 16:0 and 18:0. The acyl groups of diacyl-GPI were characterized by a high proportion of 18:0 and 20:4. The cardiolipin from mitochondria yielded acyl groups containing

mainly 18:1 and 18:2 (over 85% of the total). The acyl groups of lysolecithin were comprised mainly of 18:0, in contrast to the high proportion of 16:0 normally present in diacyl-GPC. Due to the small amount of diacyl-GPS present in the guinea pig adrenals, its acyl group composition was not determined. However, the diacyl-GPS in bovine adrenals indicated a profile quite similar to that of diacyl-GPI, except for a lower level of 20:4.

DISCUSSION

As shown from the results obtained, the phospholipids in guinea pig adrenal gland are considerably different from those in the bovine gland. In general, the guinea pig adrenal is more enriched in diacyl-GPC, whereas the phospholipids in the bovine adrenal showed a higher level of ethanolamine plasmalogens instead. It is important to consider these species differences and to relate the characteristic lipids to their physiological functions. With minor exceptions, the phospholipid composition of chromaffin granules from bovine adrenal medulla is in good agreement with that reported recently by Dreyfus et al. (5). The chromaffin granules from both guinea pig and bovine adrenals are specially enriched in ethanolamine plasmalogens. Although the functional significance of ethanolamine plasmalogens in catecholamine storage granules has not been explored, it is obvious from analysis of their acyl group composition that this type of phospholipid is highly enriched in arachidonoyl group. In the present study, some lysolecithins (up to 5%) are found in the chromaffin granules from bovine adrenal medulla, but similar level of this compound are also present in other subcellular fractions of bovine adrenal medulla. Surprisingly, only trace amounts of lysolecithins were found in the chromaffin granules as well as other subcellular fractions of the guinea pig adrenal. The apparent differences in lysolecithin content in bovine chromaffin granules (17% from the study of Blaschko et al. (2), 12% from Dreyfus et al. (5) and 5% from the present study) and the virtual absence of detectable amount in the granules from guinea pig adrenal can be explained if the accumulation is due to postmortem autolysis, since an unpredictable length of time is required for dissection of the adrenals from large size animals. Much less time is involved in dissection of the adrenals from guinea pigs. As suggested by Winkler (3) and from our study (Der and Sun, unpublished), postmortem accumulation of lysolecithin in chromaffin granules can also be attributed to enzymic factors such as postmortem activation of phospholipase A_2 activity or the lack of acyltransferase in these granule membranes to reacylate the lysolecithin back to diacyl-GPC.

Since the adrenal cortex and medulla are functionally and morphologically different, it is not surprising to find differences in membrane phospholipid composition related to the type of cells present in each region. The membrane phospholipids from bovine medulla give a closer resemblance to the phospholipids of neural tissues, especially with regard to its high sphingomyelin and ethanolamine plasmalogen content. The results are consistent with previous findings indicating that sphingomyelin was more enriched in the medullar (6) than in the cortical membranes (7). Another difference between the medulla and cortex is the relatively higher level of diacyl-GPI which is associated especially with the cortex.

The acyl groups of diacyl-GPC are high in 16:0 and 18:1, but considerable amounts of 18:0 and 20:4 (around 10% each) are also present. The acyl groups of diacyl-GPE are enriched in 18:0 and 20:4, but other types of longer chain polyunsaturated acyl groups such as 22:4 are also present in smaller amounts. Arachidonate (20:4) is prominent in alkenyl-acyl-GPE, diacyl-GPI and even in diacyl-GPS. Thus, the acyl groups of adrenal membrane phospholipids are highly enriched in arachidonoyl groups. Unlike the neural tissue, little 22:4 and practically no 22:6 are present in the adrenal membranes. Vahouny et al. (12) had recently reported a similar fatty acid profile for the phospholipids of adrenal cortical cells. Their study also indicated a low level of 22:4 in total phospholipids of these cells. On the other hand, a large amount of 22:4 (adrenic acid) as well as other longer chain polyunsaturated fatty acids were found associated with the cholesteryl esters in the adrenal gland (12). It is important to consider the differences in acyl group specificity between the phosphoglycerides and cholesteryl esters in the adrenal, since the adrenal cortex is highly enriched in cholesteryl esters and both 20:4(n-6) (from phosphoglycerides) and 22:4(n-6) (from cholesteryl esters and, to a smaller extent, ethanolamine plasmalogens) are active precursor fatty acids for prostaglandin biosynthesis. It is apparent that the characterisitc phospholipid and acyl group composition in adrenal membranes are in direct association with their cellular functions which remain to be explored.

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Synthesis of Phosphatidylcholine and Phosphatidylethanolamine at Different Ages in the Rat Brain in vitro

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ABSTRACT

The de novo synthesis of choline and ethanolamine phosphoglycerides in brain microsomes from 18 month-old male rats was investigated in vitro by using labeled cytidine-5′-diphosphate choline and cytidine-5′-diphosphate ethanolamine as lipid precursors. The rate of synthesis of the two phospholipid classes was found to be noticeably decreased, as compared to that of adult animals. The addition of exogenous diacyl glycerols to microsomes from ageing rat brain brings the rate of synthesis nearly to the adult levels. The synthesis of choline and ethanolamine phosphoglycerides is not affected in the liver microsomes of ageing rats. The molar distribution of fatty acids in brain microsomal diacyl glycerols of ageing rats is noticeably different from that of adult animals. The content of monoenoic and dienoic species is increased, whereas that of the tetraenoic species is decreased. Base exchange reaction for choline and ethanolamine incorporation into respective phospholipids is not affected in the brain microsomes of the aged rats.

INTRODUCTION

Ageing in the nervous system is characterized by a number of morphological and neurochemical alterations which occur in man and many other mammalian species. The process is undoubtedly complex, and involves also biochemical changes in neural substrates, membranes, molecules and ions which have behavioral correlates during ageing. The most important task in the study of these changes is to find out their functional significance in terms of mechanisms responsible for ageing (1,2).

A survey of current neurochemical literature (1-5) has reported only a very limited number of results dealing with changes in structure and turnover of lipid during ageing in experimental animals. Interesting results have been reported, so far, on composition and fatty acyl profile of brain lipid during ageing (6-9). It is known, on the other hand, that brain cells are continuously synthesizing new molecules of ethanolamine and choline phosphoglycerides (EPG and CPG) from low molecular weight precursors (see ref. 10) by a "net synthesis" process, and that they are able to modify hydrophobic acyl chains (10) or hydrophylic polar heads (11,12) of cellular phospholipids, respectively, by transacylations or base exchange reactions. The present work reports on the synthesis and turnover of the main rat brain phospholipids at different ages, and the parallel experiments on the same biochemical parameters in ageing rat liver.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats from our own animal house, weighing 120 ± 10 g body wt (8 weeks old), were used as adult animals. They were accurately selected from birth in order to always use specimens of the same age (56 days) which were caged in groups of 4-5 individuals. "Aged" male Wistar rats, caged individually, were obtained from I.S.F. (Milan, Italy) and were either 18 months or 20 months old; ages were accurately recorded from birth. All animals, either adult or "aged," were fed Purina Laboratory Chow and kept under identical ambiental and hygienic conditions. Water was given ad libitum. The rats were always killed by decapitation around 8 a.m. after 12 hr of fasting, and treated as follows.

Microsomes

Brain microsomes were prepared and purified, as described elsewhere (13,14). They were suspended by hand homogenization in 0.32 M sucrose containing 2 mM dithiothreitol to give a concentration of 8-10 mg protein/ml. Liver microsomes, when indicated, were prepared and purified as reported by Binaglia et al. (15). The purity of the microsomal fraction from both tissues was assessed by published procedures (13), and the microsomes used immediately.

Incubation

Unless otherwise specified, "net synthesis" of 3-*sn*-phosphatidylcholine (Ptd-Cho) and 3-*sn*-phosphatidylethanolamine (Ptd-Etn) was examined by incubating at 40 C for 40 min with labeled cytidine-5′-diphosphate-choline (CDP-Cho) and cytidine-5′-diphosphate-ethanolamine (CDP-Etn), respectively, in the following incubation medium (0.2 ml of final volume): 50 mM Tris-HCl buffer (pH 8.0), 1.24 mM labeled CDP-Cho (CMP-[1,2-^{14}C]choline-phosphate) at the specific activity of 1.95 μCi/μmole or 1.34 mM labeled CDP-Etn (CMP-

[1,2-^{14}C]ethanolamine phosphate) at the specific activity of 3.65 μCi/μmole, liver or brain microsomal protein (ca. 0.5 mg) and 10 mM $MnCl_2$. Components were added in the indicated order. Normally, incubation was carried out without addition of exogenous diacyl glycerols; when these lipid acceptors were added (see Results), diacyl glycerols were prepared as further explained.

Unless otherwise stated, base exchange reaction for Ptd-Cho and Ptd-Etn synthesis was carried out by incubating in 0.5 ml of final volume the purified brain or liver microsomes (0.5-1 mg protein) in a medium containing 2.5 mM $CaCl_2$, 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid) buffer at pH 8.1, 0.32 M sucrose containing 2 mM dithiothreitol, and 2.5 mM [1,2-^{14}C]ethanolamine (sp. act. of 0.196 μCi/μmol) or 4.5 mM [1,2-^{14}C]choline (sp. act. of 0.418 μCi/μmol). Incubation time and temperature respectively were 20 min and 37 C.

Lipid Analysis and Enzymic Assays

Incubations were terminated by adding 9 vol of chloroform/methanol (1:1, v/v), followed by a brief agitation. After one more extraction and filtration, the extracts were freed from contaminants, purified as described elsewhere (13), and finally taken to dryness under nitrogen. Intact phospholipid classes were fractionated and identified on thin layer chromatography (TLC) plates, according to Gaiti et al. (16) by using chloroform/methanol/H_2O (65:25:4, v/v/v), as the solvent system. Counting of labeled lipids was carried out, as reported elsewhere (17), in a Packard Tri-Carb Model 3330 liquid scintillation spectrometer, by the external standard method for quenching correction. Pure labeled lipid standards were used as reference compounds. Each experiment was normally done in duplicate or quadruplicate, and then mediated.

Diacyl Glycerols

Preparation from soybean lecithin. 1,2-*sn*-diacyl glycerol was prepared from purified (18) soybean lecithin (Sigma Chemical Co., St. Louis, MO) by the action of phospholipase C from *Clostridium welchii* (Sigma Chemical Co.), as described elsewhere (19). Diacyl glycerols were then purified through silicic acid columns with successive analysis of purity by TLC (18). Diacyl glycerol emulsions at 20-40 mM concentration were prepared in a solution containing 0.1% (w/v) Tween-20 and 0.05% (w/v) bovine serum albumin (BSA), by sonicating at the MSE Ultrasonic Disintegrator (100 W) for 1 min, three times each. Diacyl glycerol content was then measured exactly in the sonicate according to Wieland (20), and given amounts taken for incubation, when appropriate. Diacyl glycerol was normally kept at 4 C, if not used immediately.

Preparation from brain or liver microsomes. The neutral lipid fraction was extracted from microsomal membranes with cold acetone containing 1°/$_{oo}$ 2,6-di-*tert*-butyl-4-methylphenol (Aldrich Chemical Co., Inc., Milwaukee, WI) as antioxidant. The extract was dried, dissolved in pure chloroform, and resolved by TLC with diethyl ether/petroleum ether (50-70 C)/acetic acid (30:70:1, v/v) to isolate the diglyceride fraction. The diacyl glycerol was successively eluted from the plates with chloroform containing the antioxidant as above and then treated for 120 min at 70 C with 3% H_2SO_4 in methanol/benzene (1:1, v/v), to obtain the corresponding methyl esters.

Analysis. Microsomal diglyceride composition was estimated by gas liquid chromatography (GLC) of the corresponding methyl esters using a Fractovap Mod. G.C.V. gas liquid chromatography, (Carlo Erba, Milan, Italy). The stationary phase was 5% ethyleneglycol-adipate on sylanized Chromosorb-W (100-200 mesh) in 200 x 0.2 cm glass columns. Nitrogen flow was 20 ml/min, and column and detector temperatures were 180 C and 230 C, respectively. Quantitation was done as reported elsewhere (21). Analysis of the diglyceride fraction from soybean Ptd Cho indicated the presence of 70% 18:2.

Other Determinations

Marker enzyme assays were done as described elsewhere (13). Protein was determined according to Lowry et al. (22), using crystalline BSA as the standard. Sucrose did not interfere with the protein estimation in the concentration used. Phospholipid P content of spots was measured as reported elsewhere (15).

Materials

[1,2-^{14}C] Ethanolamine, [1,2-^{14}C]choline and CMP-[*Me*-^{14}C]phosphorylcholine (labeled CDP-Cho) were purchased from New England Nuclear Corp. (Frankfurt, West Germany). CMP-[1,2-^{14}C]phosphorylethanolamine (labeled CDP-Etn) was obtained from ICN (Cleveland, OH). Purified lipids, used as reference standards, were obtained from Pierce Chemical Co. (Rockford, IL). Before use, all unlabeled and/or labeled substrates were tested by TLC for their chemical purity and/or their isotope content and radiochemical purity. Trace contaminants have never been detected by the use of radiochromatoscanner. Organic

TABLE I

The Rate of Synthesis of Choline and Ethanolamine Phosphoglyceride Subclasses in Brain and Liver Microsomes of Adult and Aged Male Wistar Rats[a]

Lipid subclasses	Tissue	Adult rats[b]	Aged rats[c]	Decrease (%)
Diacyl-GPC	Brain	1.13 (9)	0.70 (5)[e]	-38
Alkenylacyl-GPC		0.33 (9)	0.17 (5)[d]	-49
Diacyl-GPE		1.01 (5)	0.59 (5)[e]	-41
Alkenylacyl-GPE		0.13 (5)	0.06 (5)[d]	-54
Diacyl-GPC	Liver	3.81 (8)	3.10 (7)[f]	-18
Alkenylacyl-GPC		1.18 (8)	0.92 (7)[f]	-22
Diacyl-GPE		1.02 (7)	0.86 (6)[f]	-16
Alkenylacyl-GPE		0.48 (7)	0.40 (6)[f]	-17

[a]Experiments (number between brackets) have been carried out as reported in the text. Data are expressed as nmol/mg protein/40 min. The average of the standard deviation values was ca. 10%. Diacyl-GPC, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; alkenylacyl-GPC, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphorylcholine; diacyl-GPE, 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine; alkenylacyl-GPE, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphorylethanolamine.

[b]Fifty-six day-old rats.

[c]Eighteen month-old rats.

[d]$P < 0.001$.

[e]$P < 0.01$.

[f]Statistically not significant.

solvents were all freshly distilled and freed from peroxides, and stored under nitrogen.

RESULTS

Before referring to the results of this work, it must be mentioned that all incubations have been carried out always in the presence of saturating concentrations of substrate (labeled choline, CDP-choline, ethanolamine, CDP-ethanolamine) and for time intervals so as to assure zero-order kinetic rates, as determined by preliminary experiments and with reference to previous work (16-18, 23-26).

Synthesis of Choline and Ethanolamine Lipids in Ageing Rats

The last step in the de novo synthesis of CPG and EPG in brain is carried out by the correspondent phosphotransferases (EC 2.7.8.1 and 2.7.8.2), which transfer phosphoryl-ethanolamine or phosphorylcholine from their corresponding cytidine nucleotides to the diacyl glycerol (10). The activity of microsomal membranes from ageing rat brains to form lipid material through this reaction was investigated by incubating brain microsomes from ageing rats with labeled CDP-Etn or CDP-Cho and without the addition of diacyl glycerol, and by comparing the results with those obtained with liver microsomes.

Table I shows that the rate of conversion of water-soluble intermediates to CPG and EPG subclasses is sensibly reduced in the brain microsomes of ageing rats. The variation between adult and ageing rats was highly significant. In addition to these data, preliminary experiments had shown that the kinetics of diglyceride utilization by the brain microsomes of the aged rats was exactly similar to that already reported elsewhere for adult rats (18,23). Moreover, the main concentration value of diglyceride content in the microsomes of aged rats was similar to that reported elsewhere (21), i.e., around 9-10 nmoles/mg microsomal protein. Table I shows also that the differences between the rate of synthesis of liver CPG and EPG subclasses in adult and ageing rats are not so striking as in the brain. In fact, both alkenylacyl and diacyl subclasses are synthesized in the liver at almost similar rates in both type of animals.

Subsequent experiments were carried out by incubating the brain microsomal membranes in the presence of saturating concentrations of externally added diacyl glycerols (19,23) in order to minimize the contribution of the small amount of endogenous microsomal diacyl glycerols in the overall rate of reaction in both aged and control animals. By taking into account previous work (19,23) and with preliminary experiments, it was checked that the concentrations of diacyl glycerols used were really saturating. Interestingly, no significant variation was seen in the rate of synthesis of CPG and EPG between control and aged rat brains (Table II), in contrast with the data

TABLE II

The Rate of Synthesis of Choline and Ethanolamine Phosphoglycerides in Brain Microsomes of Adult and Aged Rats in vitro, after the Addition of Exogenous Diglycerides[a]

Lipid class	Diglyceride (mM)	Adult rats	Aged rats[b]	Decrease (%)
EPG	4.0	128	121	-6
	6.5	134	118	-12
	7.8	139	117	-16
	10.5	145	126	-13
	15.7	147	114	-22
CPG	5.0	143	120	-16
	7.8	150	116	-22
	10.5	143	114	-20

[a]Sonication was carried out at room temperature with small probes, as reported in the text. Immediately after sonication, incubation with labeled CDP-Etn or CDP-Cho was carried out at 40 C for 40 min. Diacyl glycerol concentration was estimated as free glycerol, before addition. Data are expressed as nmol/mg protein/40 min and represent values from four experiments, whose deviation values were less than 10%. EPG = ethanolamine phosphoglycerides; CPG = choline phosphoglycerides.

[b]Variations in the aged rats were not statistically significant, if compared to the values obtained from the adult rats.

TABLE III

Composition of Brain Microsomal Diacyl Glycerol in Adult and Aged Rats[a]

Fatty acid	Adult rats	Aged rats	Variation (%)
16:0	24.7	23.3	---
16:1	5.7[b]	9.5	+ 67
18:0	27.0	25.0	---
18:1	17.3	20.9	+21
18:2	1.7	3.2	+88
18:3	traces	traces	---
20:4	22.7	17.9	-21
22:6	traces	traces	---

[a]Data are expressed as mole % of diacyl glycerol, and represent mean values from four experiments each. Variation for each value was within 10%.

[b]This value represents the highest among four levels, whose scatterring was larger.

reported in Table I. In separate experiments (results not shown), it was also observed that the addition of diacyl glycerols to the liver microsomal membranes did not change, on the contrary, the data of Table I.

Composition of Diacyl Glycerols in the Brain Microsomes of Ageing Rats

A series of experiments was subsequently carried out by determining the fatty acyl composition of the brain microsomal diacyl glycerols in the adult and aged rats, with the aim of relating possible variations of this composition to the different rates of the phosphotransferase reaction reported in Table I. Table III shows a noticeable variation in the fatty acyl content of microsomal diacyl glycerol of the aged rat brain, as compared to the adult animals. More precisely, the percent of the content of monoenoic and dienoic species increases noticeably in the ageing rat brain diacyl glycerol with a corresponding decrease of that of arachidonate. This variation might influence the phosphotransferase activity, since previous work (27) had reported that different reaction rates take place in brain microsomes with the use of single different diacyl glycerols as substrate.

No evident variation was noticed in the fatty acid composition of the liver microsomal diglycerides between adult and aged rats (results not shown), and this finding might help in explaining the differences found between the rate of phosphotransferase reaction in brain and liver microsomes in the adult and aged rats (Table I).

Base Exchange Reactions in Ageing Rats

As known (see refs. 11 and 12), free ethanolamine and choline are incorporated, respectively, into brain EPG and CPG subclasses, also by a Ca^{2+}-dependent base exchange mechanism particularly located at the microsomal level. Due to this localization, this pathway was also examined in the brain microsomal membranes of adult and aged rats. Figure 1 indicates that no significant variation of this activity takes place in the brain microsomes of aged rats as compared to the adult values; the data have been completed by examining also the base exchange activity in the rat brain microsomes of 12 month- and 20 month-old rats, with the aim of examining other age intervals. Table IV shows, in addition, that plasmalogen synthesis by base exchange (14) also is unaffected in the brain microsomes of the aged rats. However, a noticeable decrease in the rate of exchange of ethanolamine and choline takes place in rat liver microsomes in aging animals as compared to adults.

In order to get more information about the membrane composition of adult and aged rat brain, estimation has been carried out of the phospholipid/protein ratio of brain and liver microsomes of these animals. No variation of the ratio has been found in the microsomes of both liver and brain in the aged rats, as compared to controls.

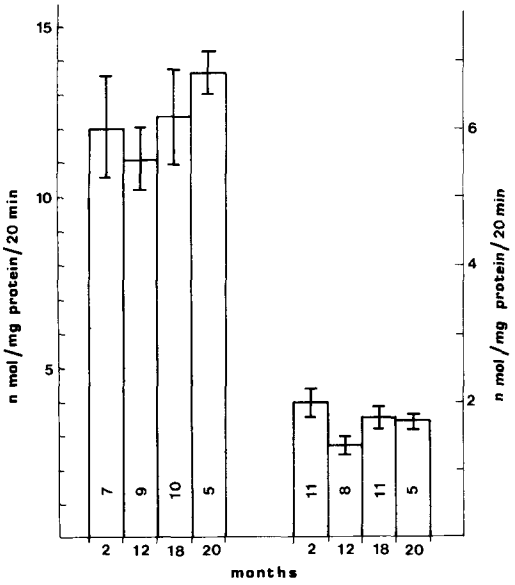

FIG. 1. Base exchange activity in brain microsomes of aged and adult rats. On the left, ethanolamine incorporation into EPG (values on the left); on the right, choline incorporation into CPG (values on the right). The exchange activity (nmol base incorporated/mg protein/20 min) is plotted against age (number of months). Bars are represented with their standard deviation values. Number of experiments for each value is inserted in the bars. See the text, for other details.

DISCUSSION

Rats of 18 months of age have been used in the present work as "ageing" animals. Although there is still much discussion about initiation of ageing, it is generally accepted, however, that in the rat, which has a life span of 2 to 3 years, the 18 months represents a time interval of reasonable length to reproduce at least the initial ageing phenomena. As known, the ageing is thought to begin at the time of reproductive and physical maturity (28) and must be particularly related to changes which possess behavioral correlates. Rats older than 18 months of age have been found often to limit experimentation due to unavoidable complications or frequent deaths and to participation of various factors which are only partially related with the ageing process. In addition, it has been found in this laboratory (unpublished observations) that male Wistar rats of 20,22, or 24 months of age

TABLE IV

Incorporation by Base Exchange of Choline and Ethanolamine into Respective Plasmalogens of Brain and Liver Microsomes of Adult and Aged Rats[a]

Source	Lipid subclass	Adult rats[b]	Aged rats[c]	Decrease (%)
Brain	Choline plasmalogen	0.162 ± 0.032(5)	0.173 ± 0.017(5)	---
	Ethanolamine plasmalogen	1.138 ± 0.17 (6)	1.023 ± 0.09 (6)	-10
Liver	Choline plasmalogen	0.330 ± 0.053(5)	0.237 ± 0.050(5)	-28
	Ethanolamine plasmalogen	2.699 ± 0.32 (5)	1.975 ± 0.21 (5)	-27

[a]Experiments (number between brackets) have been performed, as reported in the text. Data are expressed as nmol base incorporated/mg protein/20 min ± S.E.M.

[b]Fifty-six day-old rats.

[c]Eighteen month-old rats.

represent often unreliable tests of experimentation, due to the very large scattering of data to which they give rise, at least in relation to phospholipid turnover.

Interactions among cells of different origin and function in brain are very complex and critical. Liver represents, in this respect, a more homogeneous and less complex structure, although different cell types do occur also in this organ. For this reason, it has been found useful to compare some results of phospholipid metabolism obtained in the two organs.

The main findings of the present study indicate that the rate of synthesis of EPG and CPG in ageing rat brain is noticeably and significantly reduced if compared to that of adult animals (Table I). The main experimental evidence suggests that a significant variation of the microsomal diacyl glycerol composition takes place in the brain of ageing rats as compared to that of the control animals (Table III), and that this variation, rather than an enzymic defect, is the most probably cause of the different rate of the phosphotransferase reaction taking place during ageing in brain. It is known, in fact, that the molecular forms of diacyl glycerols are utilized at different rates by brain microsomes (27) and that a certain degree of specificity for different diacyl glycerols exists in rat brain (21,27). Thus, the change of molar distribution of the microsomal diacyl glycerol molecular species, which takes place in ageing brain (Table III), might contribute particularly to the change of synthetic rate. This assumption is substantiated also by the results of Table II, which indicates that much smaller variation is seen between adult and ageing brains, when their microsomes are supplemented with exogenous diglycerides. This, of course, might not be the only cause, since the phosphotransferase reaction represents only one of the various steps in the overall pathway of phospholipid synthesis in animal tissue; it is worth mentioning, in this connection, that the enzymic reaction catalyzed by the cytidylyltransferases (E.C. 2.7.7.14 and 2.7.715) is most probably the really rate-limiting step in the whole process (19,23).

It is known that the fatty acyl profile of endogenous phospholipid has some effect on the rate of the base exchange reaction in brain microsomes (29,30). It has been reported also that the fatty acid pattern of whole brain phospholipid is significantly affected during the ageing period (8), and one might expect, therefore, that base exchange activity is altered in senescent brain. On the other hand, we have been unable to find out noticeable variation of the fatty acid pattern in brain microsomal phospholipid of aged rats, as compared to adult animals (unpublished observations), and this finding might explain why the base exchange reaction (11,12) is not affected in the ageing brain microsomes (Table IV and Fig. 1).

Brain tissue consists of different cell types with various biochemical and functional features. In no other organ are the interactions among cells as complex and critical as in the brain. Therefore, a more meaningful emphasis on the relationships existing among neurons, glia and other cell types must be taken into consideration for a clear understanding of possible variations in ageing brain. Owing to these complex interactions among different cell constituents, it is feasible that significant variations encountered in the ageing brain tissue might be due to altered interactions among these tissue constituents and/or to specific enzyme changes in specialized cells or particular brain areas.

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Metabolism of Linoleic Acid in the Cat

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ABSTRACT

Cats fed a diet containing linoleate as the only polyunsaturated fatty acid showed extremely low levels of arachidonate in the plasma lipids, as well as an increase in linoleate, eicosadienoate and an unknown fatty acid. Administration of [1-^{14}C] linoleic acid and [2-^{14}C] eicosa-8,11,14-trienoic acid to cats showed that in the liver there was no conversion of the [1-^{14}C] 18:2 to arachidonate, whereas there was significant metabolism of [2-^{14}C] 20:3 to arachidonate. It was found when methyl-γ-linolenate was fed to cats that the level of 20:3ω6 and 20:4ω6 in the erythrocytes increased significantly. These results show that there is no significant Δ6 desaturase activity in the cat, whereas chain elongation and Δ5 desaturase enzymes are operative. The unknown fatty acid was isolated from the liver lipids and shown to be a 20-carbon fatty acid with 3 double bonds and which by gas liquid chromatography could be separated from 20:3ω9 and 20:3ω6. The presence of the Δ5-desaturase activity and the results of the ozonolysis studies indicated that this unknown fatty acid was eicosa-5,11,14-trienoic acid.

INTRODUCTION

In most mammalian species, the conversion of linoleate to arachidonate involves sequential Δ6-desaturation followed by chain elongation and then Δ5-desaturation.

Recent dietary studies have shown that cats have a limited ability to metabolize linoleic acid to arachidonic acid (1-3). These results showed that there was no Δ6- and Δ5-desaturase activity, whereas chain elongation was unimparied.

This communication presents data on the in vivo metabolism of [1-^{14}C] linoleic acid, methyl-γ-linolenate and [2-^{14}C] eicosa-8,11,14-trienoic acid in the cat. The results showed that Δ5-desaturase activity was present.

MATERIALS AND METHODS

Diets

Cats were maintained on three different diets: either a control diet (meat and fish-based commercial cat food), or experimental semisynthetic diets which consisted of 29.3% protein, 25.9% fat, and 38.2% carbohydrate together with vitamins and minerals at recommended levels (4). One of the experimental diets contained only hydrogenated beef fat (SAT diet) while the other contained hydrogenated beef fat plus safflower seed oil in the ratio 4.3:1 (SSO diet). Long chain polyunsaturated fatty acids (20:2ω6, 20:3ω6, 20:4ω6, 22:4ω6, 22:5ω6, 20:5ω3, 22:5ω3 and 22:6ω3) accounted for 3.8% of the dietary fatty acids in the control diet. These fatty acids were not present in either of the semisynthetic diets.

Plasma Fatty Acids

Plasma fatty acid patterns were examined from cats which had been on the control and SSO diets from weaning for at least 22 months. The lipids were extracted from the plasma to which had been added a known amount of heptadecanoic acid (Nu-Chek-Prep, Elysian, MN) (5), and the methyl esters of the lipids were formed by saponification and esterification (6). The esters were separated using a gas liquid chromatograph equipped with flame ionization detectors (Packard Becker Model 427) on a 46 Meter SCOT column packed with OV-275 (Chromalytic, Melbourne, Australia). Separations were achieved by temperature programming from 120-220 C at 2° per min with a helium carrier gas flow of 3 ml/min. The fatty acid compositions were calculated from peak areas provided by an integrator (Spectra Physics, Santa Clara, CA). Standard methyl esters (Nu-Chek-Prep) were routinely chromatographed to establish identity of unknown esters.

Initially the fatty acids were examined using conventional analytical columns (2 m x 4 mm I.D.) containing either EGSS-X, or SILAR IOCP. There was no resolution of 20:3ω9, an unknown, 22:0 and 20:3ω6, which emerged just before methyl arachidonate. This problem was overcome by use of the capillary column described above, and baseline separations were obtained between 20:2ω6, 20:3ω9, the unknown and 20:3ω6. It was found that 20:3ω3 and 20:4ω6 cochromatographed and that 22:0 and 22:1 emerged between 20:4ω6 and 20:5ω3.

Liver Fatty Acids

The livers from two cats, which had been on the SSO diet for 10 months, were extracted with chloroform-methanol (5) and phospholipids were isolated from the total lipids by

countercurrent distribution (7). The methyl esters of the phospholipid fatty acids were prepared by refluxing in benzene-methanol-H_2SO_4 (10:40:0.8), and the polyunsaturated esters were concentrated by urea fractionation (8). An aliquot (40 mg) of the urea-soluble fraction was subjected to $AgNO_3$-column chromatography (9), and twenty 10 ml fractions were collected and examined by gas liquid chromatography (GLC). Fractions 17 plus 18 were hydrogenated and rechromatographed to establish the chain length of the fatty acid esters (8). A sample of fractions 17 plus 18 was subjected to ozonolysis followed by reduction with triphenylphosphine (10). The resulting aldehydes and aldehydo esters were separated by GLC using a column (2 m x 4 mm I.D.) of 10% ethylene glycol adipate (HI-EFF-2AP, Applied Science Labs., State College, PA) on 80/100 mesh Gas Chrom Q (Applied Science Labs.). Separations were achieved by temperature programming from 90-180 C at 10° per min with a nitrogen carrier gas flow of 40 ml/min. The fractions were identified by comparison with fragments resulting from ozonolysis and reduction of standard fatty acid methyl esters (Nu-Chek-Prep).

Radioisotope Experiments

Two control and two SSO diet kittens weighing between 400 and 600 g were dosed orally with either 250 μCi of [1-^{14}C]linoleic acid or 50 μCi of [2-^{14}C]eicosa-8,11,14-trienoic acid (The Radiochemical Centre, Melbourne, Australia) dissolved in 0.5 ml triolein (Fluka, Basel, Switzerland), and the kittens were killed 48 hr after dosing. Lipids were extracted from the livers (5) and the methyl esters of the liver fatty acids were formed as described above. Duplicate samples of the methyl esters from each experiment (2-3 mg esters containing between 6,000-34,000 dpm) were separated by $AgNO_3$-thin layer chromatography (11). A standard mixture containing methyl arachidate, eicosenoate, eicosadienoate, eicosatrienoate and arachidonate (Nu-Chek-Prep) was run on each plate. In each experiment, 20 fractions were scraped from the plates into vials and counted in a scintillation counter using a toluene-based scintillator (5).

Methyl-γ-Linolenate Feeding

Two cats were weaned onto the SAT diet and after 15 weeks were fed 4.5 g of methyl-γ-linolenate (purity >99%) (Bio-oils Research, Nantwich, U.K.) over a four-week period (6 doses of 250 mg in the first 13 days followed by 6 doses of 500 mg in the next 13 days).

TABLE I

Concentration of Polyunsaturated Fatty Acids in Cat Plasma Total Lipids (mg/100 ml plasma)

Fatty acid	Control diet[a]	SSO diet[a]
18:2ω6	50.2 ± 11.2	54.3 ± 9.2
18:3ω3	2.1 ± 0.2	0.9 ± 0.2
20:2ω6	0.2 ± 0.04	0.7 ± 0.1
Unknown	0.2 ± 0.03	1.3 ± 0.4
20:3ω6	1.9 ± 0.1	0.8 ± 0.3
20:4ω6	25.1 ± 4.7	1.0 ± 0.2
20:5ω3	11.9 ± 2.2	0.1 ± 0.02
22:5ω3	1.9 ± 0.4	0.1 ± 0.01
22:6ω3	7.5 ± 1.3	0.1 ± 0.02
Total[b]	238 ± 29	151 ± 20

[a]Mean ± S.D. from three animals.
[b]Total of fatty acids from 16:0 to 22:6ω3.

Blood was collected on days 0, 14 and 28 of the γ-linolenate treatment and the lipids were extracted from the red cells to which had been added a known amount of the internal heptadecanoic acid standard. The methyl esters were formed and separated by capillary GLC as described above.

RESULTS AND DISCUSSION

The fatty acid pattern in the plasma from the SSO diet cats (Table I) was similar to that previously described for blood and tissues of cats fed diets rich in linoleate (1,3,12). The main features were a marked decrease in 20:4ω6, 20:5ω3, 22:5ω3 and 22:6ω3 and an elevation of 18:2ω6, 20:2ω6 and an unknown fatty acid.

In order to characterize the unknown fatty acid, liver lipids from the SSO diet cats were examined. The unknown was present in the liver phospholipids at approximately the same concentration as 20:3ω6 and 20:4ω6 (Table II). Urea fractionation increased the proportion of polyunsaturates and the unknown fatty acid, and separation of the urea-soluble fraction by $AgNO_3$-column chromatography showed that the unknown emerged together with the fatty acids containing 3 double bonds (18:3ω6, 18:3ω3 and 20:3ω6). Hydrogenation of fractions rich in the unknown revealed that the chain length of the unknown was 20 carbon atoms. Examination by GLC of the fragments resulting from ozonolysis then reduction of fraction 17 plus 18 revealed that 8- and 5-carbon aldehydo esters were the two major aldehydo esters. There were also lesser amounts of 9- and 6-carbon aldehydo esters present, which were derived from the four 18-carbon unsaturated fatty acid esters in these two

TABLE II

Fatty Acid Composition of Various Fractions from SSO Diet Cat Liver Lipids

Fatty acid	Fatty acid composition (Area %)			
	Phospholipid fraction	Urea-soluble fraction	$AgNO_3$-Column fractions 17 plus 18[a]	Hydrogenated column fractions 17 plus 18
16:0	14.8	2.1	0.3	1.0
16:1	5.9	4.4	---	---
18:0	16.2	1.6	0.3	13.8
18:1	33.5	18.6	0.6	0.4
18:2ω6	22.4	46.8	6.5	0.6
18:3ω6	0.05	0.5	3.4	---
18:3ω3	0.2	0.8	4.5	---
20:0	0.1	---	---	74.8
20:1	0.6	0.2	---	2.3
20:2ω6	0.8	1.1	0.1	---
20:3ω9[b]	0.05	0.1	0.7	---
Unknown	0.7	3.9	34.1	3.3
20:3ω6	0.7	5.3	48.1	3.9
20:4ω6	0.7	11.9	0.4	---

[a]Fractions 17 and 18 eluted from $AgNO_3$ column with diethyl ether/hexane (1:1).
[b]Fatty acid identified using a 20:3ω9 standard obtained from rats fed a fat-free diet.

TABLE III

Distribution of Radioactivity from [1-^{14}C] Linoleic Acid and [2-^{14}C] Eicosatrienoic Acid in Cat Liver Fatty Acids[a]

$AgNO_3$-TLC Fraction	[1-^{14}C] 18:2		[2-^{14}C] 20:3	
	Control diet	SSO diet	Control diet	SSO diet
Saturated	200	98	5	73
Monoenoic	30	117	0	
Dienoic	7603	17,497	17	105
Trienoic	85	293	4748	22,167
Band between Δ3 and Δ4	24	202	16	57
Tetraenoic	23	106	215	721
Penta- and hexaenoic	36	25	14	90
Cholesterol	126	215	27	46

[a]cpm Per fraction. Results are shown as the mean of duplicate analyses from one cat per experiment.

fractions. The only aldehyde detected was the 6-carbon fragment. The unknown 20:3 fatty acid and 8,11,14-20:3 (20:3ω6) represented 82% of the total esters subjected to this treatment, and since the 8-carbon aldehydo ester came from 20:3ω6, the 5-carbon aldehydo ester must have been derived from the unknown fatty acid. These results indicate that the unknown was 5,11,14-20:3.

The results of the radioactive feeding studies are shown in Table III. More than 94% of the radioactivity from [1-^{14}C] linoleic acid was confined to the two double bond region of the plate, with little activity associated with any other specific fraction. These results confirm the findings of Hassam et al. (2) and show that by comparison with the rat (5) there is virtually no conversion of [1-^{14}C] 18:2ω6 to 20:4ω6 in 48 hr in the cat. In the [2-^{14}C] eicosatrienoic acid experiments, from 3.5-4.3% of the radioactivity was found associated with the methyl arachidonate region of the plate. These results indicate that there is Δ5 desaturase activity in the cat which is responsible for conversion of the [2-^{14}C] 20:3 to radiolabeled 20:4ω6.

The results of the feeding experiment with methyl-γ-linolenate (Table IV) showed the existence of a rapid chain elongation mechanism since the levels of 20:3ω6 in the erythrocytes rose markedly, and also confirmed the existence of the Δ5 desaturation since the level of arachidonate also increased. However, the conversion of 20:3ω6 to 20:4ω6 in the cat is still significantly less than in the rat (13,14).

The studies by Frankel and Rivers (3) failed to show any increase in the percentage of 20:4ω6 in plasma phosphatidylcholine of cats fed a mixture of evening primrose oil (containing γ-linolenic acid) and safflower seed oil. This mixture contained ca. 1.8 g of γ-linolenic acid which was fed over a period of 5 days. Several factors may have contributed to their failure to detect a rise in the percentage of 20:4ω6 in the plasma. Expressing results as a percentage may have masked small but significant alterations in absolute amounts of arachidonic acid. Plasma total fatty acids are not a good indication of PUFA status early in refeeding experiments, and in this present experiment the increase in erythrocyte 20:4ω6 was 5 times greater than the corresponding increase in the plasma (Sinclair, McLean and Monger, unpublished observations). Furthermore, the presence of significant amounts of α-linolenic acid in the diets used by Frankel and Rivers (3) may have caused interference with the metabolism of γ-linolenic acid to 20:4ω6 by competition for the Δ5-desaturase.

Since there is little conversion of [1-^{14}C] 18:2ω6 to 20:4ω6 (Table III) but significant metabolism of 18:3ω6 to 20:4ω6 (Table IV), the major block in arachidonate synthesis in the cat must be at the Δ6 desaturation step, i.e., the conversion of 18:2ω6 to 18:3ω6.

The unknown fatty acid (20:3) is probably a metabolite of linoleate since both the unknown (20:3) and 20:2ω6 are present in considerable concentrations in tissues of SSO diet cats while being almost absent in the SAT diet cats (12). Further evidence is provided by the observation that both the unknown (20:3) and 20:2ω6 increase greatly when linoleate is fed to SAT diet cats (12).

In the rat, the Δ5-desaturase acts on 11,14-20:2 (20:2ω6) producing 5,11,14-20:3 (15,16). Since both the substrate (20:2ω6) and the enzyme (Δ5 desaturase) are present in the cat, the unknown fatty acid is probably 5,11,14-20:3, a nonmethylene interrupted fatty acid. This identification is supported by the ozonolysis studies and by examination of retention times from GLC tracings, where the unknown fatty acid (20:3) chromatographed between 20:3ω9 and 20:3ω6 (15).

TABLE IV

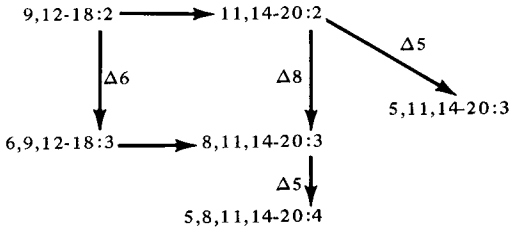
The Concentration of ω6 Fatty Acids in Cat Erythrocytes Following the Feeding of Methyl-γ-Linolenate to Cats on the SAT Diet[a]

	Days of γ-linolenate feeding		
Fatty acid	0	14	28
18:2ω6	7.2	7.7	5.2
18:3ω6	0	0.3	0.4
20:2ω6	0.2	0.2	0.1
Unknown (20:3)	0.7	0.7	0.5
20:3ω6	1.0	5.0	10.0
20:4ω6	6.8	8.7	13.0

[a]Mean results (mg fatty acid/100 ml erythrocytes) from two animals shown.

These results show that in the cat the metabolism of linoleic acid differs considerably from the rat and other mammals. In the cat there is essentially no in vivo Δ6-desaturase activity while chain elongation and the Δ5-desaturase enzymes operate. The unusual fatty acid patterns seen in cats fed diets rich in linoleic acid may be explained in the light of these results. Linoleic acid is not converted to any extent via the normal pathway to arachidonic acid, resulting in a decrease in this acid. The linoleic accumulates and is converted by chain elongation to 20:2ω6, which in turn serves as a substrate for the Δ5-desaturase, thus producing 5,11,14-20:3.

Conversion of 20:2ω6 to 20:3ω6 by Δ8-desaturation has been shown in rat testis (16), human bladder and colon (17). If this enzyme is present in the cat, it could account for the synthesis of some arachidonate from linoleate via 20:2ω6 and the Δ8-desaturation step as shown in the diagram.



In view of the limited ability of the cat to synthesize arachidonic acid, the high levels of arachidonate and long chain metabolites of linolenic acid in the plasma (Table I) and tissues (12) from control cats must largely result from the uptake of these acids from the control diet and subsequent incorporation into the tissues.

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Comparison of Lipid Composition of *Candida guilliermondii* Grown on Glucose, Ethanol and Methanol as the Sole Carbon Source

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ABSTRACT

The carbon and energy source for aerobically grown cultures of *Candida guilliermondii* profoundly influenced the neutral lipid content and the fatty acid composition of the individual lipid components. Methanol (0.80%, w/v) grown cells cultivated at 30 C in presence of 0.025% ammonium sulfate contained 12% total lipids, 67% of which was neutral lipids. Glucose (0.74%, w/v) or ethanol (0.53%, w/v) grown cells contained 21-22% total lipids, 80% of which was neutral lipids, under the same conditions. Methanol-grown cells contained a decreased 18:1 acid (52-54% of total fatty acids) and an increased 18:2 acid (23-25%), as compared with glucose- or ethanol-grown cells which contained 57-66% 18:1 acid and 8-14% 18:2 acid, in both neutral and polar lipid fractions. The relationship between methanol metabolism and desaturation of fatty acid in yeast was discussed.

INTRODUCTION

Single cell protein from methanol-utilizing yeast has attracted considerable amount of interest (1), compared to other cellular components including lipid. Our previous report, on the contrary, dealt with the cultivation factors affecting the lipid formation from methanol and changes in the lipid composition of methanol-grown *Candida guilliermondii* (2). As the content and the composition of lipids in yeast were remarkably influenced by the culture and growth conditions, including carbon source (3,4), in this paper the lipid composition of *Candida guilliermondii* grown on glucose, ethanol and methanol as the sole carbon and energy source was comparatively analyzed in order to examine the metabolic relationship between methanol metabolism and lipid biosynthesis from C_1-compound in yeast.

MATERIAL AND METHODS

Methanol-utilizing yeast *Candida guilliermondii* Y-1289 described in a preceding report (2) was used throughout the study. The yeast was maintained on slant cultures (1.7% agar) of the following methanol-mineral medium containing 0.2% (w/v) ammonium sulfate. The liquid culture medium contained (g/liter): various amounts of ammonium sulfate ranging from 0.75-0.06 as indicated; KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.001; $MnSO_4 \cdot 4H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.001; thiamine-HCL, 0.002; D-biotin, 0.00002, in addition to the following carbon source. Initial pH was adjusted to 4.6. As the carbon source, 0.74% (w/v) glucose, 0.53% (w/v) ethanol or 0.80% (w/v) methanol was added to the sterile medium in order to give approximately the same C/N ratio (ratio of carbon atom weight in added C-source to nitrogen atom weight in added N-souce). Cells were precultured aerobically for 2-3 days at 30 C in 500 ml flasks containing 100 ml of growth medium, and 10 ml samples of culture broth were transferred to 1,000 ml flasks containing 300 ml of medium. Main cultivation was performed on a rotary shaker (180 rpm) at 30 C. Cell growth was followed by reading the turbidity of the cell suspension at 660 nm. The cells grown to their early stationary phase (2 days, 3 days and 5 days after main cultivation on glucose, ethanol and methanol, respectively) were harvested by centrifugation at 6,000 x g for 10 min. Procedures for the determination of dry cell weight and for the extraction of total lipids from wet cells by a chloroform/methanol (2:1, v/v) mixture in the presence of glass beads were the same as described previously (2). The extraction process was repeated three times to collect total lipids as completely as possible. The lipids obtained were stored at 4 C in a chloroform solution.

A part of total lipids was fractionated on a silicic acid column (2 cm x 9 cm, Unisil, Clarkson Chemical Co., Williamsport, PA) in neutral and polar lipids by elution initially with 200 ml of chloroform and then with 150 ml of methanol (5). After evaporation of eluates, the amounts of each lipid were determined gravimetrically. Essentially all the lipid applied on the column was recovered in the two fractions. Neutral lipids were separated by thin layer chromatography (TLC) on precoated silica gel plate (20 cm x 20 cm, 0.25 mm thickness, Merck, Darmstadt, West Germany) in

a one dimensional, double development system at first with benzene/diethyl ether/ethanol/28% ammonium water (50:40:2:0.5, v/v) [I] and next with *n*-hexane/diethyl ether (94:6, v/v) [II], according to the method of Freeman and West (6), with a slight modification: ammonium water was used instead of acetic acid in the solvent system [I] to avoid a close or overlapped migration of free fatty acid to sterol. Quantitative analysis of neutral lipids was performed by a densitometric method (using a Shimadzu Densitometer, Model CS-910, with a zig-zag scanning mode). For standards, cholesteryl oleate, tri-, di- and monoolein, oleic acid and cholesterol (Sigma Chemical Co., St. Louis, MO) were prepared as a chloroform solution. The plates spotted with known amounts of standard mixtures and unknown samples were developed in the previous way and sprayed with 10% sulfuric acid solution. Both plates were heated simultaneously in an oven at 130 C for 15 min. The relative ratios of the individual neutral lipid components were determined by the values obtained from the individual standard curves.

Polar lipids were also separated on TLC plates developed by chloroform/acetone/methanol/acetic acid water (50:20:10:10:5, V/V) [III] for one dimensional development, and, when necessary, by chloroform/methanol/7N-ammonium water (65:35:5, v/v) [IV] prior to the system [III] for two dimensional development (7-9). Lipid spots were detected by specific spray reagents (10): sulfuric acid for all lipids, ninhydrin for amino group, molybdenium blue reagent for phosphorus, Dragendorff reagent for choline, α-naphtol reagent for reducing sugar, and iodine vapors for all lipids (for scraping corresponding silica gel). The phosphorus contents of the individual phospholipids were determined by the method of Rouser et al. (11) and expressed as the percentage of total phosphorus recovered.

Fatty acid methyl esters from total lipids, neutral and polar lipids, and the individual lipid components separated on the TLC plates, were prepared by BF_3-methanol method after saponified with 0.5 N methanolic KOH at 60 C for 5 min (12). The fatty acid composition was determined using a Gas Chromatograph (Shimadzu, Model GC-4C, PF) equipped with flame ionization detectors. The column packing used was 20% diethylene-glycol succinate on chromosorb WAW, 200 cm x 3 mm (ID). The flow rate of the nitrogen carrier gas was 40 ml/min at 180 C. Peaks were identified by comparing their retention times to those of authentic standards and quantitated by a digital integrator.

TABLE I

Lipid Composition of *Candida guilliermondii* Y-1289 Grown on Glucose, Ethanol and Methanol as the Sole Carbon Source at 30 C

Carbon source	DC (mg/300ml)	TL (mg/300ml)	NL/DC (%)	PL/DC (%)	Percent of total neutral lipids[a]						Percent of total phospholipids[b]					
					SE	TG	DG	S	UK-2	FA	CL	PE	PC	PS	PI	
G[c]	855.4	63.3	4.0	3.4	2.6	72.8	3.3	8.1	3.3	4.6	4.7	28.4	51.1	8.0	7.8	
E[c]	780.0	81.9	6.8	3.7	3.2	79.8	3.0	7.6	1.7	5.2	7.8	26.7	53.1	6.4	6.0	
M[c]	495.2	31.2	3.9	2.4	1.7	72.5	7.0	11.5	3.2	2.6	10.4	23.8	44.5	8.7	12.7	
G[d]	817.1	181.4	17.8	4.4	0.9	92.8	2.8	0.5	1.9	1.6	2.4	26.3	59.4	5.1	6.9	
E[d]	698.1	148.7	17.3	4.0	2.0	87.3	4.5	0.5	1.7	2.7	4.4	25.2	51.9	7.6	10.8	
M[d]	416.3	51.2	8.3	4.0	0.5	86.3	3.6	0.6	4.2	3.0	3.7	23.5	56.3	5.8	10.7	

[a]Trace amounts of monoglyceride and unidentified component-1 were also detected on the TLC plate. The amounts of unidentified components were calculated by the standard curve of free sterol.

[b]Tentative identification of substances after TLC with solvent sytem III. Trace amounts of glycolipid containing phosphorus were also detected.

[c]G=glucose, E=ethanol, and M=methanol. Cells were grown in 0.075% (w/v) ammonium sulfate.

[d]Cells were grown in 0.025% (w/v) ammonium sulfate.

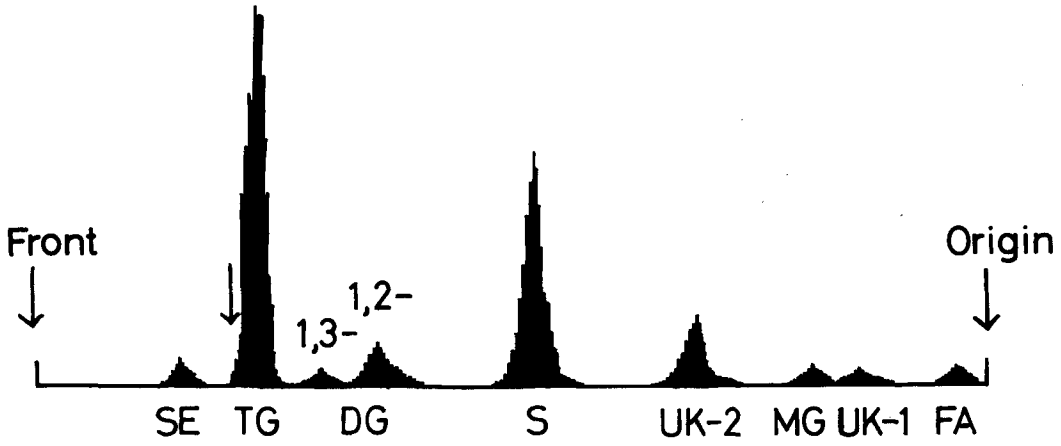

FIG. 1. Photodensitometric pattern from a TLC containing neutral lipids of *C. guilliermondii* Y-1289 grown on methanol at 30 C in 0.075% (w/v) ammonium sulfate. The silica gel precoated plate was developed at first with benzene/diethyl ether/ethanol/28% ammonium water (50:40:2:0.5, v/v) to the distance indicated by arrow and next with *n*-hexane/diethyl ether (94:6, v/v) to the solvent front. FA= free fatty acid, UK-1= unidentified component-1, MG= monoglyceride, UK-2= unidentified component-2, S= free sterol, DG= diglyceride (mixtures of 1,2- and 1,3-isomers), TG= triglyceride, SE= sterol ester.

RESULTS AND DISCUSSION

C. guilliermondii cells grown on glucose, ethanol or methanol in presence of 0.075% ammonium sulfate showed no significant differences in the neutral and polar lipid contents of dry cells (3.9-6.8% for NL/DC and 2.4-3.7% for PL/DC), though a considerable difference in the amounts of extractable total lipids (TL) were observed (Table I). By reducing ammonium sulfate from 0.075% - 0.025%, remarkable increases in the amount of TL and in the neutral lipid content of cells were recognized in any cultures of different carbon sources. As described previously, 0.025% of ammonium sulfate seemed to be the nitrogen limitation for cell growth on methanol (2), because a remarkable lipid accumulation generally occurs under the nitrogen limitation (3,13,14). It was noted, however, that the methanol-grown cells contained less than a half of the neutral lipid of cells, 8.3%, compared with 17-18% in the glucose- or ethanol-grown cells, under the lipid accumulation condition (0.025% ammonium sulfate). Polar lipid contents of cells (PL/DC), on the contrary, were almost unchanged not only by varying a nitrogen concentration but also by changing a carbon source (2.4-4.4% in Table I).

As illustrated in Figure 1, a densitometric pattern revealed the presence of at least seven kinds of neutral lipid components, but no significant differences were observed among the cells grown on three different carbon sources. Triglyceride (TG) was always a major component (73-92% of total neutral lipids) in both nitrogen concentrations (Table I). The amounts of diglyceride (DG), free fatty acid (FA) and sterol ester (SE) were 3-7%, 2-5% and 1-3%, respectively. The amount of free sterol (S), on the other hand, remarkably decreased from 8-12% to 0.5-0.6% in any cultures of different carbon sources by reducing ammonium sulfate. Small amounts of monoglyceride (MG) and unidentified components (UK-1 and UK-2) were also present in some of the lipid samples. Two dimensional TLC revealed phospholipid was a major component of polar lipids. A trace amount of glycolipid was also detected on TLC plates. Essentially no differences in the separation of major components were observed between one and two dimensional TLC. No qualitative changes were found among the cells grown on three different carbon sources. Phosphatidylcholine [PC] (45-59% of total phospholipids), phosphatidylethanolamine [PE] (24-28%), phosphatidylinositol [PI] (6-13%), phosphatidylserine [PS] (5-9%) and cardiolipin [CL] (2-10%) were tentatively identified as the principal components by comparison of Rf values and by their reactions with various reagents (7,10). Besides the above components, a minor unidentified glycolipid containing phosphorus [p-GL], phosphatidic acid[PA] and lysophosphatidylcholine [LPC] were also detected.

The fatty acid composition of neutral and polar lipid fractions indicated that 18:1, 18:2 and 16:0 acids were the predominant fatty acids with considerable amounts of 16:1,

18:3 and 18:0 acids in any cultures of different carbon sources. As compared with glucose- or ethanol-grown cells, methanol-grown cells cultivated in the same nitrogen concentration contained an increased amount of 18:2 acid and a decreased amount of 18:1 acid in both lipid fractions. For example, the neutral lipid fractions from methanol-grown cells in 0.075% ammonium sulfate contained 37% of 18:2 acid in the total fatty acids; that was 16% higher than that of 18:2 acid from cells grown on glucose or ethanol in the same nitrogen concentration. A concomitant decrease in the amount of 18:1 acid from 47-57% to 33% was observed in the neutral lipid fractions, when the glucose- or ethanol-grown cells were compared with the methanol-grown cells.

Table II showed the fatty acid composition of the individual main lipid components from cells grown on different carbon sources in 0.075% ammonium sulfate. No significant differences in the fatty acid composition of any lipid components were found between glucose-grown cells and ethanol-grown cells. Methanol-grown cells, on the contrary, contained an increased amount of 18:2 acid and a decreased amount of 18:1 acid in all lipid components, as compared with glucose- or ethanol-grown cells. Especially noted are the prominent differences in the amounts of these acids between the cells grown on homologous primary alcohols, ethanol and methanol. These differences are not considered to be caused by an indirect growth inhibition which is characteristic of toxic methanol, because the doubling times which indicate cell growth rate on the individual carbon source were almost similar in both alcoholic carbon sources (8 hr for ethanol and 9 hr for methanol). The value for glucose was much smaller (4.2 hr) than that of both alcohols, but the fatty acid profiles of glucose-grown cells resembled those of ethanol-grown cells but not of methanol-grown cells. Accordingly, the results suggested that the methanol-grown cells may have a larger activity of desaturating enzyme which converts 18:1 acid to 18:2 acid than the glucose- or ethanol-grown cells. Table II also indicated considerable differences in the fatty acid composition among the individual lipid components. In any cultures, PC, a major phospholipids, contained a larger amount of 18:1, 18:2 and total unsaturated acids, and a smaller amount of 16:0 acid, as compared with TG. PE contained a larger amount of 16:0 acid than TG or PC. The smaller amounts of 18:2, 18:3, and total unsaturated acids seemed to be a characteristic of PI.

Further, the fatty acid profiles of total lipids

TABLE II

Fatty Acid Composition (%) of Individual Lipid Components from *C. guilliermondii* Y-1289 Grown on Glucose, Ethanol and Methanol in 0.075% (w/v) Ammonium Sulfate at 30 C[a]

Lipid component	Carbon source	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	Unsaturated acids (%)
TG	G	0.2	0.1	17.2	5.6	0.6	1.6	2.4	47.5	19.5	5.4	79.4
	E	0.2	0.1	11.1	3.8	—[b]	0.2	2.7	57.7	19.8	4.5	86.0
	M	0.6	0.2	14.4	10.9	—	0.9	0.8	33.4	35.5	3.3	84.0
PC	G	T[c]	T	8.7	6.8	—	1.8	0.3	54.1	23.1	5.0	90.8
	E	0.2	T	7.2	5.2	—	0.5	0.6	51.5	27.8	7.0	92.0
	M	0.9	T	7.6	8.2	—	1.2	0.9	36.6	41.7	2.9	90.6
PE	G	0.2	0.1	24.9	6.6	0.2	1.3	1.1	47.7	14.9	2.8	73.3
	E	0.4	0.1	18.7	6.0	0.1	0.6	1.2	44.5	24.4	3.9	79.4
	M	1.3	T	17.8	5.8	0.3	0.9	0.8	37.6	33.8	1.8	79.9
PI	G	1.5	—	24.4	—	1.1	0.5	2.7	57.4	10.9	1.0	69.8
	E	1.3	—	23.7	T	0.3	T	3.6	55.6	13.9	1.6	71.1
	M	2.0	—	42.0	T	T	T	0.9	29.4	25.6	T	55.0

[a]G=glucose, E=ethanol, M=methanol, TG=triglyceride, PC=phosphatidyl choline, PE=phosphatidyl ethanolamine, and PI=phosphatidyl inositol.
[b]Not detected.
[c]T<0.1%.

from cells grown on glucose or methanol as the sole carbon source were comparatively examined as a factor of nitrogen concentration (Table III). With a stepwise decrease in the ammonium sulfate concentration from 0.075%-0.006%, an increased amount of 18:1 acid and decreased amounts of 18:2 and 18:3 acids were observed in both carbon sources. Most striking changes were found by reducing ammonium sulfate from 0.075%-0.025%. For example, the amount of 18:1 acid in methanol-grown cells remarkably increased from 32%-53%, while those of 18:2 and 18:3 acids decreased from 39%-24%, 4%-2%, respectively. Interestingly, there is a reverse tendency between two carbon sources in the amounts of 16:0 and total unsaturated acids; 16:0 acid was increasing in glucose-grown cells and decreasing in methanol-grown cells, and, vice versa, total unsaturated acids were decreasing in glucose-grown cells and increasing in methanol-grown cells by reducing ammonium sulfate. As listed in the last column of Table III, the differences in the amount of 18:2 acid between two carbon sources were found to be getting smaller from 17.6%-10.1% with a decrease of ammonium sulfate from 0.075%-0.006%. This means that the differences in 18:2 acid between two carbon sources are large in the conditions of good cell growth and bad accumulation of lipids. Accordingly, a close relationship between the methanol metabolism and the desaturation of 18:1 acid in yeast was recognized.

It is well known that the characteristics of the methanol metabolism in yeast are the increase in the activity of catalase which converts peroxidatically methanol to formaldehyde, and the formation of NADH in the oxidation of methanol to carbon dioxide (15). The fatty acid desaturation in yeast, on the other hand, requires NADH or NADPH as a reducing cofactor, in addition to the molecular oxygen and three different protein components (16,17). In this connection, an important information was recently proposed using the rat liver. The investigation of the effects of dietary conditions on the fatty acid desaturation revealed the correlation between Δ^9-desaturase and catalase in the rat liver microsomes (18). Our preliminary experiments indicated that the methanol-grown cells contained an increased activity of catalase not only in the peroxisomal and cytoplasmic fractions (19,20), but also in the microsomal fractions, as compared with the glucose- or ethanol-grown cells. Subsequent studies which are under investigation to determine whether there is a close relationship between fatty acid desaturation (especially Δ^{12}-desaturase) and catalase activity or NADH

TABLE III

Comparison of Fatty Acid Composition (%) of Total Lipids from *C. guilliermondii* Y-1289 Grown on Glucose and Methanol in Different Concentration of Ammonium Sulfate at 30 C

$(NH_4)_2SO_4$ (%, w/v)	Carbon source	DC (mg/300 ml)	TL (% of DC)	16:0	16:1	18:0	18:1	18:2	18:3	Unsaturated acids[a] (%)	18:2 [M-G][b] (%)
0.075	G[c]	855.4	7.4	15.9	5.4	1.6	47.0	21.5	6.2	81.6	17.7
	M[c]	495.2	6.3	14.3	8.7	0.8	32.1	39.1	3.6	84.3	
0.025	G	817.1	22.2	20.0	8.8	2.8	56.8	9.4	1.3	76.9	13.7
	M	416.3	12.3	13.6	6.9	1.0	52.7	23.7	1.8	85.1	
0.013	G	654.0	25.2	21.3	6.4	4.5	60.7	5.2	1.1	74.1	12.0
	M	308.2	12.5	11.4	5.3	2.1	61.2	17.2	1.2	85.9	
0.006	G	378.6	23.2	21.4	5.3	5.2	60.0	5.2	2.0	73.1	10.1
	M	278.7	13.4	10.5	5.8	1.8	63.8	15.3	1.2	87.1	

[a]Total unsaturated acids including minor amount of 17:1 acid. The difference between the sum of any row and 100% represents minor amounts of other fatty acids, such as 14:0, 15:0 and 17:0 acids.

[b]Difference in the amount of 18:2 acid between methanol-grown cells and glucose-grown cells.

[c]G=glucose, M=methanol.

formation in the methanol-grown yeast are necessary.

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Isomerization of the Double Bonds of a Conjugated Fatty Acid during β-Oxidation

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ABSTRACT

The β-oxidation of an unsaturated fatty acid containing conjugated double bonds at odd-numbered carbon atoms has not previously been studied. It is, therefore, not clear whether, during the β-oxidation of such an acid, the double bonds will be isomerized by enoyl-CoA isomerase (Δ^3 - Δ^2-enoyl-CoA isomerase) with the loss or retention of its conjugated nature. To investigate the problem, (E,E)-3,5-octadienoyl-CoA was synthesized for use as a model substrate, and enoyl-CoA isomerase was partially purified from bovine liver. The isomerization was followed by spectrophotometric and gas liquid chromatographic methods, and the results suggested that the isomerization of the model substrate proceeded with retention of a conjugated double bond system. It is, therefore, proposed that the β-oxidation intermediate of α-eleostearic acid ($\Delta^{9,11,13}$ fatty acid) will also isomerize with retention of the conjugated double bond system.

INTRODUCTION

The β-oxidation pathway for the complete oxidation of a saturated fatty acid has been studied in detail and the acyl-CoA intermediates and enzymes concerned have been identified (1-3). Unsaturated fatty acids, with double bonds at odd-numbered carbon atoms (e.g., Δ^9), are oxidized via a modified β-oxidation route and produce metabolites which are not "normal" β-oxidation cycle intermediates (2,4-6). Thus, (Z)-9-octadecenoic acid produces the "abnormal" intermediate, (Z)-3-dodecenoyl-CoA, which is isomerized to the "normal" intermediate, (E)-2-dodecenoyl-CoA, by the action of enoyl-CoA isomerase (Δ^3 - Δ^2-enoyl-CoA isomerase; ref. 5).

To our knowledge, the isomerization of fatty acid substrates with conjugated double bonds at odd-numbered carbon atoms has not previously been examined, and this paper reports on such an investigation. The identification of α-eleostearic acid [(Z,E,E)-9,11,13-octadecatrienoic acid] in edible indigenous nuts (7,8) prompted us to propose a scheme for its β-oxidation (Fig. 1). Two possible subroutes for isomerization were indicated. Similar to the β-oxidation pathway for linoleic acid, the removal of three acetyl-CoA molecules would lead to 3,5,7-dodecatrienoyl-CoA (Fig. 1). It was considered that this "abnormal" β-oxidation intermediate may be isomerized by enoyl-CoA isomerase to yield 2,4,6-dodecatrienoyl-CoA (with retention of the conjugated double bond system) or to 2,5,7-dodecatrienoyl-CoA (with partial loss of the conjugated double bond system). Both Δ^2 compounds may then serve as "normal" β-oxidation intermediates.

To distinguish between the proposed subroutes for the β-oxidation of α-eleostearic acid, it was considered sufficient to prove that $\Delta^{3,5}$ acyl-CoA compounds could serve as substrates for enoyl-CoA isomerase and to establish whether $\Delta^{2,4}$ or $\Delta^{2,5}$ acyl-CoA isomers were produced. Previous studies (9,10) indicated that both (Z)- and (E)-alkenoyl-CoA compounds could serve as substrates for enoyl-CoA isomerase, and for the present study (E,E)-3,5-octadienoyl-CoA was synthesized to serve as a

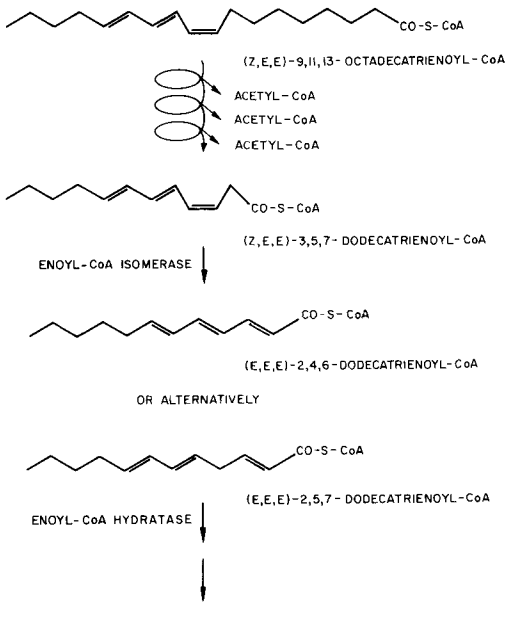

FIG. 1. Hypothetical β-oxidation route for α-eleostearic acid.

[1]Part of doctoral thesis submitted to University of Pretoria, Pretoria, 0001, Republic of South Africa.

model substrate and enoyl-CoA isomerase was partially purified from bovine liver. Alternative schemes for the isomerization of 3,5-octadienoyl-CoA are outlined in Figure 2.

MATERIALS AND METHODS

(E)-3-Octenoyl-CoA (Substrate I) and (E,E)-3,5-Octadienoyl-CoA (Substrate II)

(E)-3-octenoic was obtained from K & K Laboratories (Plainview, NY) and (E,E)-3,5-octadienoic acid was prepared by the sodium amalgam reduction of (E,E,E)-2,4,6-octatrienoic acid (11). The desired 3,5-octadienoic acid was purified by preparative gas liquid chromatography (GLC) (12), and the olefinic coupling constants (ca. 14 Hz) in the proton nuclear magnetic resonance (NMR) spectrum of this acid verified the geometry.

The method of Goldman and Vagelos (13), as modified by Struijk and Beerthuis (9), was applied to esterify (E)-3-octenoic and (E,E)-3,5-octadienoic acid with CoA-SH (Miles Laboratories, Elkhart, IN, 75% purity). The formation of the thiol ester group in substrates I and II were verified by thin layer chromatography (TLC) on Merck cellulose plates (type F 0,1 mm) with methanol/acetic acid/water (4:1:1, v/v) as mobile phase and sodium nitroprusside (14) as spray reagent. Substrates I and II both exhibited two spots (Rf 0.6, 0.37 and 0.7, 0.45, respectively) which reacted positively for thiol esters. Stadtman (15) and Pullman (16) reported similar results with other acyl-CoA compounds. Chromatographic analysis of previously purified monounsaturated acyl-CoA substrates exhibited two spots (thiol esters), and the compounds from both spots were active as substrates (15,16). Pullman (16) also found that the thiol ester impurities, as well as the enzymatic inactive byproducts, were not inhibitory when tested with a number of different enzymes.

The acyl-CoA content of the substrate reaction mixtures was determined by the hydroxamic reaction (17). Our attempts to purify substrate II by TLC (16) were unsuccessful as the highly unstable acyl-CoA decomposed rapidly. Freshly prepared substrates I and II, containing 50% and 28% acyl-CoA, respectively, were used for the study.

Enoyl-CoA Isomerase

Enoyl-CoA isomerase was partially purified according to the method described by Rilling and Coon (18). Fresh bovine liver (50 g) yielded 400 mg impure enzyme which was freeze-dried and stored under nitrogen atmosphere at -30 C. The protein content of the isolate was 68% as determined by a spectrophotometric procedure (19).

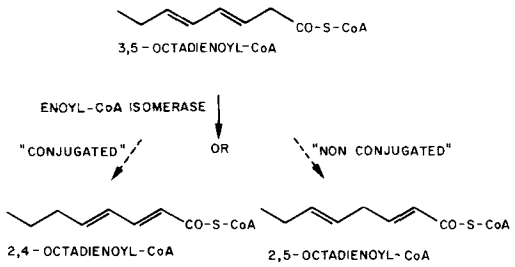

FIG. 2. Hypothetical isomerization routes for 3,5-octadienoyl-CoA by enoyl-CoA isomerase.

Ethylthio (E,E)-2,4-Hexadienoate and Ethylthio (E)-2-Octenoate

(E,E)-2,4-hexadienoic acid (sorbic acid; E. Merck, Rahway, NJ) and (E)-2-octenoic acid (K & K Laboratories) were esterified with ethanethiol (E. Merck) (20) to obtain UV absorption model compounds. Ethylthio-sorbate exhibited a λ_{max} value of 295 nm in the buffer solution (A; pH 7,4), ϵ value 16,500 (Fig. 3 I). The λ_{max} value of this compound is solvent-dependent and a λ_{max} of 271 nm in hexane solution (ϵ = 16,400) and λ_{max} 284 nm in methanol (ϵ = 16,300) were recorded. Ethylthio-2-octenoate exhibited two absorption bands, λ_{max} 220 nm (ϵ = 10,600) and λ_{max} 263 nm (ϵ = 5,900) in the buffer solution (pH 7,4; Fig. 3II).

Apart from the 295 nm absorption maximum, the UV spectrum of ethylthio-sorbate also shows a weak shoulder in the 260 nm region (Fig. 3I). We, therefore, decided to determine the A_{263}/A_{300} ratios for ethylthio-sorbate and ethylthio-2-octenoate (Gilford 2400 S) and to use these ratios to distinguish between the two possible isomerization products ($\Delta^{2,4}$ or $\Delta^{2,5}$) acyl-CoA compounds (Fig. 2). The A_{263}/A_{300} ratios for ethylthio-sorbate and ethylthio-2-octenoate were 0.62 and 11.8, respectively.

In order to simulate the absorption spectra of $\Delta^{2,4}$ and Δ^{2} acyl-CoA compounds, the UV spectra of CoA-SH (56 μ mole/ℓ) plus ethylthio-sorbate (6.1 μ mole/ℓ) and CoA-SH (61 μ mole/ℓ) plus ethylthio-2-octenoate (11.8 μ mole/ℓ) were recorded in buffer A solution (Fig. 3 IIIb and IVb).

Methyl (E,E)-2,4-Octadienoate

(E)-2-hexenal (Koch-Light Lab.) was condensed with malonic acid in the presence of pyridine to yield an octadienoic acid. The methyl ester was obtained by refluxing the acid

for 4 min in excess boron trifluoride-methanol reagent (E. Merck). The ester gave one peak on GLC analysis, and it eluted 0.35 min after methyl (E,E)-3,5-octadienoate. In the mass spectrum of the methyl octadienoate [M^+ = 154 (31%)], a low intensity McLafferty rearrangement ion (m/e 74) and a prominent m/e 111 ion, typical for methyl 2,4-dienoates (21), was observed. The double bond position ($\Delta^{2,4}$) was further verified by the UV spectrum (λ_{max} 259 nm, ϵ = 22,300), recorded in methanol, and the double bond geometry (E,E) confirmed by the results of Crombie (22).

Gas Liquid Chromatography (GLC) and Mass Spectrometry

A Hewlett-Packard 5750 instrument, equipped with dual flame ionization detectors and dual glass columns (2,2 m x 2 mm) packed with 15% DEGS on Chromosorb W, was used for analytical work. It was assumed that the response factors for the C-6 to C-12 fatty acids were the same. Peak areas were integrated with a HP 3352 B laboratory data system. Nitrogen was used as a carrier gas at a flow rate of 20 ml/min. A column temperature of 180 C (isothermally) was used for fatty acid analyses, while the methyl esters of fatty acids were analyzed at 140 C (isothermally).

Mass spectra were determined on a HP 5981 A mass spectrometer coupled to a HP 5710 A GLC instrument equipped with a XE-60 capillary (50 m x 0.3 mm) column. The column temperature was kept isothermally at 150 C.

Assay of Enzyme Activity

Enoyl-CoA isomerase activity was assayed by incubating 20 $\mu\ell$ (0.17 μ mole) substrate I and 0.4 mg enzyme (0.27 mg protein) in a buffer solution at 22 C for 10 min. The buffer solution consisted of 0.066 mℓ tris solution (pH 7.8), 0.022 mℓ 0.1 M EDTA solution (pH 7.4), 0.066 mℓ of a 0.1% bovine serum albumin solution and distilled water to a final volume of 2.5 mℓ (Buffer A). The enzyme preparation was dissolved in a phosphate buffer (0.05 M, pH 7.4; Buffer B) prior to addition to the substrate.

Conversion of 3- to 2-octenoyl-CoA was monitored by UV absorption measurements at 263 nm (23) using a Gilford 2400S instrument. The increase in the A_{263} value could be related to the formation of 0.048 μ mole 2-octenoyl-CoA from 0.16 μ mole 3-octenoyl-CoA ($\Delta\epsilon$ = 6700, ref. 15).

Using Rilling and Coon's (18) definition for enzyme unit and specific activity, the total enzyme units were determined as 6,400 and the specific activity as 23.5.

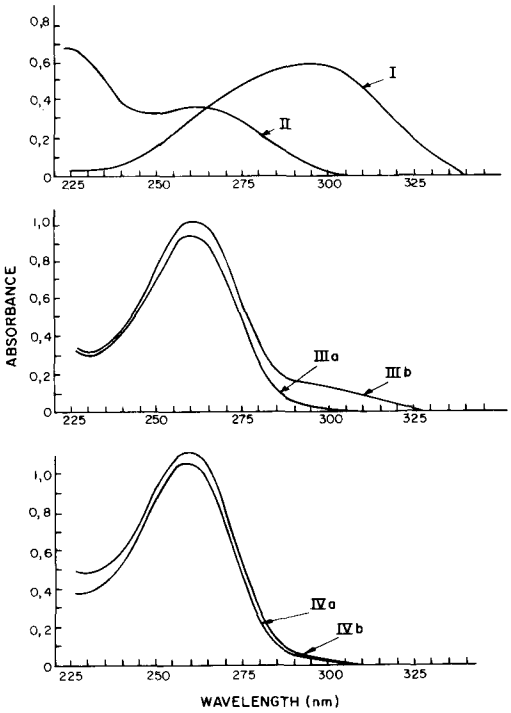

FIG. 3. UV absorption spectra of ethylthio-sorbate, 33.7 μ mole/ℓ (I); ethylthio-2-octenoate, 55 μ mole/ℓ (II); CoA-SH, 56 μ mole/ℓ (IIIa); CoA-SH plus ethylthio-sorbate, 6.1 μ mole/ℓ (IIIb); CoA-SH, 61 μ mole/ℓ (IVa); CoA-SH plus ethylthio-2-octenoate, 11.8 μ mole/ℓ (IVb) in buffer solution, pH 7.4.

For the GLC assay of enzyme activity, the same reagents were used to repeat the reaction. After 10 min, the reaction was stopped by adding 0.05 mℓ 1 M sodium hydroxide solution. Subsequently, the reaction mixture was saponified by heating at 60 C for 45 min. The pH of the solution was then lowered to pH 2.0 and the fatty acids extracted with ethyl acetate and analyzed by GLC. 3-Octenoyl-CoA isomerized to produce 27% 2-octenoic acid (derived from 2-octenoyl-CoA). It is important to note that no hydroxy fatty acids could be observed after the isomerization.

Isomerization of 3,5-Octadienoyl-CoA

Spectroscopic determination. For repetitive scanning, the sample cell (1 cm path length) contained 20 $\mu\ell$ (0.065 μ mole) substrate II solution, 0.5 mg enzyme (0.34 mg protein) and buffer A solution to a final volume of 2.5 mℓ, while the reference cell contained buffer A solution. A control reaction mixture consisted of the same reagents as for the test except that 0.5 mg denatured enzyme was employed.

Enzyme denaturation was achieved by heating one part of the enzyme preparation (0.05 mg/$\mu\ell$ buffer B) in a water bath at 90 C for 3 min. After addition of the active or denatured enzyme to the reaction mixtures, the UV absorption was scanned (Unicam SP 800) from 200-350 nm with 2 min intervals, and both reactions were terminated after 18 min (Fig. 4).

Changes in A_{263} values of a similar reaction mixture, containing substrate II (0.053 μ mole), were recorded to 3 decimal places (Gilford 2400 S) over a period of 36 min. Subsequently, A_{300} values were determined on an identical but fresh reaction mixture. The cumulative absorption values (ΔA_{300} and ΔA_{263}) were calculated by subtracting the absorption values at 0 min from each consecutive reading (Fig. 5).

A_{263} and A_{300} values were also determined on the same reaction mixture. Sample and control reaction solutions were prepared as in the previous experiment, and A_{263} and A_{300} values were recorded to 3 decimal places at 0 min and again at 33 min and the A_{263}/A_{300} ratio was calculated.

GLC determination of isomerization. A solution containing 0.42 μ mole substrate II, 5 mℓ buffer A solution and active enoyl-CoA isomerase solution (2.5 mg) was incubated at 22 C for 35 min. A similar reaction mixture containing heat denatured enzyme, instead of active enzyme, was used as a control. Both reactions were terminated by freeze-drying (Edwards EF 03 instrument), and the dried acyl-CoA components were transesterified to methyl esters by heating the mixture in 5% sulphuric acid in dry methanol for 30 min at 60 C in a reactivial. The methyl esters were taken up in hexane and the solvent carefully evaporated to concentrate the solution to 0.1 mℓ. Aliquots were taken for the GLC analysis which was commenced without delay.

RESULTS

Isomerization of (E,E)-3,5-Octadienoyl-CoA

Spectroscopic determination. Incubation of the substrate with active enzyme resulted in a moderate increase in absorption at 300 nm and a smaller increase at 263 nm (Fig. 4 II). When denatured enzyme was employed, only a very small change in absorbance with time was observed (Fig. 4 I). The UV spectra of the two thiol model compounds, especially when these compounds were analyzed mixed with CoA-SH (Fig. 3 IIIb and IVb), provided a useful basis for interpreting the spectrum in Fig. 4 II. The spectrum of CoA-SH-ethylthio-sorbate (Fig. 3 IIIb) compares favorably with that obtained after 18 min isomerization (Fig. 4 II).

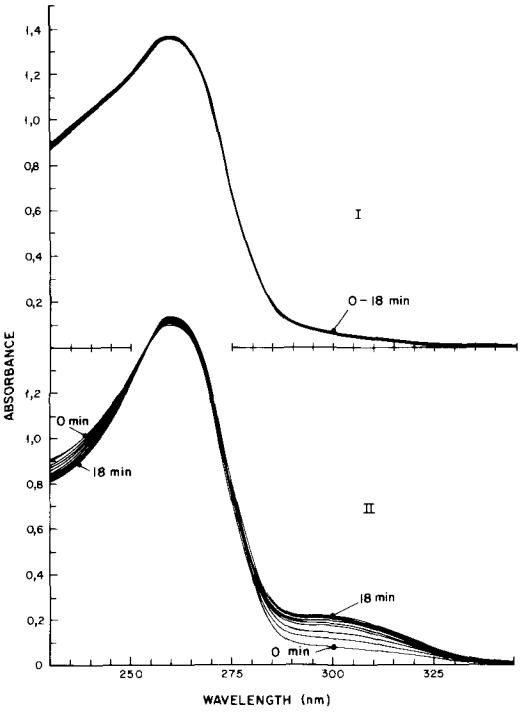

FIG. 4. Repetitive scanning of absorption during isomerization of 3,5-octadienoyl-CoA with denatured (I) and active (II) enoyl-CoA isomerase.

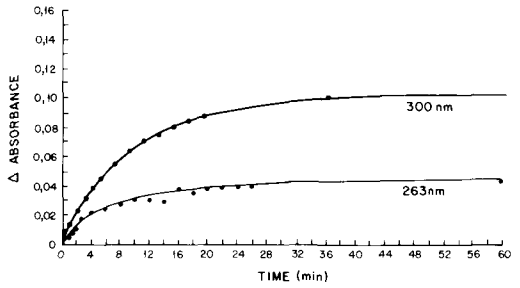

FIG. 5. ΔA per min measured during isomerization of 3,5-octadienoyl-CoA by enoyl-CoA isomerase (ΔA_{263} and ΔA_{300} values).

The results of the second isomerization experiment are given in Fig. 5, and the increase in A_{300} values is approximately twice that of the A_{263} values (Fig. 5). These A_{263} and A_{300} values were obtained from two consecutive experiments, each using a fresh reaction mixture. In a third experiment, A_{263} and A_{300} values were obtained from the same reaction mixture at 0 min and again at 33 min. The A_{263} and A_{300} values increased by 0.06 and 0.11 units, respectively, giving an A_{263}/A_{300} ratio of 0.55 for the isomerization products.

This A_{263}/A_{300} ratio is similar to that of ethylthio-sorbate (0.62) but differs markedly from that of ethylthio-2-octenoate (11.8).

GLC determination. The methyl ester mixture derived from the isomerization reaction mixture, treated with active enzyme, contained three major and five minor components (Table I). Peak 4 (t_R = 6.6 min), peak 5 (t_R = 7.06 min), and peak 6 (t_R = 7.4 min) were identifed as methyl 4,6-, methyl 3,5-, and methyl 2,4-octadienoate, respectively. The reaction mixture derived from the experiment where denatured enzyme was used yielded only peaks 4 and 5 (Table I).

TABLE I

Fatty Acid Composition Obtained after Incubation of 3,5-Octadienoyl-CoA with Active (Test) and Denatured (Control) Enoyl-CoA Isomerase

	Mass percentage of total fatty acids	
Fatty acid esters	Test	Control
1	7.9	7.0
2	2.1	3.8
3	2.8	2.6
4 (4,6-oct.)	11.6	12.1
5 (3,5-oct.)	51.0	62.0
6 (2,4-oct.)	15.5	--
7	1.2	3.5
8	7.7	7.8
Total	99.8	99.9

DISCUSSION

During the UV spectroscopic investigation of the isomerization of (E,E)-3,5-octadienoyl-CoA (substrate II), it was found that the product absorbed in the 250-310 range (Fig. 4 II). In order to determine whether the increase in absorption resulted from the formation of 2,4-octadienoyl-CoA (a $\Delta^{2,4}$ thiol ester) or 2,5-octadienoyl-CoA (a Δ^2 thiol ester), a spectroscopic analysis of the two chromophores in question was undertaken using ethylthio-sorbate and ethylthio-2-octenoate as model substances (Fig. 3). Ethylthio-sorbate exhibited a λ_{max} value of 295 nm which corresponds very well with that of sorbyl-CoA (300 nm), reported by Wakil and Hübscher (24). In addition, it was shown that the UV spectrum of ethylthio-sorbate, mixed with CoA-SH (Fig. 3 IIIb), was similar to that of the isomerization product (Fig. 4 II). It was possible to interpret the increase in A_{263} values (Fig. 5) by taking into account that ethylthio-sorbate exhibited a shoulder in the 260 nm region (Fig. 3 I). A comparison of the A_{263}/A_{300} ratios of both model compounds, with that of the isomerization product, provided further evidence that 2,4-octadienoyl-CoA, rather than 2,5-octadienoyl-CoA, had been formed.

Isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA could also be demonstrated by GLC analysis using methyl 2,4-octadienoate as reference standard. It was found that the appearance of methyl 2,4-octadienoate (15.5%) in the sample reaction mixture corresponded reasonably well with the disappearance of methyl 3,5-octadienoate (11.0%) when the sample and control reaction mixtures were compared (Table I). The sum of the unidentified minor components in the sample and control reaction mixtures were only slightly different (21.7 and 25.8%; Table I) indicating small changes or losses among them. 4,6-Octadienoate, which remained as impurity in the preparative GLC purified 3,5-octadienoate (12), served as internal standard for the fatty acids with conjugated double bonds. It is, therefore, of particular interest to note that the 4,6-octadienoate content of the sample and control reaction mixtures corresponded well, and it confirmed that only small losses of fatty acids with conjugated double bonds could have occurred.

It was possible to calculate the extent of conversion of 3,5- to 2,4-octadienoate (25%) by using the quantity of 2,4-octadienoate formed and the quantity 3,5-octadienoate which remained in the control (Table I). This conversion value (25%) is of the same order as that reported by Struijk and Beerthuis (9) for the conversion of (E)-3-dodecenoyl-CoA to (E)-2-dodecenoyl-CoA (35%).

The results obtained with the model substrate (3,5-octadienoate), therefore, suggest that the isomerization proceeded with retention of a conjugated double bond system. By virtue of this observation, it is proposed that the theoretical β-oxidation intermediate of α-eleostearic acid, namely 3,5,7-dodecatrienoyl-CoA, will isomerize to 2,4,6-dodecatrienoyl-CoA under the influence of enoyl-CoA isomerase (Fig. 1).

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METHODS

Extraction and Analysis of Lipids from Immature Soybeans

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ABSTRACT

A procedure is described for the extraction of lipids from immature soybeans that eliminates artifact formation and provides complete recovery of the lipid, including highly polar glycolipids free of nonlipid substances. The method involves pretreatment and extraction of the beans with hot dilute (0.25%) acetic acid, followed by chloroform-methanol extraction. Pretreatment and extraction of the tissue with hot dilute acetic acid destroys hydrolytic enzymes and removes organic-soluble, nonlipid substances that contaminate extracts obtained by chloroform-methanol extraction. Application of the method to immature soybeans confirmed that phosphatidic acid was largely an artifact of freezing and thawing of the beans, and phosphatidylmethanol was produced via transphosphatidylation of phosphatidylcholine and phosphatidylethanolamine upon extraction with chloroform-methanol.

INTRODUCTION

Several investigators (1-4) have shown that hydrolytic enzymes, particularly phospholipase D, must be destroyed by heat treatment to avoid the formation of artifacts in the extraction of lipids from plant tissues. Yang et al. (3) showed that this enzyme catalyzes transphosphatidylation of phosphatidylcholine (PC) with water giving phosphatic acid, and that phosphatidylethanol is produced upon extraction of fresh plant tissue with ethanol by the same reaction. These workers (3) and Douce et al. (2) showed that transphosphatidylation reactions are readily prevented by steaming the tissue prior to extraction. Wilson and Rinne (5) reported that freezing of immature soybeans adversely affected lipid composition with the formation of elevated levels of phosphatidic acid. More recently, Roughan et al. (4) reported that formation of phosphatidic acid and phosphatidylmethanol in immature soybeans during extraction with chloroform-methanol also can be prevented by pretreatment of the beans with steam.

A simple procedure for the complete extraction of the lipid of immature soybeans, including highly polar glycolipids, is reported. The procedure involves pretreatment and extraction of the beans with hot dilute 0.25% acetic acid to destroy lipolytic enzymes, and to remove organic-soluble, nonlipid substances prior to extraction of the lipid with chloroform-methanol.

MATERIALS AND METHODS

Immature soybeans of the Clay variety were picked from plants grown at the Agronomy Department of the University of Minnesota, and stored at 4 C until used.

Chloroform and methanol were purified by distillation. Reagent grade acetic acid (Mallinkrodt) was used as purchased.

Thin layer chromatography (TLC) was carried out on plates coated with 0.3 mm of Silica Gel H (Ag. Merck, Darmstadt, Germany), activated by heating for 1 hr at 110 C. Phospholipids were analyzed by one dimensional TLC with chloroform (C), methanol (M), concentrated ammonium hydroxide (NH_4OH) (65:35:5, v/v/v), and glycolipids with C/M/2.5 N NH_4OH (60:35:8, v/v/v). Two dimensional TLC was carried out with C/M/NH_4OH (65:25:2, v/v/v) in the first dimension and C/M/HAc/H_2O (85:15:10:3, v/v/v/v) in the second dimension after drying the plate in an atmosphere of nitrogen for 30 min. The phospholipid spots were detected with a molybdenum blue reagent as described by Dittmer and Lester (6); glycolipids were detected with α-napthol reagent (7); all spots were made visible by charring with chromic-sulfuric acid (8). A ninhydrin reagent (9) (0.2% in 95% butanol/5% acetic acid) was used to detect soluble, nonlipid substances and lipids containing free amines, as previously described (8).

Steam treatment of soybeans, formation of PM by incubating soybeans in methanol, as well

as aqueous washing for the purification of C/M lipid extracts were carried out essentially as described by Roughan et al. (4).

Extraction Procedure

Seeds were removed from the pods and ca. 0.5 g placed in 5 ml of hot (85 C) 0.25% aqueous acetic acid in a capped test tube and heated for 30 min. The tissue was homogenized in the hot acetic acid with a Tekmar Superdispax homogenizer (Tekmar Scientific Apparatus, Cincinnati, OH) at maximum speed for 30 sec. The homogenate was allowed to stand at room temperature for 10 min, followed by centrifugation at 12,500 rpm for 10 min. The supernatant was decanted, the pellet resuspended in 5 ml fresh 0.25% acetic acid and the suspension treated in the same manner again. This procedure was repeated twice more, then the centrifuge pellet was transferred with 10 ml of methanol to a 40 ml centrifuge tube; 10 ml of chloroform was added and the mixture homogenized 30 sec at maximum speed. The homogenate was allowed to stand 15 min and filtered. The residue was reextracted with 20 ml 1:2 C/M, filtered, and the filtrate combined with the first filtrate, evaporated to near dryness on a rotary evaporator and transferred with chloroform to a test tube fitted with a teflon-lined screw cap. The extract was evaporated to dryness with N_2 and dissolved in chloroform (1 ml per g of tissue).

RESULTS

Figure 1A shows the two dimensional TLC analysis of the lipid of fresh, immature soybeans obtained by the procedure described above. For comparison, Fig. 1B shows the analysis of the lipids obtained from a sample of the same beans by extraction with chloroform-methanol (2:1, v/v) after incubation of the homogenized tissue in methanol for 15 min at room temperature. The analysis in Fig. 1A shows that the lipid obtained by our procedure was completely devoid of PM and contained only a trace of PA. The large amount of PM and PA obtained upon incubation of the beans in methanol and extraction with chloroform-methanol demonstrated the formation of these compounds as artifacts of the extraction procedure.

Figure 2 shows the TLC analysis of ninhydrin positive material in the individual extracts obtained in the new procedure described above. The acetic acid extracts, analyses 1-4, Figure 2, contained large amounts of these substances. None of these substances was lipid inasmuch as none charred, nor was any of them identifiable as a known lipid class. In contrast, the chloroform-methanol extracts, 5 and 6, Figure 2, whose combined analysis is shown in Figure 1A, contained none of the substances detected in the acetic acid extracts indicating that they were free of nonlipid substances. PE, of course, gives a positive ninhydrin test and was present in the first chloroform-methanol extract (5, Fig. 2).

Figure 3 shows a comparison of the TLC analysis of the complex glycolipids extracted by our procedure (spots at and near the origin in Fig. 1A), and that obtained by a conventional chloroform-methanol extraction in which nonlipid substances are separated by washing

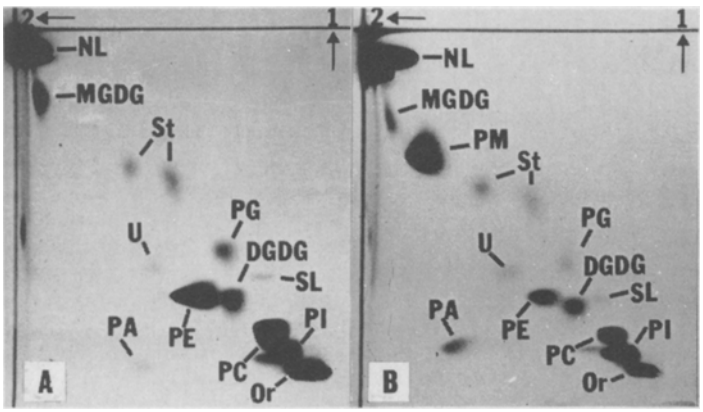

FIG. 1. Analyses of lipid extracts of immature soybean by TLC. A) Extraction with C/M after preextraction with hot acetic acid; B) extraction with C/M after incubation in methanol. NL, neutral lipid; MGDG, monoglycosyl diglyceride; PM, phosphatidylmethanol; St, steryl glucosides; PG, phosphatidylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; DGDG, diglycosyl diglyceride; SL, sulfolipid; Or, origin. First dimensional development in C/M/NH_4OH (65:25:2, v/v/v); second dimensional development in C/M/HAc/H_2O (85:15:10:3, v/v/v/v).

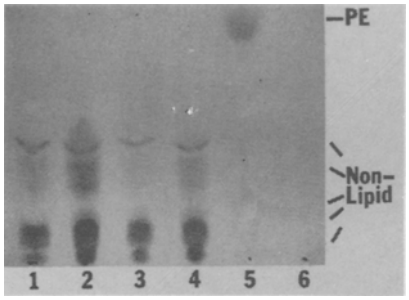

FIG. 2. Analysis of extracts of immature soybean: 1-4, extraction with dilute acetic acid (after treatment at 85 C for 30 min); 5 & 6, C/M extraction of lipid after treatment and extraction with acetic acid; PE, phosphatidylethanolamine. Developed in $CHCl_3$/MeOH/NH_4OH (65:35:5, v/v/v); visualized with ninhydrin spray.

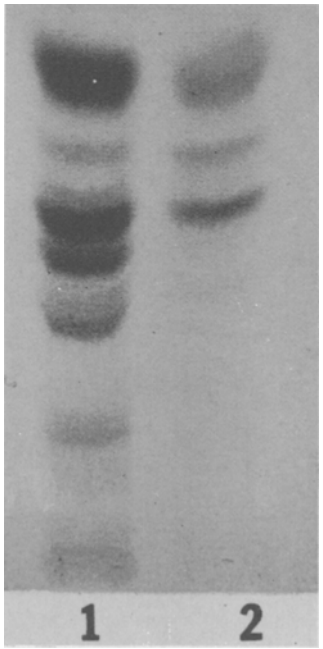

FIG. 3. Analysis of the polar glycolipid fraction of lipid extracts of immature soybeans. 1, Extract by new method; 2, extract by C/M followed by 0.9% NaCl washing. Developed in C/M/2.4 N NH_4OH (60:35:8, v/v/v).

with a 0.9% NaCl solution (4). These analyses showed that the more polar components were lost in the aqueous washing by the conventional procedure. These compounds (Fig. 3) were recovered completely by our procedure inasmuch as none were detected in the acetic acid extracts (by charring), and they were completely extracted from the tissue with chloroform-methanol. No free amine or phosphate groups were detected in these compounds (Fig. 3), but they gave a positive test for carbohydrate.

In order to demonstrate that PM and PA were artifacts of enzyme activity, upon rupture of the cells, a series of additional experiments was performed. In the first experiment, our procedure was applied to immature beans that had been frozen. Analysis of the lipid (Fig. 4A) showed that it contained phosphatidic acid, but no PM. Phosphatidic acid apparently was produced via the release of phospholipase D upon freezing and thawing of the tissue. No PM was produced in this experiment because the tissue was treated with hot acetic acid after freezing and thawing and prior to extraction with chloroform-methanol. These results (Fig. 4A) indicated that the enzyme was destroyed or inactivated by the hot acetic acid. When fresh, immature beans were extracted first with dilute acetic acid at room temperature and then with chloroform-methanol, no PM was formed, but PA was produced upon release of phospholipase D from the ruptured cells during the room temperature acetic acid extraction (Fig. 4B). In order to demonstrate further that the enzyme was destroyed by heat, a sample of fresh beans was treated with a 1:1 mixture of 0.25% acetic acid and methanol at room temperature prior to extraction with chloroform-methanol. Both PA and PM were produced in this experiment as shown in Figure 4C, indicating, indeed, that the enzyme responsible for the formation of these compounds was destroyed by the hot acetic acid treatment. When fresh tissue was treated with hot dilute acetic acid according to our procedure, no PA or PM was produced regardless of whether or not the tissue was subsequently frozen. Thus, it would appear that tissue treated in this manner could be stored indefinitely at low temperature without deterioration of the lipid.

DISCUSSION

The work reported here confirms that PA is produced in immature beans upon freezing and thawing in accord with the observations of Wilson and Rinne (5). Furthermore, the relatively large amounts of PA in immature soybeans appears to be artifacts of handling and extraction procedures as suggested by Roughan et al. (4). It also appears that contamination with PM formed by transphosphatidylation of PC and PE with methanol in conventional chloroform-methanol extraction procedures could account for the reports of large amounts of bis-phosphatidic acid (10) and N-acylphos-

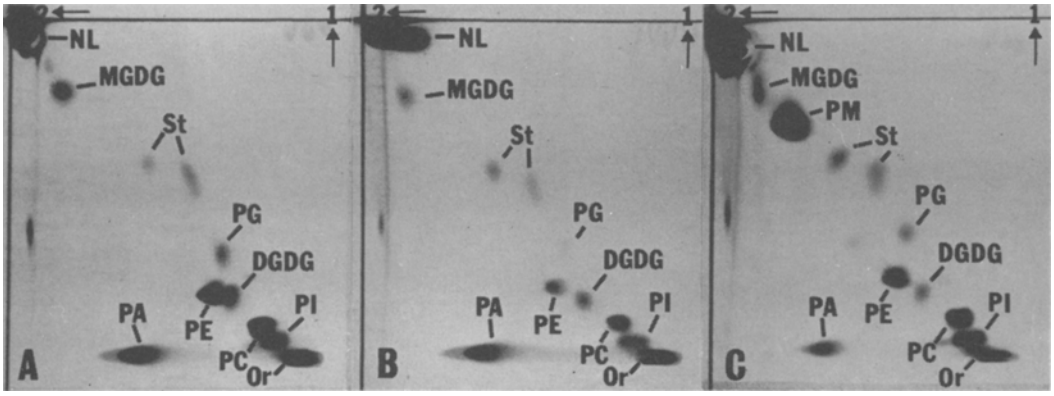

FIG. 4. Analyses of lipid extracts of immature soybeans. A) Extraction by new method after freezing and thawing; B) extraction by new method with room temperature acetic acid treatment; C) extraction with C/M after treatment with 0.25% acetic acid/MeOH (1:1, v/v) at room temperature. See Figure 1 for chromatographic conditions and spot identification.

phatidylethanolamine in immature soybeans (11,12) inasmuch as all of these compounds have similar chromatographic properties (4).

Although steaming of the beans (2-4) or a hot isopropyl alcohol treatment as described by Kates and Eberhardt (1) prevents artifact formation, considerable amounts of nonlipid substances are extracted with the lipid in these procedures. For a quantitative analysis of the lipid content of either plant or animal tissues, it is general practice to employ chloroform-methanol to insure complete extraction of the lipid, and to combine this technique with secondary purification by aqueous washing as in the Folch procedure (13) or Sephadex chromatography (14), to remove the organic-soluble, nonlipid substances. Sephadex chromatography is time-consuming and removes the highly polar glycolipids as well as the nonlipid substances. Washing with aqueous salt solutions results in loss of some phospholipids as well as a portion of the highly polar glycolipids as illustrated here (Fig. 3). Moreover, these losses can be highly variable because it is difficult to standardize the washing procedure from one laboratory to another.

The procedure described here not only eliminates artifact formation, but it also provides a complete extraction of the lipid including the complex glycolipids, free of nonlipid impurities. We have also applied the technique of preextraction of the tissue with dilute acetic acid to brain tissue (15). In this case, a hot extraction was not necessary, although we have since found that application to other animal tissue requires a hot extraction similar to that employed here. It is highly likely that the technique described here can be applied generally to plant and animal tissues, and work is in progress towards developing the technique into a general method of lipid extraction.

ACKNOWLEDGMENT

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Identification of Methyl-Branched Fatty Acids from the Triacylglycerols of Subcutaneous Adipose Tissue of Lambs

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ABSTRACT

A concentrate of branched chain fatty acids (as methyl esters) was prepared from the triacylglycerols of subcutaneous adipose tissue lipids of lambs receiving a carbohydrate-rich (cereal) diet. This was accomplished by procedures which allowed the removal of unsaturated components by peroxidation and straight chain saturated components by urea-adduct formation. The concentrate was analyzed by high resolution gas chromatography in combination with mass spectrometry and was shown to consist of a complex mixture of saturated methyl-substituted fatty acids. Methyl substitution occurred on even-numbered carbon atoms (relative to the carboxyl group) and the chain lengths of the acids ranged from 10 to 18 carbon atoms. Acids with one methyl substituent in the fatty acyl chain were most abundant; di-, tri- and tetramethyl-substituted acids were also present. The biosynthesis of these methyl-substituted acids is discussed briefly.

INTRODUCTION

Branched chain fatty acids comprise up to 10% of the total fatty acids of the triacylglycerols of subcutaneous adipose tissue lipids of lambs given diets rich in barley (1). An exploratory examination (2) into the chemical constitution of these branched chain fatty acids using the combined procedures of gas chromatography and mass spectrometry (GC-MS) showed that the major components consisted of a range of acids with monomethyl substitution on the even-numbered carbon atoms (in relation to the carboxyl group); di- and trimethyl-substituted acids were also present. It was apparent that the mixture of branched chain components was very complex and comprised upwards of a hundred components.

In a later study to facilitate the analysis of the complex mixture, a concentrate of branched chain fatty acid esters was subjected to successive fractionation with urea such that adduct formation allowed segregation of the bulk of the monomethyl-substituted fatty acids from the di- and polymethyl components (3). The fractions obtained from this procedure have now been analyzed by GC-MS, and the findings form the basis of this paper.

MATERIALS AND METHODS

Source of Fatty Acid Methyl Esters

The concentrate of branched chain fatty acid esters used in the present investigation was that described previously (3) in which triacylglycerols of the subcutaneous (rump) adipose tissue (containing 9.7% of branched chain fatty acids) of barley-fed lambs were transesterified in the presence of sodium methoxide to yield fatty acid methyl esters. Over 80% of the straight chain saturated esters (and some esters having an iso configuration) was removed by urea-adduct formation (4), and a fraction consisting essentially of unsaturated esters and branched chain esters was obtained. Unsaturated esters were removed completely by treatment of the mixture with peracetic acid (5); the saturated components, straight chain and branched chain, were isolated from the products of peroxidation by extraction into light petroleum (b.p. 40-60 C). After saponification and removal of unsaponifiable material, the resulting fatty acids were esterified with 1% (w/w) sulphuric acid in methanol, and the mixture was quantitatively distilled in vacuo, in the presence of tetracosane, to yield the concentrate of methyl esters which contained about 85% branched chain components in admixture with straight chain saturated components.

Fractionation of the concentrate (54 g) was accomplished using a mixture (by weight) of the esters (1 part), urea (5 parts) and methanol (7 parts) (6). The urea-adducted material (Fraction A) was separated by filtration from the nonadducting material (Fraction B), and both fractions were acidified with dilute sulphuric acid and extracted into diethyl ether to yield Fraction A, 36 g and Fraction B, 17 g. These manipulations occasioned very small nonspecific losses accounting for about 2% overall. Fraction B was hydrolyzed and the recovered fatty acids esterified with 1% w/w sulphuric acid in *n*-butanol to give butyl esters which were fractionated with urea in methanol as before to yield a urea-adducting fraction (Fraction C, 5.4 g) and a nonadducting fraction (Fraction D, 13 g). Both fractions of butyl esters were converted to methyl esters prior to GC-MS analysis.

Preparative Chromatography

To allow optimal GC resolution and time for MS scans to be taken, Fraction D was further fractionated by preparative GC using a 4.6 m x 0.9 mm o.d. glass column containing acid washed, silane-treated Celite 545 (80/100 mesh) impregnated with 15% (w/w) Apiezon L grease (Shell Chemicals Ltd., London); the column was operated at 200 C with argon as carrier gas at 100 ml/min. So that none of the components within a given fraction was greatly in excess of others, fractions were collected in such a way that the bulk of the major components originally present in Fraction D was collected into one series of tubes and minor components into another.

GC-MS

The collected subfractions of Fraction D along with intact Fractions A and C were analyzed by GC-MS using a 100 m x 0.25 mm i.d. stainless steel open tubular column (Perkin Elmer Ltd., Beaconsfield, Bucks, England) coated with polymerized butanediol succinate. The column was connected by means of an all-glass interface to a VG Micromass 16 F single focusing mass spectrometer (VG Micromass Ltd., Altrincham, Cheshire, England) and was operated isothermally at 170 C with helium at 1 ml/min as carrier gas. The mass spectrometer was operated at an accelerating voltage of 4 kV, an ionization energy of 70 eV, an ion source temperature of 200 C, and an interface temperature of 220 C. Mass spectra were recorded at a scan speed of 1 sec/decade either manually or with a VG 2025 twin floppy diskette data system. The resolution of the mass spectrometer was about 1000.

The equivalent chain length value and the proportion of each component in the three fractions were calculated from the chromatograms obtained when the GC column was operated in a Pye Series 104 gas chromatograph (Pye Unicam Ltd., Cambridge, England) fitted with a flame ionization detector and a splitless injection system (Scientific Glass Engineering (U.K.) Ltd., London); the GC conditions were the same as those indicated above.

RESULTS

Analyses were carried out on the concentrate of branched chain fatty acids (as methyl esters) from which the unsaturated esters and most of the straight chain saturated esters originally present were excluded; for convenience, the components in the concentrate are referred to subsequently as acids. The proportion of branched chain fatty acids in the total fatty acids of the triacylglycerols of the subcutaneous adipose tissue was 9.7%; in the concentrate, it was increased to 85%. Thus, the values in Tables I-V, which refer to the proportions of branched chain acids in the concentrate, can be readily adjusted to give the proportion of each acid in relation to the total fatty acids of the depot lipids.

Fractionation of the concentrate of branched chain fatty acids yielded Fractions A, C and D which represented 66%, 10% and 24%, respectively, of the total acids of the concentrate. In Fractions A and C, the composition of the acids was such (Table I) that the chromatographic components were well resolved. However, Fraction D was unduly complex (Table I) and required further fractionation before GC-MS analysis. Therefore, recourse was made to preparative GC, as mentioned above, and the resulting fractions yielded useful spectra on submission to GC-MS. Even so, it was found that a substantial proportion of the chromatographic peaks of the subfractions contained more than one component. Where this occurred, an estimate of the contribution of each component in a composite peak was

TABLE I

Percentage Weight Distribution of Components in the Concentrate of Branched Chain Fatty Acids Subjected to Successive Urea Fractionations[a]

Acid type	Fraction A	Fraction C	Fraction D	TOTAL
Straight chain	15.93	0.16	---	16.09
Monomethyl-substituted	50.07	5.04	1.00	56.11
Dimethyl-substituted	---	4.72	17.88	22.60
Trimethyl-substituted	---	0.08	1.85	1.93
Monoethyl-substituted	---	---	3.27	3.27

[a]See under Materials and Methods for origin of fractions.

TABLE II

Monomethyl-Substituted Fatty Acids of Lamb Triacylglycerols
Wt %[a] FCL Values[b] and Identity of Components Examined by GC-MS

Normal chain length	Position of methyl substituent																							
	2		4		6		8		10		11		12		13		14		15		16			
	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%		
10	-0.11	tr	0.43	0.61	0.43	0.26	0.71	0.06																
11			0.42	0.31	0.43	0.12	0.48	0.06																
12			0.42	1.64	0.36	0.02	0.35	0.33	0.71	0.02	0.55	0.02												
13			0.41	0.77	0.41	0.04	0.41	0.29	0.48	0.05														
14	-0.19	0.03	0.41	6.55	0.31	3.13	0.31	3.62	0.41	2.22			0.70	1.38										
15	-0.21	0.10	0.36	2.22	0.29	1.78	0.29	1.78	0.36	1.14			0.50	0.68	0.69	0.06	0.58	0.71						
16	-0.23	1.97	0.37	4.96	0.28	2.22	0.30	2.39	0.31	2.56			0.41	3.98			0.69	3.98	0.55	tr				
17			0.36	0.45	0.23	0.34	0.23	0.37	0.23	0.07			0.23	0.57			0.52	0.90	0.65	tr				
18			0.27	0.17	0.20	0.18	0.20	0.18									0.42	0.46			0.54	0.30		
Total		2.10		17.68		8.09		9.02[c]		6.04[c]		0.02		5.23		0.06		2.07[c]				0.30		

[a]Values refer to % of fatty acids in the concentrate of branched chain fatty acids.
[b]Fractional chain length. Values taken from GC of methyl esters (see under GC-MS for GC conditions).
[c]Excludes wt % values of acids with an anteiso configuration.

made from consideration of the relative intensities of diagnostic ions in the relevant mass spectra.

In Fractions A, C and D, the relative proportion of individual components was expressed as a percentage of the total area under the peaks in the mass trace of the chromatogram of each fraction. The three series of values thus obtained were adjusted to allow for the contribution which the respective fractions made to the total weight of the concentrate; summation of the adjusted values gave the fatty acid composition of the concentrate. Since the concentrate contained a number of components in proportions of the order of 0.01% to 0.09%, values presented in Tables I-V and in the text are given to two decimal places.

The percentage weight distribution of fatty acids between Fractions A, C and D (Table I) provides a useful indication of the effectiveness of urea fractionation as a means for simplifying the composition of the fatty acids in the concentrate. Thus, the first crop of urea clathrates included over 99% of the straight chain constituents and some 89% of the original content of monomethyl acids. On the other hand, the esters which were excluded by the two treatments with urea comprised 79% of the dimethyl-substituted acids originally present in the concentrate and about 96% of the total trimethyl-substituted acids. Additional to these polymethyl-substituted fatty acids, Fraction D contained a small proportion of monomethyl acids (ca. 1.9% of the total monomethyl acids) which had also escaped clathrate formation; these latter components comprised mainly medium chain fatty acids and were, therefore, confined to the earlier peaks on the chromatograms.

The structures of the components were assigned from mass spectral evidence according to established procedures (7,8) and from consideration of the gas chromatographic retention data. The identity and fractional chain length (FCL) (9) values of the monomethyl-substituted fatty acids are given in Table II; these acids represented 56.11% of the total acids in the concentrate. Acids with a substituent in the 4 position contributed 17.68% of which 4-methyltetradecanoic acid (6.55%) and 4-methylhexadecanoic acid (4.96%) were the most abundant. In contrast, other acids with mid-chain substitution were present to a lesser extent, the proportions of 6-methyl-, 8-methyl-, 10-methyl-, 12-methyl- and 14-methyl-substituted acids being 8.09%, 9.01%, 6.04%, 5.23% and 2.07%, respectively. Acids with an anteiso structure represented 5.50% of the total acids, with 14-methylhexa-

TABLE III

Dimethyl-Substituted Fatty Acids of Lamb Triacylglycerols
Wt%[a], FCL[b] Values and Identity of Components Examined by GC-MS

	Position of methyl substituents																															
Normal chain length	2,6		2,8		2,10		2,12		4,6		4,8		4,10		4,12		4,14		6,8		6,10		6,12		6,14		8,10		8,12		8,14		10,12		10,14	
	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%
9	0.31	0.02									0.97	0.11																						
10	0.27	0.02							0.61	0.02	1.00	0.25							0.86	0.01														
11	0.24	0.05	0.39	0.08					0.56	0.28	0.87	0.75	0.95	0.08					0.67	0.02	0.67	0.02												
12	0.29	0.04	0.24	tr					0.50	0.01	0.79	2.11	1.06	0.57					0.65	tr	1.00	0.05					1.06	0.05						
13											0.72	1.52	0.92	0.66							0.76	0.38					0.80	tr						
14	0.15	0.19			0.36	0.05			0.41	0.27	0.69	1.99	0.82	1.13	1.10	0.77					0.68	0.70	0.98	0.10			0.68	0.44	0.98	0.25			1.22	0.01		
15	0.15	0.05	0.20	tr	0.29	tr	0.39	0.03			0.65	1.50	0.76	0.84	0.90	0.65			0.61	0.34	0.61	0.57	0.82	tr			0.61	0.47	0.79	0.46			0.82	0.04		
16							0.33	0.03	0.33	0.02	0.62	0.91	0.74	0.58	0.77	0.88	1.09	0.22					0.74	0.38	1.04	0.09			0.67	0.47	1.04	0.13			1.02	0.22
17													0.59	0.22	0.72	0.28									0.76	0.04					0.76	0.04			0.72	0.05
Total		0.37		0.08		0.05		0.06		0.60		8.89[c]		3.51[c]		1.81[c]		0.22		0.36[c]		1.67[c]		0.38[c]		0.04[c]		0.91[c]		0.93[c]		0.04[c]		0.04[c]		0.05[c]

[a]Values refer to % of fatty acids in the concentrate of branched chain fatty acids.

[b]Fractional chain length. Values taken from GC of methyl esters (see under GC-MS for GC conditions).

[c]Excludes wt % of values of acids in which one of the methyl substituents is on the anteiso position.

TABLE IV

Trimethyl-Substituted Fatty Acids of Lamb Triacylglycerols
Wt%[a], FCL[b] Values and Identity of Components Examined by GC-MS

	Position of methyl substituents																															
Normal chain length	2,6,8		2,6,10		2,6,12		2,8,10		2,10,12		4,6,8		4,6,10		4,6,12		4,8,10		4,8,12		4,8,14		4,10,12		4,10,14		4,12,14		6,8,10		6,8,12		6,10,12		8,10,12	
	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%	FCL	wt%
10											1.55	tr																						
11	0.61	0.01											1.00	tr			1.24	tr																
13											0.86	0.11					0.92	tr																
14			0.52	0.04	0.69	0.03					0.82	0.10			1.40	0.12	1.10	tr	1.30	0.42														
15							0.46	tr			0.91	0.04	0.91	0.03					1.08	0.68			1.15	tr					0.77	0.02	0.95	tr	0.98	tr	1.33	tr
16							0.62	tr	0.62	tr									0.98	0.15	1.28	0.09			1.31	tr	1.65	0.09					1.09	tr	1.09	tr
Total		0.01		0.04		0.03						0.25		0.03		0.12				1.25		0.09						0.09		0.02				

[a]Values refer to % of fatty acids in the concentrate of branched chain fatty acids.

[b]Fractional chain length. Values taken from GC of methyl esters (see under GC-MS for GC conditions).

TABLE V

Tetramethyl-Substituted Fatty Acids of Lamb Triacylglycerols
FCL[a] Values and Identity of Components Examined by GC-MS

Normal chain length	Position of methyl substituents						
	2,4,6,8	2,6,8,12	2,6,10,12	2,6,10,14	4,6,8,12	4,6,10,12	6,8,10,12
	FCL	FCL	FCL	FCL	FCL	FCL	FCL
10	1.15						
14						1.82	1.72
15	0.56	0.75	0.75	0.78	1.68	1.68	

[a]Fractional chain length. Values taken from GC of methyl esters (see under GC-MS for GC conditions).

decanoic acid present in greatest proportion (3.98%). An anteiso acid with an odd-numbered fatty acid chain, 13-methylpentadecanoic acid, was identified; the proportion of iso acids in the concentrate was 0.73%.

The distribution of the monomethyl-substituted acids in the three fractions showed that the tendency of these fatty acids to form urea adducts increased with increasing chain length. Over 30% of the total amount of 4-methyldodecanoic acid resisted urea adduct formation and was present in Fraction D, whereas 4-methylhexadecanoic acid readily formed an adduct, the entire complement being present in Fraction A. Similarly, all the monomethyloctadecanoic acids identified were present in Fraction A only.

The identity and FCL values of dimethyl-substituted fatty acids are given in Table III; these acids comprised 22.60% of the total fatty acids of the concentrate and occurred predominantly in Fraction D (Table I). Acids in which one of the methyl substituents was in the 4 position represented 15.03% of the total acids, with the 4,8-dimethyl structure the most prominent; other mid chain, dimethyl-substituted acids accounted for only 4.42% of the total acids. The proportion of anteiso acids in which there was an additional methyl substituent in the carbon chain was 2.81%.

Trimethyl-substituted acids were present mostly in Fraction D, and their contribution (1.93%) to the total fatty acids of the concentrate was relatively small. As shown in Table IV, there was a number of trimethyl-substituted acids whose presence was readily discerible from mass spectral evidence but which constituted little more than inflexions on the chromatogram of Fraction D. The most common pattern of substitution was that in which the methyl substituents were present in the 4, 8 and 12 positions.

Tetramethyl-substituted fatty acids occurred in only trace amounts in Fraction D. Those identified are given in Table V. Other tetramethyl-substituted components were present, but these could not be identified because of low ion intensity in the mass spectra. It is apparent from Table V that acids with a substituent in the 2 position were more readily characterized.

Monoethyl-substituted fatty acids were identified in Fraction D, and these acids represented 3.27% of the total fatty acids of the concentrate. Acids with an ethyl substituent in the 4 position were present in greatest abundance. The complete range of the ethyl substituted fatty acids of lamb fat and their mass spectral characteristics are the subject of another report (10).

The ion corresponding to M-57 was significant in the interpretation of the mass spectra of methyl esters of dimethyl-substituted fatty acids. It should be noted that in the mass spectra of fatty acid methyl esters with monomethyl substitution in the 4 position, the ions corresponding to M-73 and M-57 predominate with the M-73 ion more intense than that of M-57. However, in the mass spectra of the methyl esters of 4,6 dimethyl-substituted fatty acids, the M-57 ion is absent (Fig. 1). On the other hand, when one of the methyl substituents is in the 4 position and the other in the 10, 12 or 14 positions, the M-57 ion is more intense than the M-73 ion (Fig. 2); in the mass spectra of a 4,8 dimethyl type of fatty acid methyl ester, the ratio of M-73 to M-57 is similar to that in the methyl esters of 4-methyl-substituted fatty acids.

DISCUSSION

The investigation of the concentrate of

branched chain fatty acids presented two major difficulties. One of these was occasioned by the great extent to which overlapping of gas chromatographic components occurred even under optimum conditions of separation using open-tubular columns. The other concerned the problem of obtaining useful mass spectra from minor components in the concentrate; the large discrepancy between major and minor peaks on the chromatogram of the esters represented a range of sample size from the column eluate which was frequently in excess of the linear dynamic range of the mass spectrometer. Similar problems have been encountered in the analysis of the fatty acid components of *vernix caseosa* lipid and of tissue lipids of ruminant origin (11-13).

The first of these difficulties was largely obviated by following a procedure of urea-adduct formation of methyl esters and of butyl esters which resulted in the segregation of monomethyl-substituted fatty acids from the di- and polymethyl constituents. The second problem was overcome by judicious use of preparative GC. By combining these approaches, the identity of more than 180 methyl- and ethyl-substituted fatty acids has been established in a concentrate of branched chain fatty acids prepared from the subcutaneous adipose tissue of lambs given a diet containing a high proportion of barley.

Unequivocal identification of organic compounds using GC-MS normally requires reference compounds such that known and unknown substances are eluted coincidentally and yield identical mass spectra. However, in the GC-MS analysis of complex mixtures of closely related, naturally occurring substances such as the branched chain fatty acids of *vernix caseosa* lipid (12), of uropygial gland waxes (14,15), and of lamb triacylglycerols in the present work, chemical structure was assigned on the basis of mass spectral evidence and of gas chromatographic characteristics of components.

The plethora of components in the branched chain fatty acid concentrate includes a number of homologous series of positionally isomeric mono-, di- and trimethyl-substituted fatty acids. In each homologous series, the FCL values decreased with increasing length of the

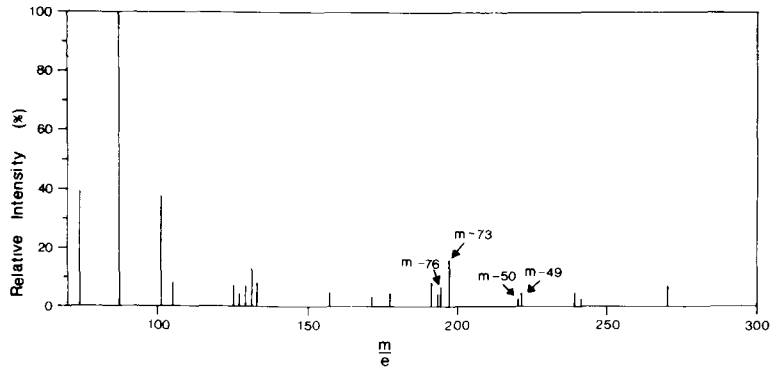

FIG. 1. Mass spectrum of methyl 4,6-dimethyltetradecanoate isolated from lamb triacylglycerols.

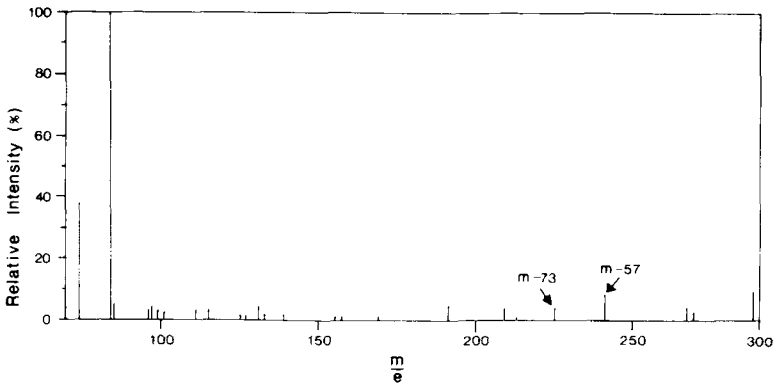

FIG. 2. Mass spectrum of methyl 4,12-dimethylhexadecanoate isolated from lamb triacylglycerols.

chain. Thus, for the 4-methyl-substituted series the values decreased from 0.43 for 4-methyldecanoic acid to 0.27 for 4-methyloctadecanoic acid. This effect is presumably associated with the shift in the position of the methyl substituent in relation to the terminal-methyl group in the fatty acid chain (16).

The chromatographic data obtained in this investigation were examined in relation to possible additive effects of methyl substituents in the prediction of relative retention times of di- and polymethyl-substituted fatty acids. When a comparison was made between methyl-substituted fatty acids of the same chain length, it was found that for dimethyl acids in which the two substituents were separated by three or more methylene groups there was close correspondence between observed and calculated FCL values. However, in fatty acids having methylene-interrupted dimethyl substitution, there was a considerable discrepancy between the respective FCL values; this is exemplified by methyl 4,6-dimethylhexadecanoate which was found to have an FCL value of only 0.41, whereas the value calculated from 4-methyl- and 6-methylhexadecanoates was 0.65 (cf. 12). It was also found that FCL values obtained for trimethyl-substituted esters were considerably lower than those expected from the contribution of substituents in the corresponding monomethyl compounds. Caution must, therefore, be exercised when using FCL values as an adjunct to mass spectrometry for the analysis of multibranched fatty acids or of dimethyl-substituted fatty acids in which one methylene group separates the two methyl substituents.

The occurrence of methyl-substituted fatty acids in adipose tissue triacylglycerols of the sheep is associated with the metabolism of the greatly enhanced proportions of propionate that are produced in the rumen as a result of the bacterial fermentation of cereal starch (17, 18). When ruminal production of propionate is in excess of that which can be oxidized or used for gluconeogenesis (via succinyl CoA), methylmalonyl CoA and its precursor, propionyl CoA, apparently become available for utilization by the fatty acid synthetase of adipose tissue (1). As was shown by studies in vitro, methylmalonyl CoA, in the presence of malonyl CoA, can be utilized by fatty acid synthetase preparations from ovine adipose tissue to produce a complex mixture of methyl-substituted fatty acids (19). Also, the concomitant availability of propionyl CoA as primer unit would account for the presence of odd-chain as well as even-chain components in the mixture of branched chain fatty acids from lamb adipose tissue.

Not all the branched chain fatty acids in the concentrate are of endogenous origin because the presence of iso acids, which could not have been formed biosynthetically by utilization of methylmalonyl CoA, is indicative of a contribution arising from assimilation from the gut of the component lipids of rumen micro-organisms (20). These iso acids will almost certainly have been accompanied by anteiso acids since they also form part of the structural lipid of rumen bacteria, though the extent of the contribution to the anteiso acids in the concentrate is likely to be small. The origin of the anteiso acid, 13-methylpentadecanoic acid, is not known although it has been identified among the fatty acids of baboon liver lipids (21).

Among the components in the concentrate of branched chain fatty acids, the lower limit of chain length is apparently 10 carbon atoms. The possibility exists that the lipids from which the concentrate was prepared contained fatty acids with less than 10 carbon atoms in the chain because among the procedures used in the segregation of branched chain fatty acids was that of solvent removal under vacuum; the attendant risk of loss of short and medium chain fatty acid methyl esters would be considerable. In this connection, medium chain methyl-substituted fatty acids have been identified in the steam-volatile fraction of mutton fat (22,23).

The occurrence of branched chain fatty acids in considerable proportions in ruminant tissue presents an opportunity to study several aspects of the physiological and nutritional significance of these acids when they are included in the diets of rats. These investigations will be the subject of a later report.

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: V. Photosensitized Oxidation[1]

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ABSTRACT

The role of singlet oxygen in oxidation was studied by analyzing hydroperoxide isomers in unsaturated fats and esters by gas chromatography-mass spectrometry (GC-MS). On oxidation photosensitized with methylene blue at 0 C, methyl oleate produced a 50-50% mixture of 9- and 10-hydroperoxides, linoleate a mixture of 66% conjugated (9+13) and 34% unconjugated (10+12) hydroperoxides, and linolenate a mixture of 75% conjugated (9+12+13+16) and 25% unconjugated (10+15) hydroperoxides. Cottonseed, safflower, and corn oil esters showed, as in soybean esters, the presence of varying amounts of 12-hydroxy esters derived from the corresponding hydroperoxide at low peroxide values. Since these oils do not contain linolenic acid, a likely source of the 12-hydroperoxide is linoleic acid by photosensitized oxidation. Several lines of evidence support the conclusion that singlet oxygen may contribute to the unique hydroperoxide composition of vegetable oil esters at low levels of oxidation. In the presence of photosensitizers such as methylene blue and chlorophyll, the unique hydroperoxide composition (high levels of 10- and 12-hydroperoxides) obtained in soybean esters was similar to that produced by oxidation at low peroxide values. In contrast, a normal hydroperoxide composition was produced, as expected from the fatty acid composition of soybean oil esters, when singlet oxygen quenchers such as β-carotene and α-tocopherol were used and when the esters were treated with carbon black to remove natural photosensitizers. GC-MS analyses of the derived unsaturated alcohols provided indirect evidence for 12-hydroperoxy-9,13-diene in soybean esters as expected by photosensitized oxidation of linoleate.

INTRODUCTION

There is increasing evidence that photosensitized oxidation may be important in initiating or propagating normal free radical autoxidation of unsaturated fats (1-5). Although autoxidation of linoleate and linolenate produces only conjugated hydroperoxides, photosensitized oxidation by singlet oxygen produces a mixture of conjugated and nonconjugated hydroperoxides (1,3,4). Our preceding paper reported that an unexpectedly high concentration of the 12-hydroperoxide isomer was found by gas chromatography-mass spectrometry (GC-MS) as the derived hydroxystearate in weakly oxidized soybean oil esters (6). Photosensitized oxidation of methyl linoleate was suggested as a possible source of this hydroperoxide. The present paper extends these studies to other vegetable oil esters and presents further evidence on the effect of singlet oxygen promoters and quenchers.

EXPERIMENTAL

The methyl esters of vegetable oils were prepared as before by methanolysis (6). Additives such as methylene blue (zinc free, Matheson Coleman & Bell, Cincinnati, OH), β-carotene (General Biochemicals, Chagrin Falls, OH), and α-tocopherol (Eastman Chemicals, Rochester, NY) were obtained commercially. A mixture of chlorophyll A and B was prepared chromatographically from soybean leaves (7).

The same procedures as previously reported were used for autoxidation, analyses, KI reduction, catalytic hydrogenation, silylation, and GC-MS (8-10). KI was used instead of $NaBH_4$ to reduce hydroperoxides to the allylic alcohols to avoid the formation of 10- and 12-hydroxymonoenes as minor artifacts from 9- and 13-hydroperoxides of linoleate (11,12). Unsaturated hydroxy derivatives from KI-reduced samples were concentrated by the same procedure as the saturated hydroxy derivatives from hydrogenated samples (6). Photooxidations were carried out in methanol solutions at 35 C in 25 ml Erlenmeyer flasks shaken in a photochemical Warburg apparatus illuminated at the bottom of the flasks with a bank of ten 75 watt tungsten bulbs giving light energy of 2.3×10^5 erg/cm^2 sec (measured with a Kettering radiant power meter, Laboratory Data Control, Riviera Beach, FL). For control dark experiments, oxidations were carried out under the same conditions in flasks protected with aluminum foil. The oxidation of pure oleate,

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[2]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

linoleate, and linolenate photosensitized with methylene blue (9 mg/2 g ester) and oxygen was done at 0 C in methanol solution (10 ml) in a cooled jacketed flask exposed to a 1000 watt tungsten light source (25 x 10^5 erg/cm^2 sec) through a 1 in. layer of water to filter infrared radiation.

To remove any photosensitizers, soybean oil esters were treated with carbon black by the following procedure. A hexane solution of 25 g esters was passed through a column (2 cm outside diameter) packed with a hexane slurry of 1:1 activated carbon black (Darco S51, Atlas Chemical Inc., Wilmington, DE) and diatomaceous earth to a height of 8 cm plus a layer of 1 cm activated silicic acid on top of the column. The soybean esters eluted with 200 ml hexane showed no significant change in fatty acid composition (14% palmitate, 4.8% stearate, 23.1% oleate, 51.2% linoleate, and 6.9% linolenate).

RESULTS

Oxidation of Vegetable Oil Esters

Different vegetable oil esters were oxidized under the same conditions as soybean oil esters (6) to compare the isomeric hydroperoxide composition at different peroxide values (Table I). The products show the expected greater contribution of linoleate hydroperoxides (9-+13-OOH) than oleate hydroperoxides (8-+9-+10-+11-OOH). Cottonseed, safflower, and corn oil esters showed, as in soybean oil esters, varying amounts of the 12-hydroxy derived ester when oxidized at low levels. To check whether low levels of linolenate might contribute to the 12-hydroperoxide formation, we searched for the presence of 16-hydroperoxide but found no evidence for it. The unexpectedly high concentration of 12-OOH in cottonseed oil esters compared to safflower oil esters, which have more linoleate, may be due to higher levels of natural photosensitizers in cottonseed oil. The 10-hydroperoxide is another isomer expected by photosensitized oxidation of linoleate (3,4,13). Although the level of 10-hydroxy ester was invariably larger than the 11-hydroxy ester derived from oleate-hydroperoxides, the difference was not always significant.

Photooxidation

According to the "ene"-type mechanism recognized for the reaction of singlet oxygen and unsaturated compounds (14), oleate produces a mixture of 9- and 10-hydroperoxides, linoleate a mixture of 9-, 10-, 12-, and 13-hydroperoxides, and linolenate a mixture of 9-, 10-, 12-, 13-, 15-, and 16-hydroperoxides (3,4,13). Although the relative amounts of these isomers are well established for oleate (50% each of the 9- and 10-isomers), no reliable quantitative study has yet been

TABLE I

GC-MS Analysis[a] of Isomeric Hydroxystearate from Vegetable Oil Esters Oxidized at 40 C

Methyl esters[b]	Peroxide value	Relative percent					
		8-OH	9-OH	10-OH	11-OH	12-OH	13-OH
Corn	22.7	1.1	45.3	3.0	1.4	4.3	44.8
	61.2	1.8	47.7	1.2	1.0	2.3	45.9
	96.2	1.4	48.2	1.7	0.8	2.6	45.2
	516	2.5	47.4	1.6	2.5	0.4	45.5
Cottonseed	23.0	0.5	41.4	1.0	0.4	19.7	37.1
	87.4	1.6	45.8	1.0	0.8	7.0	43.8
	120	0.7	48.4	0.7	0.7	5.2	44.3
	484	1.9	48.9	1.2	0.6	1.3	46.0
Safflower	23.7	1.0	43.1	5.6	1.2	6.6	42.5
	41.3	1.2	45.6	2.8	1.6	5.1	43.7
	131	1.0	47.1	2.6	1.8	2.2	45.4
	518	1.8	49.7	1.3	0.7	0.8	45.8

[a]By computer summation method (8), all analyses are based on two or more replications.

[b]GC analyses; Corn oil esters: 12.0% palmitate, 1.9% stearate, 26.0% oleate, 50.1% linoleate. Cottonseed esters: 22.4% palmitate, 2.1% stearate, 16.9% oleate, 58.6% linoleate. Safflower esters: 7.6% palmitate, 2.5% stearate, 14.5% oleate, 75.5% linoleate.

reported for linoleate and linolenate. Quantitative GC-MS analyses were therefore carried out on pure fatty esters photooxidized at 0 C in the presence of methylene blue (Table II). The formation of the expected equal mixture of 9- and 10-hydroperoxides from oleate without any evidence of 8- and 11-hydroperoxides demonstrated that no free radical autoxidation occurred under our conditions of photooxidation. Furthermore, control experiments in the dark with photosensitizer showed no increase in peroxide value under these conditions. For linoleate, the ratio of conjugated (9-+13-OOH) to nonconjugated (10-+12-OOH) hydroperoxides was 2:1. For linolenate, the outer 9- and 16-hydroperoxides were the major isomers (48%) and the inner 10-, 12-, 13-, and 15-hydroperoxides were all formed in approximately equivalent amounts (12 to 14%). The nonconjugated 10- and 15-linolenate hydroperoxides amounted to 25%.

Several experiments were carried out to elucidate how photosensitizers, such as methylene blue and chlorophyll, and singlet oxygen quenchers, such as β-carotene and α-tocopherol, affect rates of oxidation and hydroperoxide composition of soybean oil esters. Methylene blue and chlorophyll behaved as photosensitizers because their catalytic effect was virtually eliminated when the oxidation was carried out in the dark (Table III). α-Tocopherol was much more effective in diminishing rates of oxidation than was β-carotene. Although both of these additives are known quenchers of singlet oxygen (15-18), α-tocopherol reacts also with singlet oxygen (19). The effect of β-carotene is also complicated by its ready oxidation as indicated by its bleaching during the reaction. The oxidation products of β-carotene would be expected to catalyze free radical oxidation. The carbon black-treated sample was more stable to photooxidation and showed less peroxide development in the dark than the control. The study of isomeric composition provided a more reliable

TABLE II

GC-MS Analysis of Isomeric Hydroxystearates from Hydroperoxides Produced by Photosensitized Oxidation at 0 C[a]

Methyl esters	Peroxide value (time, hr)	Relative percent					
		9-OH	10-OH	12-OH	13-OH	15-OH	16-OH
Oleate	1727 (6)	47.7	52.3				
Linoleate	1124 (3)	31.9	16.7	17.0	34.5		
Linolenate	1566 (2)	22.7	12.7	12.0	14.0	13.4	25.2

[a]Two gram esters + 9 mg methylene blue + 10 ml methanol, oxygen bubbling, exposed to 1000 watt tungsten lamp (see Experimental).

TABLE III

Effect of Photosentitizers and Quenchers on the Oxidation of Soybean Oil Esters at 35 C

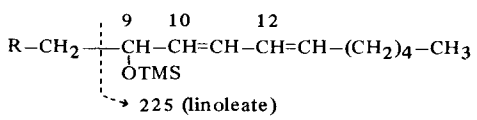
Samples[a]	Peroxide value		
	4 hr	6 hr	12 hr
Control	32.2	41.9	90.2
Control, dark	24.0	30.6	33.3
Methylene blue (2 mg)	291	460	---
Methylene blue (2 mg), dark	26.3	30.4	---
Chlorophyll (6 mg)	132	207	---
Chlorophyll (6 mg), dark	26.3	31.4	29.2
β-Carotene (2 mg)	19.4	25.5	74.3
α-Tocopherol (3 mg)	3.6	9.2	10.6
Carbon black	16.1	20.3	58.4
Carbon black, dark	12.4	20.0	22.6

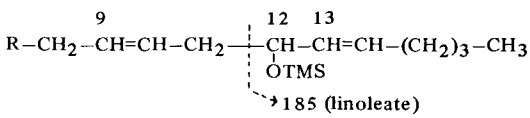
[a]Two gram esters + 2 ml methanol, shaken in air in a photochemical Warburg apparatus. All analyses are based on duplicate oxidations.

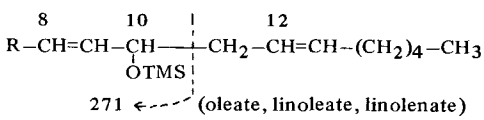
diagnostic measure of photosensitized oxidation occuring concurrently with free radical autoxidation.

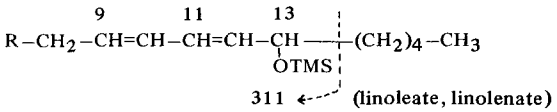
Quantitative GC-MS analyses based on hydrogenated samples are summarized in Table IV. Initially high levels of 12-hydroxy esters were found in both light exposed and dark control samples. The 10-hydroxy esters was also found in higher concentrations than the 11-hydroxy ester at low peroxide values. Similar results were obtained in the oxidations photosensitized with methylene blue and chlorophyll. In the presence of these photosensitizers, higher concentration of the 12- and 10-hydroxy isomers were produced at all levels of oxidation studied. The amount of 15-hydroxy ester that would be expected from linolenate photosensitized oxidation (Table II) was not significantly increased. The amounts of 12- and 10-hydroxy isomers were significantly decreased at low peroxide levels in the presence of β-carotene, α-tocopherol, and in samples treated with carbon black. Both α-tocopherol and β-carotene apparently act by quenching the formation of singlet oxygen (15-18). Treating soybean oil esters with carbon black would remove natural photosensitizers. Therefore, traces of photosensitizers in soybean oil esters apparently catalyze the oxidation of linoleate by singlet oxygen at low peroxide values.

Additional evidence for the 12-hydroxy ester derived from the corresponding 12-hydroperoxy-9,13-diene expected by photosensitized oxidation of methyl linoleate (4) was obtained by GC-MS analysis of KI-reduced samples. Although the method has not been standardized with known ccompounds, the relative intensities were measured for m/e values 225, 185, 271, and 311 corresponding to the respective trimethylsilyl allylic ether derivatives of the 9-hydroxy (conjugated), 12- and 10-hydroxy (unconjugated) and 13-hydroxy (conjugated) esters:









[R=–$(CH_2)_6COOCH_3$]

Mass 185 is the only unique fragment expected from photosensitized oxidation of linoleate. Mass 271 can come from either oleate autoxidation or linoleate and linolenate photosensitized oxidation. The GC-MS analyses are complicated by the fact that fragments 225 and 185, which contain one oxygenated function, have higher intensities than fragments 271 and 311, which have two oxygenated functions (20). This bias is compensated, however, by adding the relative intensities due to the conjugated isomers (m/e 225 + m/e 311) and those due to the nonconjugated isomers (m/e 185 + m/e 271). The results in Table V confirm those in Table IV showing: high levels of the unconjugated 12-hydroxy isomer in control samples at low peroxide values; a significant increase in the 12- and 10-hydroxy isomers resulting from added methylene blue and chlorophyll; and a corresponding decrease in these isomers by added β-carotene, α-tocopherol, and carbon black treatment. That methylene blue acts as a photosensitizer is again demonstrated by elimination of its effect when oxidation was carried out in the dark. This evidence provides further support to our GC-MS approach for diagnosing the effect of singlet oxygen by analyzing hydroperoxide isomers in unsaturated fats.

DISCUSSION

The GC-MS method used in this study for hydroperoxide analysis has provided a useful tool to follow the unique products from oxidation with singlet oxygen formed concurrently during free radical autoxidation. Several lines of evidence support the conclusion that singlet oxygen may contribute to the unique hydroperoxide composition of vegetable oil esters oxidized at low peroxide values. Although cottonseed, safflower, and corn oil esters do not contain linolenic acid, their oxidation products included the 12-hydroperoxide determined as the derived hydroxy esters at low peroxide values. This hydroperoxide isomer is expected from linoleate by photosensitized oxidation. When soybean esters were treated with oxygen in the presence of methylene blue and chlorophyll as photosensitizers, the unique hydroperoxide composition was similar to that produced by oxidation at

TABLE IV

GC-MS Analysis of Isomeric Hydroxystearates from Soybean Oil Esters Oxidized at 35 C

Samples[a]	Peroxide value	Relative percent							
		8-OH	9-OH	10-OH	11-OH	12-OH	13-OH	15-OH	16-OH
Control, light	7.8	2.1	32.2	10.6	2.6	10.2	29.2	4.0	9.1
	19.2	2.7	34.4	8.5	2.1	7.9	32.1	4.2	8.1
	30.5	3.6	37.0	5.0	3.8	7.4	31.9	2.8	8.5
Control, dark	10.0	3.8	32.3	4.3	4.5	12.3	34.3	2.2	6.3
	13.2	3.0	34.7	5.7	4.2	10.7	34.1	1.8	5.8
	36.3	2.8	35.1	4.2	3.6	7.1	38.2	1.9	7.0
Methylene blue, light	35.0	1.6	29.3	18.2	2.0	12.5	23.0	3.4	10.0
	429	1.1	32.4	16.1	2.4	11.1	30.2	2.6	3.7
	837	1.5	33.9	14.7	2.8	11.6	28.2	1.4	5.9
Chlorophyll, light	108	1.7	36.8	13.1	3.1	8.7	28.8	2.7	5.0
	152	2.2	38.7	8.6	1.5	5.9	36.0	2.2	5.0
β-Carotene, light	31.7	1.6	42.4	1.7	1.5	2.7	43.3	1.2	5.6
	434	2.2	45.6	1.1	1.1	1.9	41.6	2.0	4.6
α-Tocopherol[b], light	14.8	2.9	41.0	5.1	4.5	3.0	36.0	2.1	5.4
	42.3	1.2	42.2	0.7	0.2	2.6	43.6	1.0	8.5
	60.0	0.4	43.8	0.4	1.0	3.0	43.6	1.8	6.0
Carbon black, light	13.7	3.3	38.0	3.7	3.3	3.1	41.3	1.0	6.2
	20.3	2.6	41.3	6.2	3.3	4.1	35.8	0.4	6.3
Carbon black, dark	13.6	3.0	39.5	2.2	3.1	1.5	40.7	1.8	8.3
	20.4	3.6	39.6	3.0	5.0	2.3	38.3	1.5	6.9

[a]See legend in Table III.

[b]Oxidation temperature 60 C.

low peroxide values. The increased levels of 10- and 12-hydroperoxides are those expected from oxidation of linoleate with singlet oxygen (Table II). The possibility of allylic rearrangement of the 10- and 12-hydroperoxides by 1,3-shifts (21,22) is ruled out by our failure to detect the corresponding allylic 8- and 10-hydroxy isomers. When soybean esters were treated with carbon black to remove natural photosensitizers, a normal hydroperoxide distribution was obtained, as expected, from the fatty acid composition of the starting material. This evidence is, however, ambiguous because other materials can be removed by carbon black. A normal isomeric hydroperoxide composition was also produced when α-tocopherol and β-carotene were added to quench singlet oxygen. Finally, GC-MS analyses of KI-reduced samples provided indirect evidence for a 12-hydroxy-9,13-diene (m/e 185) from the corresponding hydroperoxide expected by photosensitized oxidation of linoleate. All these results are consistent with the view that traces of photosensitizers in soybean and other vegetable oil esters apparently catalyze the oxidation of linoleate by singlet oxygen at low peroxide values.

There remains the problem of explaining why the concentration of the 12-hydroperoxide was high when the oxidations were carried out in the dark (Table IV). Singlet oxygen can be generated in a great variety of ways. Two recent reviews summarize 12 different potential sources of singlet oxygen (23,24). In addition to the photosensitized reaction with oxygen, there are several chemical methods to activate oxygen to the singlet state. Certain molecules are known to form unstable adducts with oxygen and can produce singlet oxygen by a transfer reaction (25). Another process involves the termination of secondary hydroperoxides via a tetroxide intermediate which decomposes into a ketone, an alcohol, and oxygen (26). There is evidence that the oxygen thus produced is activated in the singlet state (27). Therefore, in soybean and other vegetable oil esters, there are possible ways to mimic sensitized oxidation without requiring light. Such processes may involve either unstable endoperoxides (23) from linolenate autoxidation or metal oxygen complexes (28). The role that singlet oxygen and related species play in the hydroperoxidation of unsaturated lipids is now under intensive investigation (17,23,24,29). Problems for further research include ascertaining more directly whether or not singlet oxygen is involved in catalytic processes by unstable oxygen adducts, endoperoxides, and metal complexes.

ACKNOWLEDGMENT

We are grateful to K. Eskins for samples of chlorophyll and for useful discussions.

TABLE V

GC-MS Analyses of KI-Reduced Samples from Soybean Oil Esters Oxidized at 35 C

Samples[a]	Peroxide value	m/e[b] (Relative intensity)				Relative percent[c]	
		225	185	271	311	Conjugated	Nonconjugated
Control, light	11.0	55	22	4	19	74	26
	29.0	49	24	4	23	72	28
	468	65	4	4	27	92	8
Control, dark	13.2	53	27	5	15	68	32
	26.7	56	16	7	21	77	23
	36.3	49	23	8	20	69	31
Methylene blue, light	20.3	28	31	26	15	43	57
	41.5	21	39	31	9	30	70
	429	27	32	30	11	38	62
Methylene blue, dark	26.3	50	17	14	20	69	31
Chlorophyll, light	108	31	27	23	19	50	50
	152	35	25	23	18	53	47
β-Carotene, light	23.0	60	15	5	20	80	20
α-Tocopherol, light	30.0	62	8	10	20	82	18
Carbon black, light	16.1	57	13	5	25	82	18
	21.5	57	12	5	20	83	17
Carbon black, dark	20.4	58	14	4	24	82	18

[a]See legend in Table III.

[b]See text for fragmentation.

[c]Conjugated: m/e 225 + m/e 311; nonconjugated: m/e 185 + m/e 271.

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Ethanolamine Kinase Activity and Compositions of Diacylglycerols, Phosphatidylcholines and Phosphatidylethanolamines in Livers of Choline-Deficient Rats

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ABSTRACT

These experiments were performed to find the reasons for the increased concentrations of docosahexaenoyl phosphatidylethanolamines (PE) in livers of choline-deficient rats. We measured the activity of ethanolamine kinase, which catalyzes the first step in PE formation. We also measured the compositions of PE and phosphatidylcholines (PC) and concentrations and fatty acid compositions of diacylglycerols (DG), which are precursors of PE. Young male rats were fed for one week a low-methionine, choline-deficient diet, or the same diet supplemented with choline. Ethanolamine kinase activity was measured in liver cytosol (100,000 g supernatant). Fatty acids were measured in total liver diacylglycerols and in microsomal PE and PC. Ethanolamine kinase activities were equal in choline-deficient and choline-supplemented rats. Concentrations of DG were elevated 6-fold by choline deficiency. The percentage of docosahexaenoic acid (22:6n-3) in microsomal PE was nearly doubled by choline deficiency. Although the increased concentrations of PE in choline-deficient livers cannot be attributed to increased activity of ethanolamine kinase, the rate of PE formation probably was increased by increases in concentrations of its precursors, including DG. The disproportionate increase in 22:6n-3 PE probably was caused by a selective formation of PE from DG that contain 22:6n-3.

INTRODUCTION

In the livers of rats, choline deficiency causes accumulations of triacylglycerols (TG) and esterified cholesterol, and an increase in the ratio of phosphatidylethanolamines (PE) to phosphatidylcholines (PC). Because rats can biosynthesize choline by transmethylation of PE, a diet low in methionine must be used to produce changes in liver lipid compositions characteristic of choline deficiency (1).

In choline-deficient liver, the proportion of PE as percentage of total lipid phosphorus is always higher than normal. The increased percentage of PE is due largely to a disproportionate increase in molecular species that contain docosahexaenoic acid (22:6n-3) (2-5). Since it has been shown that choline or choline phosphate inhibits ethanolamine kinase activity (6-8), and that concentrations of these compounds are greatly depressed in livers of choline-deficient rats (9,10), it seemed probable that the activity of ethanolamine kinase should be elevated above normal in these livers. An increase in its activity could increase the rate of PE formation (11). The selective accumulation of 22:6n-3 PE could be caused either by an increased supply of 22:6n-3 diacylglycerol (DG) or by selective formation of PE from 22:6n-3 DG. Chen et al. (12) noted a small increase in DG concentration in microsomes from choline-deficient rat liver, but the fatty acid components of these DG were not reported.

Therefore, to find the reasons for increased proportions and amounts of 22:6n-3 PE in livers of choline-deficient rats, we measured the activity of ethanolamine kinase and the concentrations and fatty acid patterns of DG in choline-deficient livers. We also measured fatty acid patterns in microsomal PE and PC.

Choline deficiency did not elevate ethanolamine kinase activity, a surprising result that also was observed by Schneider and Vance (13) while our work was in progress. However, choline deficiency did elevate concentrations of total liver DG about 6-fold, and those of 22:6n-3 DG about 4-fold.

MATERIALS AND METHODS

Experimental Design

Male rats were used because choline deficiency produces more severe responses in males than in females (14,15). We used dietary conditions similar to those used earlier (5) to produce fatty livers and alterations of liver phospholipid distributions. The following experiments were performed twice, each time with 3 control and 3 deficient livers, because we could process only 6 livers at a time. The results agreed and data from both experiments have been combined.

Chemicals

Adenosine triphosphate, disodium salt (ATP), cysteine hydrochloride, O-phosphorylethanolamine (ethanolamine phosphate), Tris buffer (Sigma 7-9), and ninhydrin were purchased from Sigma Chemical Co., St. Louis,

MO. Ethanolamine hydrochloride was obtained from U.S. Biochemical Corp., Cleveland, OH. Sucrose, magnesium chloride, chloroform, ammonium chloride, methanol, diethyl ether, toluene, petroleum ether, and butanol-1, all reagent grade, were from Mallinkrodt, Inc., St. Louis, MO. Ethanol, reagent grade, was obtained from Ashland Chemical Co., Newark, CA. Petroleum ether, B.R. 30-60 C, and ethanol were redistilled before use. Cesium chloride, ultra-pure grade, was obtained from Nutritional Biochemicals, Irvine CA. Eastman Organic Chemicals, Rochester, NY, supplied 2′,7′-dichlorofluorescein. Cytidinediphosphoethanolamine (CDPE) was obtained from Boehringer Mannheim, Indianapolis, IN. Ethylenediaminetetraacetic acid (EDTA) was obtained from Mann Research Labs. Inc., New York, NY. Glacial acetic acid, reagent grade, was obtained from Allied Chemical Co., Morristown, NJ. Labeled ethanolamine, 2-[^{14}C]-ethan-1-ol-2-amine hydrochloride, 50 mCi/mmol, was supplied by Amersham/Searle, Arlington Heights, IL. The radiopurity of this material was checked by thin layer chromatography (TLC) as described before (16) and found to be 98.5% or greater. Heptadecanoic acid (internal standard) and methyl esters of fatty acids for calibration of the gas liquid chromatograph were obtained from NuChek Prep., Elysian, MN, and Applied Science Laboratories, State College, PA. PPO (2,5-diphenyloxazole) was obtained from Research Products Industries, Elk Grove Village, IL, and Triton X-100 was obtained from Central Solvents, Hayward, CA.

Rats and Diets

Male Long-Evans rats (Simonsen Laboratories, Gilroy, CA), ca. 150 g, were fed Purina rat chow (Dean's Supply Co., Belmont, CA) for one week. They were then given for one week a low-methionine, choline-deficient diet, or the same diet supplemented with choline (Table I). Unfasted, unanesthetized rats were killed by decapitation, and their livers were removed and processed immediately.

Preparation of Liver Homogenate, Cytosol and Microsomes

Livers were rinsed in 0.9% NaCl solution, blotted, weighed and homogenized for 5 sec with 0.25 M sucrose in 0.001 M tris buffer, pH 7.5, 3 ml/g liver, in a Polytron PCU-2 homogenizer (Brinkmann). An aliquot of homogenate was taken for immediate lipid extraction. Homogenates were centrifuged at 17,000 g at 4 C for 10 min in a Sorvall RC-5 refrigerated centrifuge. Supernatants were made 0.015 M in CsCl by addition of 0.30 M CsCl solution. These mixtures were centrifuged for 1 hr at 100,000 g in a SW 27 rotor in a Beckman L2-65B ultracentrifuge. Floating fat was removed, and the supernatant (cytosol) was decanted. The pellet (microsomes) was dispersed in water, 1 ml/g liver, with the Polytron homogenizer. Protein concentrations in cytosol and microsomal preparations were measured by the Lowry procedure with bovine serum albumin as standard (17). Cytosol preparations were dialyzed under vacuum against 0.001 M tris buffer, pH 7.4, in a collodion bag apparatus with a molecular weight cutoff of ca. 75,000 (Schleicher and Schuell, Inc., Keene, NH).

TABLE I

Composition of Diets

Ingredient	Percent by weight
Soy protein[a]	20
Sucrose	66.25 - 66.5
Corn oil[b]	10
Minerals UCB 1Rb[c]	3.5
Choline	±0.25
Vitamins[d]	

[a]Tecklad Test Diets, Madison, Wisconsin.

[b]Mazola, Monarch Foods, Brisbane, California.

[c]Williams et al. (32).

[d]Provided as mg/kg of diet: Riboflavin, 20; niacinamide, 120; Ca-d-pantothenate, 60; pyridoxine HCl, 19.2; folic acid, 4.0; biotin, 2.0; menadione, 0.80; inositol, 200; thiamine, 10. Provided as U/kg of diet: Vitamin A (500,000 U/g), 20,000; vitamin D (400,000 U/g), 2,000; vitamin E (250 U/g), 400.

Assay of Ethanolamine Kinase Activity in Cytosol

Ethanolamine kinase activity was assayed by measuring the radioactivity converted from ethanolamine to ethanolamine phosphate.

Within an hour of preparation, cytosol was incubated at 37 C for 10 min in a medium with the following composition: Tris HCl buffer, 0.114 M, pH 8.6; EDTA, 1.97 mM; cysteine HCl, 6.73 mM; ATP, 6.17 mM; $MgCl_2$, 20.2 mM; and ^{14}C-ethanolamine (10^6 dpm), 0.21 mM. The total incubation volume was 1.0 ml. The amount of protein was 3 to 4 mg. The reaction was started by addition of cytosol to a vial in which the incubation medium had been brought to 37 C in a shaker bath. The reaction was stopped by heating the vial in boiling water for 2 min. Zero-time samples were heated during addition of cytosol. Under these conditions, incorporation of label into ethanolamine phosphate was proportional to time for at least 30 min.

The reaction mixture was filtered through a Millipore, HA 0.49 mμ, and a 25 μℓ-aliquot of filtrate was separated by thin layer chromatography (TLC) on 0.25 mm precoated silica gel 60 plates (Merck). To the sample on the plate was added 40 μg each of ethanolamine, ethanolamine phosphate, and CDPE, so that ethanolamine compounds could be detected with ninhydrin spray reagent. The plate was developed for 5 hr in 2.7 M ammonium acetate, pH 5.0/95% ethanol, 3:7 (6). Plates were sprayed with 0.3% ninhydrin in 3% acetic acid in butanol-1, and heated at 110 C for 10 min for color development. Regions corresponding to the origin, ethanolamine phosphate (R_f 0.16), CDPE (R_f 0.29), ethanolamine (R_f 0.48) and the space above ethanolamine were scraped into scintillation vials. Water, 2 ml, and counting cocktail, 10 ml, were added to the sample, which was counted in a Beckman LS-100 scintillation counter. The counting cocktail was 2.7 g PPO in 370 ml Triton X-100 plus 630 ml toluene. Counting efficiency was 85%, and quenching was measured by adding a known number of dpm as ^{14}C-toluene.

Enzyme activities were measured before and after dialysis to remove substances such as choline or choline phosphate which could affect enzyme activity, and endogenous ethanolamine, which would dilute the radioactive ethanolamine. Cytosol preparations were stored for 2 to 8 days at -15 to -20 C. To determine the effects of storage, equal numbers of control and deficient samples which had been frozen for equal lengths of time were assayed or dialyzed and assayed on the same day.

Lipid Extraction of Liver Homogenate

An aliquot of homogenate equivalent to 1.3 g liver was extracted with 18.75 ml chloroform/methanol, 1:2. After 1 hr, 6.25 ml chloroform and 6.25 ml water were added and mixed. The mixture was centrifuged, the upper layer was removed, and the pellet and lower layer were washed with "synthetic upper layer." After centrifugation, the lipid-containing lower layer was recovered, evaporated to dryness under nitrogen, and dissolved in petroleum ether.

Analysis of 1,2-Diacylglycerols in Liver Homogenates

Duplicate aliquots of liver homogenate extract, containing 50-150 μg 1,2-diacylglycerols, were streaked on 0.35 mm Silica Gel H (Merck) TLC plates, which had been heated 1 hr at 110 C. Plates were developed in petroleum ether/diethyl ether/acetic acid, 70:20:4, for 50 min, sprayed with 2′,7′-dichlorofluorescein solution (0.4% in methanol), and viewed under ultraviolet light. A standard mixture containing 1- or 2-acyl glycerols, 1,2- and 1,3-diacylglycerols and triacylglycerols (Applied Science TLC Mixture 8) was applied to each plate. The 1,2-diacylglycerol bands were scraped into screw-capped culture tubes and a measured amount of heptadecanoic acid was added as an internal standard. Methyl esters were prepared by sulfuric acid-catalyzed transesterification, isolated and analyzed by gas liquid chromatography (GLC) as described earlier (18).

Analysis of Microsomal Phospholipids

An aliquot of microsomal suspension equivalent to 5 g liver was extracted as described for total liver homogenate. The extract was analyzed for lipid phosphorus (19). Aliquots containing 125-50 μg lipid phosphorus were streaked (7 cm) on 0.35 mm Silica Gel H TLC plates which were developed in chloroform/methanol/acetic acid/water, 25:15:4:2. Phospholipid bands were located with 2′,7′-dichlorofluorescein spray, as above. PC and PE were eluted from the silica gel (20) and their acyl groups were converted to methyl esters as described earlier (18). Eluates were analyzed for phosphorus (19).

Gas Liquid Chromatography

Methyl esters were analyzed on a Varian Model 2100 gas chromatograph with glass columns (1/8 in. i.d. x 6 ft.) packed with 10% SP 216 PS on 100/200 Supelcoport (Supelco, Inc., Bellefont, PA). The instrument was calibrated with a mixture of methyl esters of 16:0, 18:0, 18:1, 18:2n-6, 18:3n-3, 20:0, 20:1, 20:4n-6, 22:0, 22:1, 22:6n-3, 24:0 and 24:1. Other components were tentatively identified by comparison with data in the literature or by use of log plots (21).

Statistics

Statistical significances of differences between diet groups were calculated according to the t-test (22).

RESULTS

Ethanolamine Kinase Activity

Choline deficiency had no effect on ethanolamine kinase activity (Table II). Our values for undialyzed preparations are similar to those found by Schneider and Vance in livers from control and choline-deficient female Wistar rats (13). Storage reduced enzyme activity significantly only in choline-deficient livers. Dialysis increased radioactive ethanolamine phosphate formation, presumably by removing inhibitors or endogenous substrate. The presence of 0.5

TABLE II

Ethanolamine Kinase Activity in Cytosol from Livers of Control and Choline-Deficient Male Rats

Condition of cytosol	nmol Ethanolamine phosphate formed/min/mg protein[a]	
Diet:	Control	Deficient
Fresh, undialyzed	0.640 ± 0.081	0.649 ± 0.080
2-8 Days storage, undialyzed	0.609 ± 0.088	0.582 ± 0.067[b]
2-8 Days storage, dialyzed	0.818 ± 0.085[c]	0.798 ± 0.061[c]
2-8 Days storage, dialyzed, 0.5 mM choline	0.690 ± 0.109[d]	0.672 ± 0.106[d]

[a]Mean ± S.D., duplicate assays on each of 6 rats per diet group.

[b]Significantly different from fresh, undialyzed preparation, $P<0.005$.

[c]Significantly different from stored, undialyzed preparations, $P<0.001$.

[d]Significantly different from stored, dialyzed preparations with no added choline, $P<0.005$.

TABLE III

Microsomal Phosphatidylethanolamines in Livers from Choline-Deficient and Control Male Rats[a]

Fatty acid	Fatty acid composition				
	Weight % of total methyl esters		nmol FA/mg microsomal protein[b]		Increase in FA = Deficient minus Control (nmol/mg microsomal protein)
	Control	Deficient	Control	Deficient	
14:0	0.3 ± 0.1	0.2 ± 0.06[c]	0.4	0.4	0
16:0	15.5 ± 0.6	17.0 ± 1.0[c]	21.7	37.4	15.7
16:1	0.4 ± 0.1	0.4 ± 0.1	0.6	0.9	0.3
18:0	26.1 ± 1.2	27.5 ± 1.4	36.5	60.5	24.0
18:1n-9	4.1 ± 0.7	2.3 ± 0.3[c]	5.7	5.1	-0.6
18:2n-6	9.9 ± 1.4	5.8 ± 0.7[c]	13.9	12.8	-1.1
20:3n-9	1.0 ± 0.2	0.8 ± 0.1	1.4	1.8	0.4
20:4n-6	28.1 ± 1.8	22.5 ± 0.8[c]	39.3	49.5	10.2
22:4n-6	1.4 ± 0.1	0.8 ± 0.3	2.0	1.8	-0.2
22:5n-6	2.3 ± 1.3	3.6 ± 1.5	3.2	7.9	4.7
22:5n-3	1.0 ± 0.2	0.7 ± 0.1[c]	1.4	1.5	0.1
22:6n-3	9.9 ± 1.3	18.5 ± 2.3[c]	13.9	40.7	26.8
Phosphatidylethanolamine concn., nmol/mg microsomal protein			70 ± 14	110 ± 14[c]	

[a]Mean ± S.D.; 6 animals per group.

[b]Estimated from nmol PE and mean weight percentages of methyl esters.

[c]Significantly different from control, $P<0.05$ or less.

mM choline reduced enzyme activity, as has been observed by other workers using enzyme preparations from normal rats (7,8).

Concentrations and Fatty Acid Compositions of Microsomal PE and PC

Choline deficiency significantly increased the PE/protein ratio in liver microsomes and decreased that of PC (Tables III, IV). Similar changes were observed by Chen et al. (12) in washed microsomal preparations from control and choline-deficient rats.

Choline deficiency also significantly changed fatty acid patterns in both PE and PC. The increase in μmol of PE was due to increases in 16:0, 18:0, 20:4n-6 and 22:6n-3, with little change in 18:1n-9 or 18:2n-6 (Table III). The reduction in PC was due mainly to decreases in μmol of 16:0, 18:1n-9 and 18:2n-6, with little change in other components (Table IV).

Concentrations and Fatty Acid Compositions of Liver Total 1,2-Diacylglycerols

Choline deficiency increased the concentration of 1,2-diacylglycerols about 6-fold in liver total lipid (Table V). Our value for control liver, 96 μg fatty acid/g liver, is equivalent to about 0.3 μmol diacylglycerol, and agrees well with values reported by Elovson (0.2 - 0.25 μmol/g liver) (23) and Akesson (0.34 μmol/g liver) (24). In DG of choline-deficient liver, amounts of all fatty acids increased, but by far the greatest increases occurred in μmol of 16:0, 18:1n-9 and 18:2n-6. Thus, the increases in fatty acids of DG mirrored the decreases in fatty acids of PC.

DISCUSSION

Ethanolamine Kinase Activity

Choline deficiency did not change the activity of ethanolamine kinase in rat liver cytosol, in agreement with the findings of Schneider and Vance (13). Nevertheless, choline deficiency probably did increase the rate of PE formation by increasing the concentrations of its precursors. We found increased concentrations of DG, and Haines and Rose (9) found increased concentrations of ethanolamine, ethanolamine phosphate and cytidine diphosphoethanolamine in livers of choline-deficient rats.

The reason for elevated concentrations of ethanolamine (9) in choline-deficient liver is unknown. The concentration of serine, an important precursor of ehtanolamine, may be increased in choline-deficient rat livers because liver serine concentrations are inversely related to dietary methionine levels (25,26). The concentration of methionine would be expected to be lower in choline-deficient livers than in controls because controls would utilize less of it in the transmethylation of PE. The concentration of serine in choline-deficient livers has not been reported as far as we know.

Liver Diacylglycerols and Phosphatidylcholines

Choline deficiency increased the concentration of liver total DG about 6-fold, and the concentration of 22:6n-3 DG increased 4-fold (Table V). The increased concentration of 22:6n-3 DG could provide more substrate for increased formation of 22:6n-3 PE. Perhaps the reduced *percentage* (not amount) of 22:6n-3 in DG of choline-deficient livers reflects selective increased utilization of these species for PE formation.

Accumulation of DG indicates that their rates of formation have increased or that their

TABLE IV

Microsomal Phosphatidylcholines in Livers from Choline-Deficient and Control Male Rats[a]

	Fatty acid composition				
	Weight % of total methyl esters		nmol FA/mg microsomal protein[b]		Decrease in FA = Control minus Deficient
Fatty acid	Control	Deficient	Control	Deficient	(nmol/mg microsomal protein)
14:0	0.4 ± 0.1	0.3 ± 0.1	1.4	0.9	0.5
16:0	19.8 ± 1.4	18.6 ± 1.1	71.3	58.8	12.5
16:1	0.7 ± 0.2	0.6 ± 0.1	2.5	1.9	0.6
18:0	23.2 ± 1.2	27.6 ± 1.7[c]	83.5	87.2	-3.7
18:1n-9	5.0 ± 0.7	3.7 ± 0.2[c]	18.0	11.7	6.3
18:2n-6	18.8 ± 2.2	13.6 ± 1.7[b]	67.7	43.0	24.7
20:3n-9	1.6 ± 0.3	1.7 ± 0.4	5.8	5.4	0.4
20:4n-6	25.4 ± 3.2	29.3 ± 2.0[b]	91.4	92.6	-1.2
22:4n-6	0.3 ± 0.1	0.2 ± 0.1	1.1	0.6	0.5
22:5n-6	0.7 ± 0.1	0.8 ± 0.3	2.5	2.5	0
22:5n-3	0.4 ± 0.1	0.2 ± 0.04[c]	1.4	0.6	0.8
22:6n-3	3.6 ± 0.7	3.4 ± 0.8	13.0	10.7	2.3
Phosphatidylcholine concn., nmol/mg microsomal protein			180 ± 18	158 ± 15[c]	

[a]Mean ± S.D.; 6 animals per group.

[b]Estimated from nmol PC and mean weight percentages of methyl esters.

[c]Significantly different from control, $P<0.05$ or less.

TABLE V

Total Liver 1,2-Diacylglycerols in Choline-Deficient and Control Male Rats[a]

Fatty acid	Weight % of total methyl esters[b]		μg Fatty acid/g liver[c]		Increase in μg FA = Deficient minus Control
	Control	Deficient	Control	Deficient	(μg FA/g liver)
14:0	1.5 ± 0.6	1.4 ± 0.3	1.4	8.1	6.7
16:0	26.4 ± 2.6	32.2 ± 2.8[d]	25.4	185.8	160.4
16:1	1.5 ± 0.5	2.2 ± 0.5[d]	1.4	12.7	11.3
18:0	7.9 ± 1.8	5.3 ± 0.8[d]	7.6	30.6	23.0
18:1n-9	13.2 ± 2.0	16.8 ± 1.5[d]	12.7	96.9	84.2
18:2n-6	31.4 ± 3.0	31.1 ± 2.3	30.3	179.4	149.1
18:3n-3	0.3 ± 0.1	0.4 ± 0.1	0.3	2.1	1.8
20:0	0.4 ± 0.1	0.7 ± 0.4	0.4	3.9	3.5
20:3n-9	1.1 ± 0.4	0.5 ± 0.1[d]	1.1	2.9	1.8
20:4n-6	10.8 ± 1.9	5.3 ± 0.7[d]	10.4	30.6	20.2
22:4n-6	0.5 ± 0.1	0.3 ± 0.1	0.5	2.0	1.5
22:5n-6	0.7 ± 0.2	0.5 ± 0.3	0.7	3.1	2.4
22:5n-3	0.6 ± 0.3	0.5 ± 0.3	0.6	2.9	2.3
22:6n-3	3.1 ± 0.6	2.2 ± 0.5[d]	3.0	12.7	9.7
Diaclyglycerol concn., μg FA[c]/g liver			96.4 ± 42.3	577 ± 96	

[a]Mean ± S.D.; 6 rats per group.

[b]Some minor components are omitted.

[c]As methyl esters.

[d]Significantly different from control, $P<0.005$ or less.

rates of removal have decreased, or both. DG can be formed by the splitting of PC to DG and CDPcholine and can be removed by the reversal of this reaction. The activity of the enzyme responsible for this reaction is not changed by choline deficiency (13).

If incorporation of DG into PC is impaired in choline deficiency, the fatty acids in accumulated DG should be those normally incorporated into PC most abundantly by the cytidine pathway. These are 16:0, 18:1n-9 and 18:2n-6, as shown by the rapid in vivo incorporation of $^{14}CH_3$-choline into liver PC that contain 16:0, 18:1n-9 and 18:2n-6, and its much slower incorporation into PC that contain 18:0, 20:4n-6 or 22:6n-3 (27). The labeling pattern is apparently due to enzyme selectivity rather than abundance of different diglyceride substrates, because formation of PC from individually labeled 1,2-diglycerides by rat liver microsomes showed the following substrate preference: 16:0 - 18:1n-9 > 16:0 - 18:2n-6 > 16:0 - 22:6n-3 > 16:0 - 20:4n-6 > all stearoyl homologs (28). An accumulation of 16:0, 18:1n-9 and 18:2n-6 was, in fact, observed (Table V), and a corresponding deficit was observed in PC (Table IV). These changes are consistent with an impairment in PC formation from DG, but do not exclude a possible increase in rate of DG formation.

Liver Phosphatidylethanolamines

Choline deficiency increased the concentration of PE in microsomes (Table III) and decreased that of PC (Table IV). The increase in PE was due mainly to increases in 16:0, 18:0, 20:4n-6 and 22:6n-3 (Table III), changes that are consistent with an increased rate of PE formation. In vivo, radioactive ethanolamine is incorporated most extensively into rat liver hexaenoic PE, followed by tetraenoic, dienoic and monoenoic PE in that order (5,20,29,30). This incorporation pattern is due to selectivity of the enzyme rather than abundances of different substrates, as shown by the preference of rat liver microsomes for incorporation of individually labeled diglycerides: 16:0 - 22:6n-3 > 18:0 - 22:6n-3 > 16:0 - 18:1n-9 = 18:0 - 20:4n-6 > 16:0 - 20:4n-6 = 16:0 - 18:2n-6 > 18:0 - 18:1n-9 (28). The increased amount of liver PE and particularly 22:6n-3 PE is, therefore, consistent with an increased rate of PE synthesis.

A selective impairment in transmethylation probably does not contribute significantly to the selective accumulation of 22:6n-3 PE for the following reasons: first, the incorporation of $^{14}CH_3$-methionine into PC does not seem to be selectively impaired in choline-deficient rats (4,31); secondly, the amount of transmethyla-

tion in both control and choline-deficient rats is probably severely reduced by the low-methionine diet, as shown by the occurrence of fatty liver, etc., in animals not supplemented with choline. It is possible that the rate of transmethylation is even greater in choline-deficient rats than in controls, as suggested by an increase in phosphatidylethanolamine-S-adenosylmethionine methyl transferase activity in choline-deficient livers (13).

The accumulation of 22:6n-3 PE in livers of choline-deficient rats appears to be due to an increased rate of PE formation, impairment of transmethylation, and selectivity for 22:6n-3 DG of the enzyme CDP-ethanolamine:1,2-diacylglycerol ethanolamine phosphotransferase. The increased rate of PE formation is probably caused by increased concentrations of its precursors, including ethanolamine phosphate and 1,2-diacylglycerols and not to an increase in ethanolamine kinase activity. The reasons for increased concentrations of these precursors remain to be determined.

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Incorporation of Dietary *cis* and *trans* Octadecenoate Isomers in the Lipid Classes of Various Rat Tissues

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ABSTRACT

The percentage distribution of the geometrical and positional isomers in the hexadecenoates and octadecenoates isolated from triglycerides, phosphatidylcholines, and phosphatidylethanolamines of brain, heart, kidney, liver, lung, muscle, spleen, and adipose tissues from rats maintained four weeks on a semipurified diet supplemented with 15% partially hydrogenated safflower fatty acids, has been determined. Except for brain, octadecenoate percentages were increased in each of the lipid classes of all the tissues by the dietary fat. Although the diet did not contain detectable hexadecenoates, the 16:1 fraction from the lipid classes of all the tissues was composed of 10-70% of the *trans* isomers, indicating chain shortening of the dietary octadecenotes. Distribution of *cis* and *trans* positional isomers in triglyceride hexadecenoates was approximately the same in all tissues. Relatively high percentages of the Δ9, Δ10, and Δ11 isomers were observed, but the Δ8 was the predominating *trans* hexadecenoate isomer, indicating preferential chain shortening of the *trans* Δ10 octadecenoate. *Trans* octadecenoates were found in all tissues, but concentrations were dependent on tissue and lipid class. The distribution of the *cis* and *trans* octadecenoate isomers was similar in all the tissue triglycerides, with the distribution of the *trans* isomers resembling the diet. In contrast, the percentage distribution of the *trans* octadecenoates in the phospholipid classes differed dramatically from the diet, and the distribution was dependent on both the tissue and lipid class. The Δ12, Δ13, and Δ14 *trans* octadecenoates were present in both phospholipid classes of most tissues in higher percentages than the diet, suggesting an accumulation of these isomers. Although the *cis* Δ10 octadecenoate was a significant dietary component, this isomer was not incorporated significantly into any lipid class of any tissue. The metabolic fate of this isomer remains unknown.

INTRODUCTION

The origin of the geometrical and positional octadecenoate isomers, the possible relation of these dietary fatty acids to some diseases, and the earlier work in this area have been reviewed (1). In our earlier studies, we reported on the distribution of dietary geometrical and positional octadecenoate isomers in the major lipid classes of rat liver (2,3). The data showed the following: (a) the octadecenoates of the major lipid classes contained different amounts of the *trans* isomers in addition to the normal cis isomers; (b) incorpoation of octadecenoate isomers at the 1- and 2-positions of glycerol exhibited both geometrical and positional isomer specificity; (c) preferential incorporation or exclusion of some positional isomers was observed for both the *cis* and *trans* octadecenoates; and (d) chain shortening of some *cis* and *trans* octadecenoate isomers occurred to yield hexadecenoates.

The present studies were aimed at determining whether the incorporation of the dietary *cis* and *trans* octadecenoate isomers in the lipid classes of various other tissues was similar to that of liver. A preliminary report of these studies has appeared (4).

EXPERIMENTAL PROCEDURES

Male Buffalo strain rats (175-225 g) were fed a semipurified fat-free diet supplemented with 15% (by weight) partially hydrogenated safflower fatty acids for four weeks as reported previously (2). The use of free fatty acids instead of intact glycerides allows adsorption and incorporation of the various isomers at the positions of glycerol to be compared without the problem of partial glycerides resulting from digestion. Brain, heart, kidney, liver, lung, muscle, spleen and adipose tissues were excised, lyophylized, and extracted twice by the Bligh and Dyer procedure (5). Triglycerides, phosphatidylcholines, and phosphatidylethanolamines, the major lipid classes, were isolated by thin layer chromatography (TLC); methyl esters were prepared and analyzed quantitatively by gas liquid chromatography (GLC); *cis* and *trans* monoene esters were isolated by argentation TLC; hexadecenoates and octadecenoates were isolated by preparative GLC; and the double bond positions were determined by GLC analysis of the ozonide cleavage products as described previously (6-10).

The source and purity of lipid standards, solvents, chemicals, and reagents were the same as given previously (9).

RESULTS

Dietary Lipids

The fatty acid and isomer content of the

TABLE I

Percentage Distribution of Positional Isomers of *Cis* and *Trans* Octadecenoate Fatty Acids Isolated from Triglycerides of Rats Fed a Diet Containing 15% Partially Hydrogenated Safflower Fatty Acids

Diet and tissue	Isomeric percentages[a]						
	Δ8	Δ9	Δ10	Δ11	Δ12	Δ13	Δ14
				trans isomers			
Diet	---[b]	12.6	32.7	34.3	13.1	4.6	2.5
Heart	5.4 ± 1.9	17.6 ± 1.4	27.8 ± 0.7	29.0 ± 2.6	12.4 ± 0.8	4.9 ± 0.1	2.5 ± 0.3
Kidney	---	18.4 ± 1.0	33.3 ± 1.8	30.9 ± 0.8	14.4 ± 0.9	4.1 ± 0.1	---
Liver	4.0	22.7	32.2	23.8	11.9	3.5	1.9
Lung	4.7 ± 0.6	17.6 ± 1.4	28.3 ± 0.3	30.0 ± 1.7	13.2 ± 0.4	4.2 ± 0.1	1.8 ± 0.1
Muscle	4.2 ± 0.7	17.3 ± 0.7	31.0 ± 1.9	29.4 ± 3.8	14.4 ± 1.0	4.9 ± 0.1	2.3 ± 0.2
Spleen	3.2 ± 1.9	16.5 ± 1.3	30.4 ± 1.3	27.6 ± 2.0	14.7 ± 1.2	4.9 ± 0.1	2.3 ± 0.1
Adipose	5.5 ± 1.7	16.7 ± 0.1	28.7 ± 1.8	28.6 ± 1.2	13.6 ± 0.4	4.7 ± 0.3	1.8 ± 0.1
				cis isomers			
Diet	---	61.8	10.7	11.0	16.1	0.3	---
Heart	---	71.0 ± 3.2	3.6 ± 1.2	13.5 ± 1.1	10.6 ± 1.9	1.0 ± 0.2	---
Kidney	---	79.5 ± 2.0	---	11.2 ± 0.7	9.1 ± 1.7	---	---
Liver	1.3	74.8	---	14.3	9.6	---	---
Lung	---	75.4 ± 1.3	2.0 ± 0.2	11.4 ± 0.2	10.0 ± 0.8	0.9 ± 0.1	---
Muscle	---	72.3 ± 2.8	2.4 ± 0.2	12.1 ± 0.3	12.1 ± 2.0	---	---
Spleen	---	77.4 ± 1.9	1.9 ± 0.5	10.9 ± 0.3	8.5 ± 0.9	1.1 ± 0.5	---
Adipose	2.5 ± 0.3	70.0 ± 1.2	2.3 ± 0.3	12.1 ± 0.4	11.7 ± 0.5	1.0 ± 0.1	0.20 ± 0.04

[a]Percentages represent the mean ± standard deviation of three rats. Percentages without standard deviations represent duplicate determinations of a pooled sample from three animals.

[b]--- Denotes undetectable quantities or levels too low to measure.

partially hydrogenated lipids used in these studies has been reported previously (1,2), but because of the numerous comparisons to be made with the dietary fat, it is given again. The partially hydrogenated safflower fatty acids were composed of: 16:0, 5.7%; 18:0, 6.2%; 18:1, 76.0%; and 18:2, 12.2%. The octadecenoate fraction consisted of 66.6% *trans* isomers, with the *cis* isomers making up the balance. Percentages of the positional isomers in each of the geometrical forms are given in Table I. The 18:2 fraction consisted of 69% *cis cis*, 29% of *cis trans* and *trans cis*, and 2% of *trans trans* isomers. The 15% partially hydrogenated fatty acids in the diet represented approximately one-third of the calories. This level of free fatty acids had no effect on feed consumption or weight gain of the animals relative to chow fed animals.

Fatty Acid Composition

The hexadecenoate and octadecenoate percentages of triglycerides, phosphatidylcholines, and phosphatidylethanolamines from the various tissues are given in Table II. The complete fatty acid composition of these tissue lipid classes will be published elsewhere (1). The partially hydrogenated fatty acid diet produced some changes in the fatty acid composition relative to the percentages from chow-fed animals reported recently (11). Except for liver, triglyceride octadecenoate percentages represented ca. 50% of the fatty acid in all the tissues, some 10-20% higher than chow fed animals. The increased 18:1 percentages replaced mostly the 18:2 fraction. Except for the higher percentage of 16:1 in kidney, the triglyceride hexadecenoates were affected little by the partially hydrogenated fat diet. In contrast to the octadecenoate composition of the triglycerides, the phospholipids from the various tissues contained different percentages of 18:1 that ranged between 20 and 50% of the total fatty acids. Except for brain, the percentages of 18:1 in the various tissue phospholipids were elevated substantially, in some cases up to 300%, over percentages found in chow-fed animals (11). Unlike the triglycerides in which the increased percentage of 18:1 replaced primarily the 18:2 fraction, the increased percentage of the octadecenoates in the phospholipids came at the expense of saturated fatty acids in addition to 18:2 in some tissues. The increased percentage of 18:1 fatty acids was probably not due solely to the *trans* isomers in the diet, since similar increases have been observed in the serum and adipose tissue lipids of rabbits fed a diet high in *cis*

TABLE II

Octadecenoate and Hexadecenoate Percentages Found in the Various Tissues of Rats Maintained on a Fat-Free Diet Supplemented with 15% Partially Hydrogenated Safflower Fatty Acids[a]

	Percentages of the total fatty acids[b]					
	TG[c]		PC		PE	
Tissue	18:1	16:1	18:1	16:1	18:1	16:1
Brain	50.1 ± 1.0	5.8 ± 0.4	30.6 ± 0.2	1.0 ± 0.0	26.5 ± 0.6	T[d]
Heart	26.0 ± 1.5	8.6 ± 1.7	26.3 ± 0.9	1.3 ± 0.1	20.9 ± 1.5	0.5 ± 0.1
Kidney	48.8 ± 3.1	12.8 ± 1.9	23.4 ± 1.2	3.1 ± 0.3	19.1 ± 1.1	0.9 ± 0.1
Liver	35.5	3.0	29.5 ± 0.8	3.2 ± 0.7	23.6 ± 0.8	2.4 ± 0.4
Lung	49.4 ± 1.7	6.4 ± 0.1	18.0 ± 0.5	14.7 ± 1.0	29.4 ± 3.9	3.0 ± 1.3
Muscle	48.9 ± 2.5	8.6 ± 1.6	21.1 ± 0.4	3.8 ± 0.5	17.8 ± 2.1	0.8 ± 0.2
Spleen	47.3 ± 1.8	8.7 ± 1.4	32.2	4.7	35.2	1.8
Adipose	51.9 ± 4.2	6.2 ± 0.8	43.1	2.8	50.7	2.0

[a]The dietary fatty acids consisted of 76% octadecenoates, but no detectable hexadecenoates.

[b] Percentages represent the mean ± standard deviation from analyses of four rats individually. Means without standard deviation represent duplicate analyses of a pooled sample consisting of 3-6 animals.

[c]TG = triglycerides; PC = phosphatidylcholines; PE = phosphatidylethanolamines.

[d]T Denotes detectable amounts less than 0.5%.

TABLE III

Distribution of *Cis* and *Trans* Hexadecenoate Isomers in the Major Lipid Classes Obtained from Several Tissues of Rats Maintained on a Fat-Free Diet Supplemented with 15% Partially Hydrogenated Safflower Fatty Acids[a]

	Geometrical isomer percentages[b]					
	TG		PC		PE	
Tissue	*% cis*	*% trans*	*% cis*	*% trans*	*% cis*	*% trans*
Brain	---	---	61.1	38.9	---	---
Heart	76.0 ± 5.9	24.0 ± 5.9	29.9	70.1	---	---
Kidney	84.5 ± 14.0	15.5 ± 14.0	31.7	68.3	54.8	45.2
Liver	62.0	38.0	69.0	31.0	79.0	21.0
Lung	75.5 ± 12.7	24.5 ± 12.7	48.6 ± 9.2	51.4 ± 9.2	45.8	54.2
Muscle	90.8 ± 3.6	9.2 ± 3.6	50.2	49.8	---	---
Spleen	88.6 ± 3.7	11.4 ± 3.7	35.0	65.0	75.2	24.8
Adipose	83.1 ± 4.2	16.9 ± 4.2	55.5	44.5	81.7	18.3

[a]The diet did not contain detectable quantities of hexadecenoates.

[b]Percentages represent the mean ± standard deviation from analyses of three rats individually. Means without standard deviations represent analysis of a pooled sample consisting of three animals.

octadecenoates as well as the *trans* isomers (12). Generally, the hexadecenoate percentages were higher in the phospholipids from the animals fed the partially hydrogenated fat diet than the chow-fed animals, but the percentages remained relatively small except for lung phosphatidylcholines.

Geometrical Isomer Composition

The percentage of the *cis* and *trans* hexadecenoate isomers from the major lipid classes of the various tissues is given in Table III. Except for liver, the triglycerides of the various tissues consisted of 75% or more of the *cis* isomer. The hexadecenoates of heart, kidney, lung, and spleen phosphatidylcholines contained more than 50% of the *trans* isomers. The percentage distribution of the hexadecenoates in liver, spleen and adipose phosphatidylethanolamines was similar to the triglycerides in which the *cis* isomers predominated.

Table IV gives the percentages of the octa-

TABLE IV

Distribution of *Cis* and *Trans* Octadecenoate Isomers in the Major Lipid Classes Obtained from Several Tissues of Rats Maintained on a Fat-Free Diet Supplemented with 15% Partially Hydrogenated Safflower Fatty Acids[a]

	Geometrical isomer percentages[b]					
	TG		PC		PE	
Tissue	*% cis*	*% trans*	*% cis*	*% trans*	*% cis*	*% trans*
Brain	—	—[c]	98.4 ± 0.1	1.6 ± 0.1	97.4 ± 0.1	2.6 ± 0.1
Heart	72.6 ± 1.8	27.4 ± 1.8	50.4 ± 5.6	49.6 ± 5.6	39.9 ± 3.3	60.1 ± 3.3
Kidney	76.3 ± 6.5	23.7 ± 6.5	66.5 ± 3.4	33.5 ± 3.4	58.9 ± 2.4	41.1 ± 2.4
Liver	84.7	15.3	45.4	54.6	31.7	68.3
Lung	68.6 ± 2.4	31.4 ± 2.4	72.8 ± 2.2	27.2 ± 2.2	66.9 ± 3.8	33.1 ± 3.8
Muscle	72.4 ± 4.3	27.6 ± 4.3	72.5 ± 0.8	27.5 ± 0.8	44.4 ± 3.6	55.6 ± 3.6
Spleen	70.8 ± 4.8	29.2 ± 4.8	58.1	41.9	53.9	46.1
Adipose	65.6 ± 0.9	34.4 ± 0.9	58.4	41.6	56.9	43.1

[a]The octadecenoates represented 76% of the total fatty acids and consisted of 33.4% *cis* and 66.6% *trans* isomers.

[b]Percentages represent the mean ± standard deviation from analyses of three rats individually. Means without standard deviations represent analysis of a pooled sample consisting of three animals.

[c]Quantities were too small to permit analysis.

decenoate geometrical isomers found in the major lipid classes of the various tissues of animals fed the partially hydrogenated fat diet for one month. Preferential lipid class and tissue distributions of the *cis* and *trans* isomers were observed. The triglycerides consisted of 65-85% of the *cis* isomers. The octadecenoates of brain phospholipids were almost exclusively the *cis* isomers; however, detectable quantities of the *trans* isomers were observed. Octadecenoates from the other tissue phospholipids contained much larger percentages of the *trans* isomers; in some cases more than half of the octadecenoate fraction.

Positional Isomer Composition

The distribution of the *cis* and *trans* hexadecenoates from the triglycerides of the various tissues of rats fed the partially hydrogenated fat diet is shown in Table V. There was little evidence of preferential incorporation of any positional isomer in any of the tissues. The *cis* Δ9 hexadecenoate represented more than 94% of the total in all the tissues. The remainder of the *cis* fraction contained predominately small percentages of the Δ8, Δ10, and Δ11 isomers. The Δ8 was the predominant *trans* hexadecenoate isomer (35-50%) in all the tissue triglycerides. The Δ9 isomers accounted for 15-20% of the *trans* hexadecenoates, followed by 10% each of the Δ10 and Δ11 isomers. Smaller percentages of the Δ7, Δ12, Δ13, and Δ14 made up the balance of the *trans* hexadecenoates in the triglycerides.

The percentage distribution of the positional isomers in the *cis* and *trans* octadecenoates isolated from the triglycerides of various tissues of rats fed the partially hydrogenated fat diet is given in Table I. The percentage distribution of the positional isomers for the *cis* and *trans* octadecenoates in the diet is also given in the Table for comparison. Oleate, the Δ9 isomer, represented 70-80% of the triglyceride *cis* octadecenoates. The Δ11 and Δ12 isomers were the other major positional isomers of this fraction. Except for the Δ8 and Δ9 isomers, the distribution of the *trans* octadecenoate positional isomers in all the tissue triglycerides was similar to the positional isomer distribution in the diet.

The distributions of the positional isomers found in the *cis* and *trans* octadecenoate fractions isolated from the two major phospholipid classes of various tissues from rats maintained on the partially hydrogenated fat diet for one month are given in Tables VI and VII. Lipid classes and tissues exhibited preferential incorporation of the positional isomers from both geometrical forms. *Cis* octadecenoates from the brain phospholipids were predominately the Δ9 and Δ11 isomers. The Δ12 isomer percentages of all tissue phosphatidylcholines and four of the eight tissue phosphatidylethanolamine *cis* octadecenoates were higher than was present in the diet. Heart and spleen phosphatidylcholine contained a higher percentage of the Δ12 than the oleate or vaccenate, which predominate in all tissue

TABLE V

Percentage Distribution of Positional Isomers of *Cis* and *Trans* Hexadecenoate Fatty Acids Isolated from Triglycerides of Rats Fed a Diet Containing 15% Partially Hydrogenated Safflower Fatty Acids

Tissue	Isomeric percentages[a]							
	Δ7	Δ8	Δ9	Δ10	Δ11	Δ12	Δ13	Δ14
	trans isomers							
Heart	4.7	42.7	20.2	12.1	10.8	5.6	2.6	0.9
Kidney	4.1	43.6 ± 2.1	18.9 ± 1.0	14.4 ± 1.0	13.8 ± 3.6	6.4 ± 1.0	2.9	1.2
Liver	4.7	54.4	16.4	9.9	7.9	5.6	1.0	---[b]
Lung	6.6	41.6	20.8	11.7	10.8	5.6	1.3	1.3
Muscle	2.6	36.6	20.2	12.7	13.2	6.9	4.5	2.9
Spleen	6.2	45.2	18.6	11.1	9.4	5.2	2.6	1.2
Adipose	4.7	39.4	17.7	11.8	10.3	6.1	5.1	4.5
	cis isomers							
Heart	---	1.9 ± 0.4	93.9 ± 2.5	2.9 ± 2.6	0.9 ± 0.1	0.3 ± 0.1	---	---
Kidney	---	2.6 ± 1.0	94.6 ± 2.2	1.8 ± 1.2	0.6 ± 0.4	0.3	---	---
Liver	4.4	1.4	88.1	2.7	2.4	0.9	---	---
Lung	---	1.7 ± 0.7	98.2 ± 1.5	1.0	0.6	---	---	---
Muscle	---	1.9 ± 0.5	96.0 ± 0.6	1.3 ± 0.5	0.5 ± 0.1	0.1 ± 0.1	---	---
Spleen	---	0.9 ± 0.6	97.9 ± 1.9	1.1 ± 0.4	0.6 ± 0.1	0.2 ± 0.1	---	---
Adipose	---	2.4 ± 1.3	95.5 ± 2.2	1.1 ± 0.6	0.7 ± 0.1	0.3	---	---

[a]Percentages represent the mean ± standard deviation of three rats. Percentages without standard deviations represent duplicate determinations of a pooled sample from three animals.

[b]--- Denotes undetectable quantities or levels too low to measure.

phospholipids from animals fed diets containing natural fats (11). The percentages of the *trans* octadecenoate positional isomers in most of the tissue phospholipids differ dramatically from the diet: the Δ10 isomer was reduced significantly in most tissues, whereas the Δ12, Δ13, and Δ14 isomers were increased.

DISCUSSION

The occurrence of *trans* hexadecenoate isomers in the lipid classes of the various tissues is of considerable interest, since the diet did not contain detectable quantities of hexadecenoates and the lipids of animals maintained on chow diets do not contain more than trace amounts of *trans* hexadecenoates (11). The *trans* isomers were present in all the tissues examined, but the percentages were dependent on the tissue and the lipid class. The origin of the *trans* hexadecenoates would appear to be chain shortening of the *trans* octadecenoates as we suggested earlier (2) that occurs in liver. Chain shortening by two carbon atoms or "retroconversion," as it is called by some investigators, has been reported to occur in polyunsaturated fatty acids of intact rats (13) and rat liver mitochondria (14). Cell-free preparations of rat liver have also been reported to partially degrade stearoyl-CoA (15,16). Examination of the positional isomers of the 16:1 fraction from triglycerides (Table V) indicates preferential chain shortening. The predominance of the *trans* Δ8 hexadecenoate may be related to the conformation of either the substrate (*trans* Δ10 octadecenoate) or the product. Conformational and configurational changes might make the molecule undesirable or incompatible with the beta-oxidation system, resulting in the release of the *trans* Δ8 hexadecenoate after removal of only one acetate. Some chain shortening of the *cis* octadecenoate isomers also occurred (Table V), but to a much smaller degree than with the *trans* octadecenoates. This conclusion is based upon the assumption that the *cis* Δ9 hexadecenoate that predominated in the cis fraction of the triglycerides was not derived to any significant extent from the chain shortening of the *cis* Δ11 octadecenoate isomer. These data appear to represent an exception to the long held belief that there is no partial degradation of fatty acids (17,18).

Although chain shortening of the octadecenoates probably accounts for most of the unnatural *cis* and *trans* hexadecenoates, it does not appear likely to explain the occurrence of the Δ13, Δ14 and possibly some of the Δ12 *trans* hexadecenoates (Table V), which are absent from most tissues of chow-fed animals (11). The dietary octadecenoates (Table I) did

TABLE VI

Percentage Distribution of Positional Isomers of *Cis*-Octadecenoate Fatty Acids Isolated from Phospholipids of Rats Fed a Diet Containing 15% Partially Hydrogenated Safflower Fatty Acids

	Isomeric percentages[a]						
Tissue	Δ8	Δ9	Δ10	Δ11	Δ12	Δ13	Δ14
			Phosphatidylcholines				
Brain	0.6 ± 0.4	72.1 ± 1.0	---[b]	24.5 ± 0.6	1.5 ± 0.0	0.6 ± 0.1	0.7 ± 0.3
Heart	0.6 ± 0.0	26.5 ± 1.2	2.6 ± 0.7	37.7 ± 2.2	28.3 ± 1.5	2.5 ± 0.1	1.8 ± 0.3
Kidney	---	52.5 ± 1.3	---	20.7 ± 0.6	21.6 ± 3.6	2.2 ± 0.6	0.8 ± 0.3
Liver	T[c]	43.2 ±	1.0	23.5	29.0	2.5	0.8
Lung	0.6 ± 0.2	54.9 ± 3.4	1.9 ± 0.6	16.6 ± 1.0	23.7 ± 2.4	1.7 ± 0.2	0.8 ± 0.1
Muscle	---	37.9 ± 3.7	2.5 ± 0.3	29.2 ± 3.3	26.7 ± 1.9	2.4 ± 0.2	1.2 ± 0.6
Spleen	0.4	32.3	1.6	22.8	39.5	2.6	0.7
Adipose	0.3	62.6	1.5	11.2	22.3	1.3	0.7
			Phosphatidylethanolamines				
Brain	4.9 ± 2.0	75.6 ± 3.1	---	18.2 ± 1.5	0.6 ± 0.1	---	0.7 ± 0.5
Heart	1.4 ± 0.8	32.7 ± 3.0	3.3 ± 0.6	36.9 ± 2.1	22.1 ± 1.1	1.6 ± 0.4	2.4 ± 2.0
Kidney	---	74.3 ± 1.7	0.9 ± 0.2	12.7 ± 0.7	9.8 ± 1.3	0.7 ± 0.2	1.6 ± 1.3
Liver	1.9	36.8	1.7	31.4	21.5	3.7	1.8
Lung	---	73.1 ± 1.6	0.9 ± 0.2	11.8 ± 1.6	10.3 ± 1.7	0.6 ± 0.1	3.4 ± 4.3
Muscle	0.7 ± 0.3	46.4 ± 3.2	2.3 ± 0.3	28.0 ± 5.4	19.0 ± 2.0	1.5 ± 0.2	2.3 ± 0.8
Spleen	---	45.3	3.0	22.7	26.9	1.4	0.7
Adipose	4.9	69.7	1.6	14.6	7.8	0.7	0.6

[a]Percentages represent the mean ± standard deviation of three rats. Percentages without standard deviations represent duplicate determinations of a pooled sample from three animals.

[b]--- Denotes undetectable quantities or levels too low to measure.

[c] T Denotes detectable quantities less than 0.5%.

TABLE VII

Percentage Distribution of Positional Isomers of *Trans*-Octadecenoate Fatty Acids Isolated from Phospholipids of Rats Fed a Diet Containing 15% Partially Hydrogenated Safflower Fatty Acids

	Isomeric percentages[a]						
Tissue	Δ8	Δ9	Δ10	Δ11	Δ12	Δ13	Δ14
			Phosphatidylcholines				
Brain	---[b]	---	---	---	---	---	---
Heart	0.6 ± 0.1	14.2 ± 0.7	7.1 ±0.8	30.6 ± 1.0	24.6 ± 0.4	8.7 ± 0.5	13.2 ± 0.7
Kidney	---	11.2 ± 2.0	12.6 ± 2.4	22.1 ± 3.2	31.4 ± 2.2	9.0 ± 2.2	12.0 ± 3.8
Liver	3.2	15.5	5.8	26.9	33.9	6.7	6.8
Lung	1.4 ± 0.3	7.4 ± 1.7	24.2 ± 0.9	22.7 ± 0.6	25.2 ± 2.0	4.4 ± 0.1	14.0 ± 1.2
Muscle	---	19.4 ± 1.6	23.8 ± 2.3	20.7 ± 1.2	15.5 ± 1.1	5.8 ± 0.8	12.8 ± 2.6
Spleen	0.6	10.3	21.8	29.8	27.6	5.8	3.5
Adipose	0.4	13.2	13.7	28.1	32.8	5.6	5.4
			Phosphatidylethanolamines				
Brain	--- 0.1	---	---	---	---	---	---
Heart	1.2 ± 0.1	15.2 ± 2.8	6.4 ± 1.7	31.5 ± 1.3	24.0 ± 1.0	8.4 ± 1.0	12.2 ± 3.7
Kidney	0.8 ± 0.0	13.6 ± 1.0	9.9 ± 0.9	25.0 ± 1.5	32.3 ± 1.1	6.3 ± 0.3	11.3 ± 2.2
Liver	2.4	14.4	7.8	24.4	35.4	6.9	7.3
Lung	0.7 ± 0.1	12.1 ± 0.1	23.5 ± 2.7	27.0 ± 1.3	23.4 ± 1.4	5.2 ± 0.4	7.7 ± 4.1
Muscle	1.6 ± 0.2	18.2 ± 1.3	8.7 ± 1.2	25.8 ± 1.0	22.3 ± 0.9	8.5 ± 0.4	14.2 ± 1.0
Spleen	1.7	9.4	17.9	31.1	27.6	6.9	4.5
Adipose	3.3	16.4	16.1	28.1	26.3	5.1	4.0

[a]Percentages represent the mean ± standard deviation of three rats. Percentages without standard deviations represent duplicate determinations of a pooled sample from three animals.

[b]Low concentrations did not permit analysis of *trans* positional isomers in brain.

not contain detectable quantities of the Δ15 and Δ16 isomers and only 2.5% of the Δ14 isomer which would have been the precursor of the Δ12 hexadecenoate. Some rough calculations indicate that if the *trans* Δ15 and Δ16 octadecenoates had been present at the 0.25% level, which would have been near the limits of detection, nearly all of these isomers would have had to have been deposited after chain shortening to equal the amounts observed. If this were to be the case, then the lack of turnover of these isomers might lead to accumulation which might be of concern. This will be taken up again later in the discussion. However, it seems unlikely that these hexadecenoate isomers would not undergo some turnover. This sequence of events might give rise to a small amount of these isomeric fatty acids, but it does not seem reasonable to explain the observed levels of the Δ12, Δ13, or Δ14 *trans* hexadecenoates. A similar situation exists in the octadecenoates in which the triglycerides (Table I), and to a lesser degree the phospholipids (Table VII), contained a much higher percentage of the Δ8 isomer than was present in the diet. This also suggests that either trace amounts of this isomer in the diet were not turned over after being incorporated into the lipids or that there is a metabolic process at work which has not been described. Such a metabolic process might involve a microbial or mammalian isomerase capable of shifting a double bond at specific locations one or more carbons with retention of configuration. Movement of the double bond toward the methyl end before or after chain shortening could account for the Δ12 to Δ14 hexadecenoates, whereas movement towards the carboxyl group would give rise to the *trans* Δ8 octadecenoates found in the tissue lipids. If such an isomerase is present, it would appear that the *trans* isomer is the primary product since the *cis* hexadecenoates and octadecenoates from the triglycerides (Table I and V) did not contain more than trace amounts in most tissues of the Δ12, Δ13, Δ14 hexadecenoates and the Δ8 octadecenoates.

Triglycerides from the various tissues exhibited approximately the same: fatty acid composition (Table II); percentage distribution of *cis* and *trans* octadecenoate isomers (Table IV); and percentage distribution of the positional isomers from each of the geometrical octadecenoate fractions (Table I). These similarities in the triglycerides among various tissues have been noted in chow-fed animals, and the metabolic conditions that might explain this observation (11) have been discussed. Except for a slight enrichment of the Δ8 and Δ9 isomers, the distribution of the *trans* octadecenoate positional isomers in the tissues was similar to that of the diet (Table I). In addition to the triglycerides, the cholesterol esters of liver have been shown to contain a distribution of *trans* octadecenoate isomers similar to the diet (1,2). These results indicate that most of the *trans* positional octadecenoate isomers were absorbed, transported, and incorporated into triglycerides, and presumably cholesterol esters, without appreciable selectivity or specificity. This remarkable phenomenon does not include the incorporation of *trans* fatty acids into phospholipids or some of the positional isomers of the *cis* octadecenoates.

Although the interpretation of the distribution of the dietary *cis* octadecenoates into the tissue lipids is somewhat complicated by the two naturally occurring isomers, oleate and vaccenate, it can still be recognized that the distribution differs from the diet. The *cis* Δ10 octadecenoate isomer, a significant component of the diet, was not incorporated to any significant extent in either the triglycerides or the phospholipids (Tables I and VI). When the liver lipids were first shown to be practically devoid of this isomer (2), it was thought that this might be a peculiarity of liver, but the present data indicate that the isomer is low in all the tissues examined. The lipids of the feces from the animals on this diet have been examined, but the *cis* Δ10 isomer was not present in higher amounts than the other dietary isomers (1). At the present, the metabolic fate of this isomer is unknown. *Cis* Δ12 octadecenoate was incorporated in the lipids of all tissues, but the percentages were higher in the phospholipids of some tissues than the percentage of this isomer in the diet. The possible importance of the apparent accumulation of this unnatural isomer will be discussed later.

In contrast to the triglycerides where the distribution of the positional isomers in the tissues differed little from the diet, the phospholipids exhibited positional isomer selectivity (Table VII). *Trans* Δ10 octadecenoate, a major isomer of the diet, was restricted to a low percentage in the phospholipids of some tissues, especially heart and liver. The low percentage incorporation of this isomer probably resulted from more stringent enzyme specificities in some tissues than others. Preferential or rapid oxidation of the Δ10 isomer in some tissues can probably be discounted because as noted in the earlier discussion the triglycerides of all the tissues examined contained a high level of this *trans* isomer. Some of the ideas advanced on how the preferential distribution of the

naturally occurring octadecenoate isomers occurs in different tissues may apply to the unnatural isomers as well (11).

In addition to the *cis* Δ12 isomer, the *trans* Δ12, Δ13, and Δ14 octadecenoates appear to accumulate in the phospholipids of most tissues. The word "accumulate" is used here to indicate a substantially higher percentage of an isomer in the tissue lipids than was present in the diet. Some, and perhaps most, of the unnatural isomers are probably incorporated into complex lipids and turn over at rates similar to natural fatty acids. A slower turnover rate of some isomers could lead to their accumulation and perhaps, eventually altered membrane and tissue function. A slow turnover might be implicated if the isomers that accumulate were selectively or preferentially reincorporated into the phosphoglycerides after being hydrolyzed in normal turnover, but one would not expect unnatural isomers to be preferentially incorporated to natural fatty acids. Decreased enzymatic activity that would be necessary for the isomers to accumulate has been observed. Both geometrical and positional isomers have been shown to affect fatty acid activation (19,20), acyl transferases (21-23), sterol ester hydrolases (24,25), phosphatidylcholine/cholesterol ester hydrolase (26), mitochondrial oxidation (27) and incorporation into egg lipids (28). Generally, enzymatic activities were reduced as the double bond is moved in either direction from the Δ9 position.

The data reported here on the distribution of the geometrical and positional octadecenoate isomers in various tissues are new and add to our basic information regarding the metabolic fate of these dietary components. At the present, the data indicate that the incorporation and metabolism of these fatty acid isomers are dependent upon the tissue, the lipid class and the nature of the isomer.

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The Lipid Composition of Erythrocytes in European Cattle and Buffalo Steers

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ABSTRACT

This study compared the lipid composition of the red blood cells of European cattle and of buffalo steers at the same level of feed intake in a thermoneutral environment. The mean volumes of the erythrocytes and their lipid content were greater in buffalo than those in cattle. However, the amounts of phospholipid and cholesterol in cells of equal volume were higher in buffalo than in cattle. In contrast, the phospholipid level at a given cholesterol level was higher in cattle than in buffalo. The distribution of the different molecular species of phospholipids in the red cells of the two breeds were similar, but there were significant distinctions in their fatty acid patterns, notably in the levels of 24:0 and 24:1 in the sphingomyelin fractions. The proportion of total monounsaturated acids in the erythrocytes were similar from both breeds. However, there was a higher percentage of polyunsaturated fatty acids with a corresponding lower content of total saturated fatty acids in the red cells from buffalo than in those from cattle. The breed differences in erythrocyte lipid composition are discussed in relation to breed differences in red cell characters and could lead to a better understanding of the mechanisms of environmental adaptation.

INTRODUCTION

It is well established that different breeds of bovines vary in their reactions to environmental stresses, e.g., *Bos indicus* (zebu) breeds of cattle are more tolerant of high ambient temperatures than *B. taurus* (European) breeds. However, there is little known about the biochemical processes involved in acclimatization to heat in contrast to the extensive literature on the mechanisms involved in the acclimatization of mammals to cold (1).

The cells of poikilothermic organisms can adapt to changing temperature by altering their lipid composition in such a way as to restore membrane fluidity (2), and there is some evidence that the cells of normally homeothermic mammals can also exhibit this kind of temperature response (3). Since alterations in membrane lipid composition can influence membrane permeabilities and the activities of membrane-bound enzymes, it is possible that differences among bovines in their ability to acclimatize to tropical environments are associated with variations in membrane lipid composition. In this regard, it might be noted that studies on genetically different types of cattle showed that prolonged exposure to high environmental temperatures causes derangements in their lipid metabolism (4).

The red blood cells (rbc) are an easily accessible source of material for the study of membrane biochemistry in large animals such as cattle and might be a suitable model system for investigating membrane temperature adaptation, since Kuiper et al. (5) have shown that chronic exposure of hamsters to heat causes a change in the lipid composition of their rbc. However, if a role for lipid metabolism in heat adaptation in bovines is to be defined, it is necessary to separate genetic differences from associated differences in environmental effects. This paper presents a comparison of the lipid composition of rbc in European cattle with that in buffalo, at the same level of feed intake in a thermoneutral environment.

MATERIALS AND METHODS

Animals

The experiment was conducted during the winter with mean maximum and minimum daily temperatures of 23 C and 11 C, respectively, and the animals were housed indoors in individual stalls. Five buffalo steers and five Simmental x Hereford (cattle) steers were kept on a daily food intake of 6 kg of lucerne hay. Water was freely available throughout. Blood samples were obtained by jugular venepuncture after the morning feed. The mean body weights (kg ± standard errors) at the time of blood sampling were: buffalo 480 ± 11; cattle 470 ± 10.

Haematology

Red blood cell count, haematocrit and haemoglobin concentrations were measured in a Coulter Counter (Model F, Coulter Electronics Ltd., Herts, England). Erythrocyte sedimenta-

tion rate was determined in a Wintrobe tube after 1 hr.

Extraction and Analysis of Lipids

The erythrocytes were separated from plasma by centrifuging for 30 min at 1200 G, washing three times in aqueous 0.9% sodium chloride. They were then haemolyzed with an equal volume of water, and this preparation was blended with 7.5 vol of methanol, heated at 55 C for 1 hr and then 14 vol of chloroform was added and shaken. After standing for 30 min, the mixture was filtered through paper (Whatman No. 1), the residue was washed with chloroform/methanol (2:1 v/v) and the washings added to the filtrate. The extract was shaken with 0.2 vol of aqueous 0.88% potassium chloride to remove nonlipid contaminants and allowed to stand until the solvent layers separated. The lower layer was withdrawn and taken to dryness in a rotary vacuum evaporator; the dried residue of total lipids was taken up in chloroform/methanol(9:1, v/v).

The total lipid extract was examined for neutral lipids by thin layer chromatography (TLC) using glass plates coated with Kieselgel H (E. Merck, Darmstadt, Germany); the solvent system was hexane/diethyl ether/acetic acid (90:30:2, v/v). Only free cholesterol was detected and this was scraped from the plates, extracted with acetic acid and determined by the method of MacIntyre and Ralston (6). The lipid extract was fractionated into individual phospholipids on TLC plates coated with Kieselgel H with sodium carbonate; the solvent system was chloroform/methanol/acetic acid/water (25:15:4:2, v/v) as described by Skipski et al. (7). All TLC plates were sprayed with an 0.1% w/v solution of 2,4-dichlorofluorescein in methanol and the lipid bands were visualized under U.V. light. The bands were scraped from the plates and the phospholipids were eluted with methanol. Identification was by comparison with known standards. The phosphorus of phospholipids was analyzed by the method of Bartlett (8) after separation on TLC plates. The amount of individual phospholipids was expressed as a percentage of total phospholipid phosphorus. Methyl esters of the fatty acids were prepared with methanolic HCl and analyzed by gas liquid chromatography in a Varian 3700 apparatus with a hydrogen flame ionization detector and a CDS III Chromatography Data System. A 1.8 m stainless steel column of 3 mm i.d. was packed with Chromosorb W, HP (80–100 mesh, acid washed) coated with 15% EGSS-X. The column temperature was 174 C and nitrogen was the carrier gas. Peak identifications were based on relative retention times relative to methyl palmitate and cochromatography with known standards of methyl ester mixtures (Nu-Chek Prep, Inc., Elysion, MN). Some samples were analyzed before and after hydrogenation. Hydrogenation of methyl esters was accomplished in methanol solution at room temperature with PtO_2 catalyst at 2.5 atm H_2 with vigorous shaking for 3 hr. Where ever possible, reactions were carried out under nitrogen to minimize autoxidation.

Statistical Analysis

The data were subjected to analyses of variance and covariance based on general linear models using the method of least squares solved by fitting constants (Seebeck, R.M., SYSNOVA Version 9 Reference Manual, C.S.I.R.O., Division of Animal Production).

RESULTS

The results are given in Tables I-IV, which show mean values with standard errors of differences between breed means.

There were significant breed differences in all haematological parameters measured except the mean corpuscular haemoglobin concentration (Table I). No sedimentation of cattle erythrocytes was detected during the period of 1 hr in the Wintrobe tubes.

The total phospholipid and cholesterol contents calculated on a per cell basis (Table II) were higher in buffalo than in cattle. Since there were significant breed differences in mean corpuscular volumes, the lipid content per cell was analyzed using cell volume as covariate. Between breeds, there were no significant differences in regression coefficients, but both the phospholipid and cholesterol contents corresponding to a given cell volume were higher in buffalo than in cattle ($P<0.01$). On the other hand, the phospholipid content corresponding to a given cholesterol level expressed in moles was higher ($P<0.01$) in cattle than in buffalo.

The phospholipid composition expressed as a percent of total phospholipid phosphorus was not significantly different between breeds (Table II). In contrast, the fatty acid distribution of each major phospholipid class showed highly significant differences between breeds (Tables III–IV). The levels of 18:1 in the phosphatidylethanolamine and phosphatidylserine fractions were higher in cattle than in buffalo. In the phosphatidylethanolamine fraction, the lower content of 18:1 in buffalo compared to cattle was associated with increased levels of the saturated acids 14:0, 16:0

TABLE I

Haematological values[a] in cattle (Simmental x Hereford) and Buffalo steers

	Cattle	Buffalo	SE[b]
Haematocrit	0.38	0.53	0.02***
Haemoglobin (g/l blood)	131.4	183.0	7.9***
Red cell count (x 10^{12}/l)	7.27	8.94	0.4**
Mean corpuscular volume (fl)	51.8	58.3	1.2***
Mean corpsucular haemoglobin content (pg)	18.3	20.5	0.6**
Mean corpuscular haemoglobin concentration (g/l)	340	339	4.6
Erythrocyte sedimentation rate (mm/h)	0	22	

[a]Mean values for five steers.

[b]Standard error of the differences between breeds. Asterisks indicate significant breed differences: **P<0.01; ***P<0.001.

TABLE II

The Lipid Content and Phospholipid Composition[a] of Erythrocytes from Cattle (Simmental x Hereford) and Buffalo Steers

	Cattle	Buffalo	SE[b]
Lipid content (mg x 10^{-10}/cell):			
Phospholipid	1.43	1.52	0.01***
Cholesterol	0.86	1.02	0.02***
Phospholipid/cholesterol mole ratio	0.89	0.80	0.01***
Phospholipids (percent of total)[c]			
Phosphatidylethanolamine	32.5	35.9	4.1
Phosphatidylserine	13.7	10.8	4.2
Phosphatidylcholine	10.7	9.6	3.7
Sphingomyelin	38.7	40.9	8.6
Lysophosphatidylcholine	2.9	2.0	1.1
Other phospholipids	1.7	0.8	1.2

[a]Mean values for five steers.

[b]Standard error of the differences between breeds. Asterisks indicate significant breed differences: ***P<0.001.

[c]Measured as phospholipid phosphorus.

and 18:0. While the levels of 14:0 and 18:0 in the phosphatidylserine fractions were also higher in buffalo than in cattle, the content of 18:0 was similar in both breeds and much higher than in the phosphatidylethanolamine fraction.

The sphingomyelin fractions showed significant breed differences in the proportions of 24:0 and 24:1, and these fatty acids were absent from all other phospholipids except the phosphatidylcholine fraction in buffalo. The essential fatty acids, linoleic and linolenic (18:2ω6 and 18:3ω3), were found in all fractions but phosphatidylethanolamine and phosphatidylserine were the main carriers of these acids; however, arachidonic acid (20:4ω6) was not detected in any phospholipid fraction. The level of total unsaturated fatty acids was higher (P<0.01) in buffalo (64.4%) than in cattle (60.2%), but the levels of the monounsaturated acids were similar in the erythrocytes from both breeds (Table IV). Hence, there was a higher percentage of total polyunsaturated fatty acids and a lower content of total saturated fatty acids in red cells from buffalo than in those from cattle.

DISCUSSION

Normal bovine erythrocytes do not participate in rouleau formation (9) and, therefore, little or no sedimentation was expected in the Wintrobe tube during the period of 1 hr. This result was confirmed for the cattle, but the buffalo blood showed a remarkably high sedimentation rate. Comparisons of the lipid content of erythrocytes made on the basis of

TABLE III

Fatty Acid Composition (wt % of total)[a] of Phosphatidylethanolamine, Phosphatidylserine and Phosphatidylcholine in the Erythrocytes of Simmental x Hereford (SX) and Buffalo (BUF) Steers

Fatty acid	Phosphatidylethanolamine			Phosphatidylserine			Phosphatidylcholine		
	SX	BUF	SE[b]	SX	BUF	SE[b]	SX	BUF	SE[b]
14:0	1.5	5.3	0.1***	1.1	4.2	0.4***	2.2	10.3	1.6***
16:0	5.8	10.5	0.2***	7.4	12.5	0.4***	29.2	16.8	1.4***
16:1	2.3	2.9	0.3	1.2	2.7	0.2***	1.2	3.1	0.6***
17:0				1.3	0.9	0.2		0.7	
17:1	1.2	0.4	0.1***	0.5	0.3	0.1			
18:0	6.1	13.8	0.3***	29.0	28.4	0.6	15.5	9.5	0.7***
18:1	56.6	39.0	0.7***	42.5	32.7	1.0***	25.7	11.1	1.7***
18:2(ω6)	10.7	9.0	0.3***	9.2	9.5	0.4	13.7	7.6	1.3***
18:3(ω3)	2.7	2.6	0.2	1.1	1.3	0.1	3.1	1.1	0.3***
20:3(ω6)	0.6	0.5	0.1	1.3	2.4	0.3**	1.5	0.4	0.1***
20:3(ω3)	7.4	9.9	0.3***	4.4	3.1	0.2***	4.3	3.2	0.6
20:5(ω3)	4.6	4.8	0.6	0.6	0.5	0.1		10.2	
21:0				0.3	0.5	0.1			
24:0								2.4	
24:1								14.4	
Others	0.8	1.2		0.4	0.9		3.6	9.1	

[a]Mean values for five steers.

[b]Standard error of the differences between breeds. Asterisks indicate significant breed differences: **P< 0.01; ***P<0.001.

lipid weight per unit volume may be misleading when red cells which differ markedly in size are compared. Since the volumes of red cells from buffalo were greater than those from cattle, the lipid content per cell was considered to be a more appropriate method of expression.

There has been much interest in the role of lipids in determining a number of properties of the red blood cell, e.g., its osmotic fragility and the mechanisms for the maintenance of ionic gradients across the membrane. Sagawa and Shiraki (10) demonstrated that in rats the fragility of red cells is influenced by their lipid content, and Claret et al. (11) have shown that membrane cholesterol alters the cation translocation rate and the selectivity of the sodium pump for internal Na in human red cells. The breed difference in the amount of lipid per unit cell volume and in the phospholipid/cholesterol mole ratios found in this study point to the possible involvement of lipids in differences in red cell characters of bovines. In this regard, Evans and Turner (12) reported that the mean corpuscular fragility was lower in *B. indicus* than in *B. taurus* cattle, and it is well established that the red cells of cattle and buffalo exhibit a polymorphism in their potassium concentrations.

The erythrocyte membranes of several species of ruminants including cattle are characterized by a low phosphatidylcholine and high sphingomyelin content, and the present results extend these findings to buffalo. Although the distributions of the different molecular species of phospholipids in the red cells of the two breeds were similar (Table II), there were significant distinctions in the fatty acid patterns. In the erythrocytes from buffalo, both choline-containing phospholipids (lecithin and sphingomyelin) contained C24 acids, but these acids were exclusive to the sphingomyelin fraction in cattle red cells. The levels of 24:0 and 24:1 in the sphingomyelin fraction of cattle erythrocytes, 38 and 9%, respectively, were similar to those reported by Hanahan et al. (13) for the cow, but the values of these acids for buffalo sphingomyelin, 3 and 52% respectively, agree with those reported by Nelson (14) for sheep.

The dietary intake of lucerne hay, rich in polyunsaturated fatty acids, was similar for all animals, but biohydrogenation reactions within the rumen limit the availability of essential fatty acids. Phosphatidylserine and phosphatidylethanolamine were the main carriers of C18 polyunsaturated acids in the erythrocytes. The levels of 18:2ω6 and 18:3ω3 together formed ca. 13% of the total fatty acids in the red cells from both breeds, but 20:4ω6 was absent. However, the derivatives of 18:2ω6, 20:3ω6 and of 18:3ω3, 20:3ω3 and 20:5ω3, together accounted for 14% of the total fatty

TABLE IV

Fatty Acid Composition (wt % of Total)[a] of Sphingomyelin and Total Phospholipids in the Erythrocytes of Simmental x Hereford (SX) and Buffalo (BUF) Steers

Fatty acid	Sphingomyelin			Total phospholipids		
	SX	BUF	SE[b]	SX	BUF	SE[b]
14:0	1.4	4.5	0.1***	2.0	0.5	0.5*
16:0	10.6	24.3	2.0***	11.2	9.5	1.0
16:1				1.7	1.2	0.2
17:0	0.6	1.3	0.3	0.6	0.9	0.1**
17:1				0.7	0.4	0.1**
18:0	2.3	4.1	0.4**	10.5	14.0	0.7**
18:1	1.4	3.3	0.2***	26.9	17.1	1.3***
18:2(ω6)	1.1	0.8	0.2	8.1	7.7	0.3
18:3(ω3)	1.6			4.5	5.8	0.8
20:0	1.8					
20:3(ω6)				3.7	4.2	0.3
20:3(ω3)	14.6	2.7	0.7***	6.3	8.6	0.2***
20:5(ω3)	8.5	1.3	0.3***	4.2	5.7	0.3***
21:0	5.7			3.3	4.9	0.7
24:0	37.6	2.9	0.9***	11.0	3.0	0.7***
24:1	8.8	52.3	3.4***	4.1	14.2	0.8***
Others	4.0	2.0		1.3	2.8	
Saturated fatty acids				38.5	32.7	1.0***
Monounsaturated fatty acids				33.5	33.0	0.8
Polyunsaturated fatty acids				26.7	31.4	1.2**

[a]Mean values for five steers.

[b]Standard error of the differences between breeds. Asterisks indicate significant breed differences: *P<0.05; **P< 0.01; ***P<0.001.

acids in cattle and 19% in buffalo. While the present results showing low levels of essential fatty acids in cattle and buffalo agree with those reported for sheep (14), they do not support the general conclusion that in ruminants the polyunsaturated fatty acid content of the erythrocytes is reduced compared to other species. In fact, the percentages of total polyunsaturated fatty acids reported here for both breeds are similar to the literature values for several monogastric species cited by Nelson (15).

While there was a significant breed difference in the percentage of total unsaturated fatty acids of phospholipids, the levels of total monounsaturated acids were similar in the erythrocytes from both breeds. Hence, there was a higher percentage of polyunsaturated fatty acids with a corresponding lower content of total saturated fatty acids in red cells from buffalo than in those from cattle. Phospholipids generally contain saturated fatty acids in the 1-position with the unsaturated acids being restricted to the 2-position. However, examination of the proportions of saturated and unsaturated fatty acids (Tables III and IV) suggests that the cattle phospholipids are partially 1,2-diunsaturated and that the content of these phospholipid species is even higher in buffalo. Thus, the greater levels of 1,2-diunsaturated phospholipids in buffalo red cells could mean greater membrane fluidity amplified by the increased amounts of total unsaturated fatty acids (16). On the other hand, the lower amount of phospholipid relative to cholesterol in buffalo red cells will also be important in exerting a significant effect on the mobility of the membrane (17). Since membrane bound enzymes have been shown to be modulated by alterations in the physical properties of membranes (18), and because there is strong evidence from many organisms that among membrane lipids the fatty acyl chain is an important factor for temperature acclimation (19), the breed differences in erythrocyte lipid composition reported here may be of practical significance.

The present results showing that the fatty acid composition of each phospholipid is characteristic and significantly different between breeds suggests genetic differences in the fatty acids initially incorporated in the red cell membranes during erythropoiesis. Since many physiological processes depend on the func-

tional integrity of membrane systems, it is possible that differences in membrane lipid composition between cattle and buffalo might reflect the particular environment in which each breed has evolved. If this proves to be the case, then the lipid composition of the red blood cell membrane could be a useful guide in assessing the ability of different breeds of bovines to thrive in tropical areas.

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Volatile Hydrocarbon and Carbonyl Products of Lipid Peroxidation: A Comparison of Pentane, Ethane, Hexanal, and Acetone as in vivo Indices

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ABSTRACT

A study was undertaken to determine whether respiratory hexanal and acetone as well as pentane and ethane could be measured as potential indices of lipid peroxidation in vivo. The tests of induction of lipid peroxidation in rats included injection of iron-dextran and the vitamin E deficiency status. Injection of 460 mg of iron/100 g body wt over a 28-day period increased pentane and ethane production 4- and 6-fold, respectively. Hexanal production was increased 7-fold after injection of 60 mg of iron/100 g body wt, and then it fell back to the preinjection level in spite of continued injection of iron-dextran. Acetone production was lower in iron-injected rats than in controls, and it was ca. 10-fold higher in fasted vitamin E-deficient rats than in vitamin E-supplemented rats, being ca 48 and 5 nmol/100 g/min, respectively. It was observed that halomethane injection did not increase hexanal production, while acetone and pentane production were increased. Pentane and hexanal, but not acetone, were found to arise from decomposition of linoleic acid hydroperoxide in vitro. It was concluded that hydrocarbon gases are better indices of lipid peroxidation than hexanal, which is enzymatically metabolized, and acetone, the production of which is dominated by factors such as altered carbohydrate metabolism.

INTRODUCTION

Since Riley et al. (1) first reported ethane to be a new index of lipid peroxidation in vivo, numerous investigations have been undertaken to show that ethane and pentane are both useful indices. Hydrocarbons have been measured as lipid peroxidation products formed during vitamin E (2) or vitamin E and selenium deficiency (3,4); during ethanol (5,6), carbon tetrachloride (5,7–10), and ozone (11) toxicity; as products of iron-stimulated peroxidation (12); as a function of feeding a synthetic dietary antioxidant (13); and during exercise in the human (14). In vitro, the quantitative amounts of hydrocarbon gases formed during decomposition of linoleic, linolenic, and arachidonic acid hydroperoxides have been determined, and the formation of these hydroperoxides during peroxidation has been correlated with the disappearance of fatty acids and formation of conjugated dienes (15).

There is a vast amount of literature on lipid peroxidation in oils and food systems wherein pentane and carbonyl compounds, primarily hexanal, have been correlated with flavor scores as an index of rancidity (16–20). Acetone has also been detected in oxidizing lipids of food systems (19–23). Based upon this literature, a study was undertaken to determine whether these carbonyl compounds could be measured in the breath of rats and, if so, whether they could be correlated quantitatively with respiratory hydrocarbon gases produced under conditions in vivo known to initiate lipid peroxidation, including injection of iron and the status of vitamin E deficiency in the rat. An observation on the effect of injected halomethanes is also reported.

METHODS

Chromatographic Measurement of Hydrocarbons and Carbonyl Compounds

The methods for collection of rat breath samples and subsequent gas chromatographic analysis of hydrocarbon gases have been described (2). Briefly, rats were placed individually into a holding chamber with a 120 ml/min flow rate of hydrocarbon-free air for a minimum of 10 min prior to collection of 300–400 ml samples of air plus breath. Collection of samples normally required 5–8 min. A comparison of peak areas, measured by triangulation, of the standard sample and the breath sample allowed calculation of the molar amount of pentane or ethane produced. The following computation was applied:

$$\frac{(\text{pmol pentane})\,(\text{incoming air flow rate})}{(100\text{ g rat body wt})\,(\text{sample volume})} = \text{total pentane produced/min/100g body wt.}$$

Carbonyl compounds, namely hexanal and acetone, were measured on a 4 ft x 1/8 in. Poropak Q column. Samples of 300 ml of breath were collected as previously described (2), except that the sample trap was filled with glass beads rather than alumina. The sample was

injected into the gas chromatograph via a 6-way gas sampling valve while the trap was heated to ca. 200 C by immersion in a mineral oil bath. With a detector temperature of 300 C and an injector temperature of 230 C, the samples were analyzed either isothermally at 170 C or during temperature programming. For making a programmed analysis, an initial temperature of 75 C was held for 2 min followed by a 3 C/min rise for 28 min, and then a 33 C/min rise to 155 or 160 C. Standards of hexanal in methanol, pentane as a mixed gas standard (Scott Research Laboratories), and acetone were injected directly through the septum. Based upon a comparison of detector response to pentane and ethane standards on 30 different days, the response to ethane was shown to average 2.5 times less than the response to an equimolar amount of pentane. Using the daily instrument response to pentane standards, this ratio was used to calculate the response to ethane.

Animals, Diets, and Treatments

For studies of the rate of disappearance of injected hexanal from the breath, or the effect of injected iron-dextran, or observations on the effect of injected halomethanes, male Sprague-Dawley rats were obtained at 3 wk of age and fed a standard laboratory rat diet. These rats were housed in plastic cages on wood shavings.

To determine the rate of metabolism of an injected dose of hexanal, 4 rats of ca. 200 g each and 4 wk of age were injected intraperitoneally with 208 μmol of hexanal in 0.1 ml of mineral oil/100 g body wt. Hexanal in the breath was measured 5 times over a period of 75 min.

To determine the effect of injected iron on production of hydrocarbons and carbonyl compounds, 5 rats were injected over a period of 28 days, beginning at 3 wk of age, with a total of 460 mg of iron as iron-dextran (Burns-Biotec)/100 g body wt. The initial dose was 20 mg iron/100 g body wt. At day 21, the dosage was increased to 60 mg iron/100 g body wt in order to increase the rate at which peroxidation occurred in these rats fed an adequate level of vitamin E. Breath measurements were made over a course of 45 days beginning the day of the first injection of iron.

For the study of the effect of vitamin E deficiency, beginning at 3 wk of age, 4 male Sprague-Dawley rats were fed for 7 months a casein-based, 10% corn oil, vitamin E-deficient diet (24) obtained from Teklad Test Diets. Another 5 rats were fed the same diet supplemented with 11 i.u. dl-alpha tocopherol acetate for 7 months. Hydrocarbon and carbonyl compounds in the breath were measured after 7 months of feeding the rats. These rats were housed in hanging wire cages for the duration of the study. Rats were all fasted overnight prior to measurement of breath samples, except to show the effect on nonfasting on acetone production.

Measurement of Pentane, Hexanal, and Acetone Following Decomposition of Linoleic Acid Hydroperoxide

The in vitro system for decomposition of linoleic acid hydroperoxide and subsequent hydrocarbon gas analysis has been described by Dumelin and Tappel (15). The same conditions were used except that decomposition was carried out at 50 C and analyses were done on a Poropak Q column rather than on an alumina column. The 2 ml reaction volume contained 15.3 μmol linoleic acid hydroperoxide and the head space volume in the flask was 26.5 ml.

RESULTS

Metabolism of Hexanal in vivo as Measured by the Rate of Its Disappearance from Breath Following Intraperitoneal Injection

The rate of disappearance of an injected dose of hexanal, presumably via the action of aldehyde-metabolizing enzymes, is shown in Figure 1. Five min postinjection hexanal was being expired at an average rate of ca. 11.6 nmol/100 g body wt/min, or less than 0.006% of the injected dose/100 g/min. Between 35 and 75 min post injection, the rate of decrease was much more gradual than between 5 and 20 min. By 75 min. post injection, the rate of elimination was ca. 2.8% of the 5 min. rate. The percentage of the injected dose expired is an average value since the absolute amount of hexanal injected varied from 366 to 466 μmol because of the individual rat weight differences. A rat injected with only mineral oil gave off ca. 1–2 pmol hexanal/100 g body wt/min over a 2 hr time period.

Effect of Iron Injection on Pentane, Ethane, Hexanal, and Acetone Production

Figure 2 summarizes the rate at which rats fed a standard laboratory diet supplemented with 66 i.u. vitamin E/kg produced pentane, ethane, and hexanal over the course of injection of iron and the subsequent decline in their production after cessation of iron injections. The variation of the standard error of the mean values (S.E.M.) for the data obtained from the 5 rats is a reflection of the usually observed individual animal variation, but the trend for the production of each compound was the same

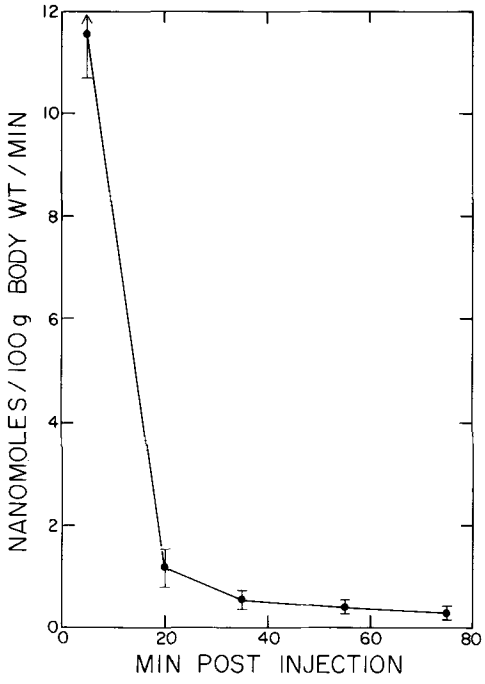

FIG. 1. Hexanal in the breath of rats following its intraperitoneal injection. The plotted values are the mean ± S.E.M. for 4 rats. The weights of the rats varied from 176 to 224 g and the injected dose was 208 μmol/100 g body wt.

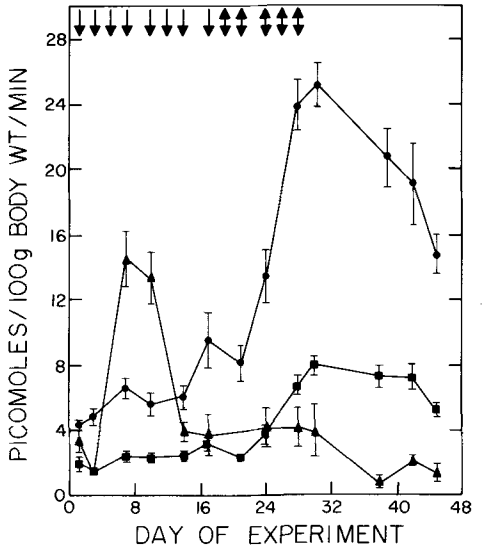

FIG. 2. Effect of iron injections on the production of pentane (■), ethane (●), and hexanal (▲) by rats. Injections of 20 mg of iron, as iron-dextran, per 100 g body wt were given on the days indicated by the downward pointing arrows; 60 mg of iron per 100 g body wt were given on the days indicated by double arrows. The plotted values represent the means ± S.E.M. for 5 rats.

by all rats. Pentane and ethane levels were highest in the iron-injected rats 2 days after the last injection of iron, being increased on the average ca. 4-fold and 6-fold over initial levels, respectively. By ca. day 30 of the experiment, pentane and ethane levels were ca. 4-fold and 7-fold higher than those of the rats not injected with iron, as shown in Figure 3.

Hexanal production followed an entirely different time course from that of hydrocarbon production. Maximum hexanal production was seen on day 7 of the experiment, following injection of a total of 60 mg of iron/100 g of body wt. The level dropped fairly rapidly to about the preinjection level and remained there in spite of further injections of iron being administered. Acetone production was lowest in the group of rats injected with iron, being about one-fourth that by the correponding control rats (Fig. 4).

Effect of Vitamin E Deficiency on Production of Pentane, Ethane, Hexanal, and Acetone

Figure 3 is a bargraph presentation of data showing pentane, ethane, and hexanal levels in the breath of rats fed the basal 10% stripped corn oil diet with and without vitamin E for 7 mo. The values are shown in comparison with those of control rats fed the standard laboratory diet and those of the iron-injected rats at the time of peak production of pentane and ethane (day 30, Fig. 2). Pentane levels were highest in vitamin E-deficient rats, followed by the iron-injected rats. The rats fed the standard diet and the basal 10% stripped corn oil diet supplemented with 11 i.u. vitamin E/kg produced about the same level of pentane. Iron injection stimulated ethane production to the greatest extent, followed by vitamin E deficiency. The rats fed 11 i.u. vitamin E/kg of the Draper diet had the lowest ethane production. Hexanal production was greatest by the vitamin E-deficient rats, being about twice the level of the other 3 groups of rats. As with acetone production, hexanal production tended to be lowest by the iron-injected rats. The almost 10-fold higher production of acetone by fasting vitamin E-deficient rats than by the other groups of rats is shown in Figure 4.

In Vitro Measurement of Pentane, Hexanal, and Acetone from Iron-Catalyzed Decomposition of Linoleic Acid Hydroperoxide

This experiment was done to determine whether hexanal and acetone in vivo could arise from iron-catalyzed decomposition of linoleic

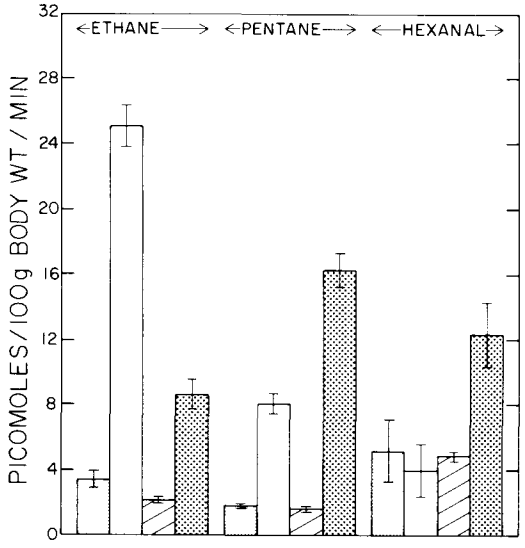

FIG. 3. Comparison of the effects of vitamin E and iron injection on production of pentane, ethane, and hexanal by rats. The light-stippled bars represent values for a group of 4 rats fed a standard laboratory diet; the blank bars, values at day 30 (Fig. 2) for a group of 5 rats fed the standard diet and injected with iron-dextran; the diagonal-lined bars, a group of 5 rats fed a casein-based, 10% stripped corn oil diet supplemented with 11 i.u. vitamin E/kg for 7 mo; and the heavy-stippled bars, a group of 4 rats fed a vitamin E-deficient diet for 7 months. The plotted values represent the means ± S.E.M.

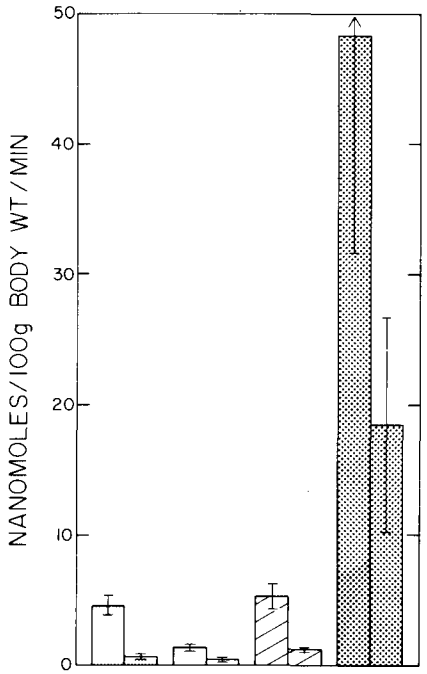

FIG. 4. Acetone production by rats. The bars on the left of each pair represent values obtained for rats fasted overnight prior to measurements and bars on the right represent values for nonfasted rats. The plotted values represent the means ± S.E.M. Identification of bars is the same as in Figure 3.

acid hydroperoxide as is known to be the case for pentane (15). When a 15.3 μmol sample of linoleic acid hydroperoxide in buffer was held for 5 min at 50 C, the headspace above the mixture contained the following in mol %: hexanal, 0.06%; acetone, 0.12%; and pentane, 0.005%. At 90 min following decomposition of the hydroperoxide by addition of 2 μmol of iron and 72 μmol of ascorbic acid, the following in mol% was found: hexanal, 0.35%; acetone, 0.05%; and pentane, 1.06%. This showed that some hexanal and no acetone derived from iron-catalyzed decomposition of linoleic acid hydroperoxide, while a similar amount of pentane as previously reported (15) was observed.

DISCUSSION

In the past few years, a great amount of interest has been expressed in methods for measurement of lipid peroxidation products in vivo. The choice of measurement of the hydrocarbons pentane and ethane had as a background the vast literature on lipid peroxidation and rancidity in food systems. This same literature, only some of which is cited here (16–23), served as a basis for a search for other lipid peroxidation products that might also serve as in vivo indices. Among the carbonyl compounds measured in food systems, hexanal and acetone were selected for study in vivo because of the relatively large amounts reported to be found in vitro.

Our first question during this study was: "If hexanal is produced in vivo via a lipid peroxidation pathway, can it be measured in the breath of animals?" Pentane is an easily measured lipid peroxidation product that derives from linoleic acid hydroperoxide decomposition, whereas hexanal, if formed in vivo, is more likely to be metabolized. The conclusion reached from this study was that any hexanal measured in breath must represent the almost immediate formation in vivo because of the presence of aldehyde metabolizing enzymes in liver cytosolic and subcellular fractions. Recently, Nakayasu et al. (25) described a rat liver microsomal aldehyde dehydrogenase that exhibits increasing activity against aldehydes as the aldehyde carbon chain length increases. They also described a cytosolic enzyme with highest activity against hexanal. In human blood, an aldehyde dehydrogenase was

described as probably playing a quantitatively important role in clearance of blood acetaldehyde deriving from ethanol metabolism (26). Although the activity of the enzyme(s) was not measured, it was probably the rat tissue aldehyde metabolizing enzymes that rapidly removed the injected dose of hexanal. This conclusion is based upon the rapid decline in breath levels of the hexanal following its intraperitoneal injection.

In vivo (1,7,9,10) and in vitro (1) CCl_4 causes rapid peroxidation of lipids in the liver with release of hydrocarbon gases. Recknagel et al. (27) have recently reviewed the mechanism of CCl_4 toxicity. An observation was made during the course of these studies that rats injected with CCl_4 or CCl_3Br did not produce more hexanal than mineral oil injected controls, but that pentane and acetone levels were increased. This observation suggests that the mechanism of hexanal formation is different from that of hydrocarbon formation. The observation that acetone increased is of interest, but an explanation for its formation on the basis of lipid peroxidation cannot be made at this time.

Based upon an earlier report by Dougherty and Hoekstra (12) that ethane production increased in iron-injected rats, a comparison of hydrocarbon and carbonyl compound formation in vivo was made in rats injected with a large amount of iron. The type of iron overload accomplished by parenteral injection is called reticuloendothelial iron-overload because the reticuloendothelial system is the site of the initial distribution of the iron (28). Iron tolerance in piglets, mice, and chicks in vivo is known to be increased by injections of vitamin E (29). Dougherty and Hoekstra (12) found a 6-fold increase in ethane production over that by vitamin E-supplemented rats when a single 5 mg dose of iron/100 g body wt was injected into vitamin E- and selenium-deficient rats. The large amount of iron, 450 mg/100 g body wt, injected into rats fed a standard laboratory diet over a period of 28 days caused a 6-fold increase in ethane and a 4-fold increase in pentane production. The very different profile for the production of hexanal over the 45-day time course of the experiment and the very low amount of acetone produced by iron-injected rats both indicate a different mechanism for the production of these carbonyl compounds from that involved in the production of hydrocarbons. The lack of release of acetone following decomposition of linoleic acid hydroperoxide by ascorbate reduced iron strongly suggests that any acetone that might be produced during lipid peroxidation would arise as a reaction intermediate and not via hydroperoxide decomposition. In vitro, iron is a known catalyst of hydroperoxide decomposition, which yields small amounts of hydrocarbon gases (15). Others (18) have also noted that the mechanisms of production of hexanal and pentane are different; for example, in peanut homogenates their formation as a function of time after homogenization was different, with hexanal being produced almost immediately and pentane being produced over a longer period of time. Heimann et al. (30) have pointed out that there are two possible paths for formation of volatile substances from oils. One is formation from linoleic acid cleavage products and a second is formation from other free radical reaction intermediates. They found that the majority of volatile substances arise from reaction intermediates (peroxide radical). However, n-hexanal was formed from hydroperoxide cleavage in addition to the reaction intermediate mechanism, with the hydroperoxide path accounting for only ca. 20% of the total hexanal. Linoleic acid hydroperoxide was also shown in this study to give rise to hexanal following decomposition by addition of iron. The drop in hydrocarbon production soon after injections of iron ceased may be due to a redistribution of the injected iron or a change in its form so that it wasn't as active a lipid peroxidation initiator or peroxide decomposer as is Fe^{++} in vitro. Wills (31) has shown that iron injection increases the rate of NADPH-stimulated microsomal lipid peroxidation. Similarly, we noted (unpublished observation) that the initial rate of pentane production during NADPH stimulated lipid peroxidation in microsomes prepared from a rat injected with iron was twice that of microsomes prepared from a noninjected rat. Acetone was produced by the control peroxidizing microsomes but not by the microsomes from iron-injected rats, while hexanal was produced by both.

As has been previously shown for rats fed a vitamin E-deficient diet for 7 weeks (2), the rats fed this same diet for 7 months had greater pentane production than ethane production, with the difference being about 2-fold for each study. Hydrocarbon gas production by rats fed adequate vitamin E remains low unless a strong prooxidant is administered. The level of hexanal production by vitamin E-deficient rats was quantitatively between that of ethane and pentane production. Of the three comparison conditions tested, iron injection, halomethane injection, and vitamin E deficiency, hexanal production was at about the same level as hydrocarbon production only in the vitamin E-deficient rats. Since the level of hexanal in

rats fed 11 i.u. vitamin E/kg of diet for 7 months was over twice that of the ethane and pentane levels in these same rats, hexanal production appears to be less affected by vitamin E than is production of ethane and pentane. Vitamin E may more efficiently stop hydroperoxide formation than formation of other reaction intermediates. The levels of hexanal in rats fed the standard diet with 66 i.u. vitamin E/kg and in the rats supplemented with 11 i.u. vitamin E/kg diet were about the same. Rats injected with iron only briefly, and very early in the series of injections, produced higher hexanal levels. In relation to acetone production, the rats fed the standard diet and the basal 10% stripped corn oil diet supplemented with vitamin E had levels that were considerably lower than that of the vitamin E-deficient rats. Iron appeared to have an influence on depression of the metabolism of fats; at least the breath acetone levels would indicate a lower level of ketosis in rats injected with iron.

Vitamin E is required for mitochondria to sustain oxidation of Krebs cycle intermediates in vitro (32–35), and it is also an effective inhibitor of mitochondrial lipid peroxidation (36). In skeletal muscle of vitamin E-deficient rats, there is a defect in energy production (37). Membranes of muscle cells from these rats also become damaged and release pyruvate kinase into the plasma (38); therefore, membrane damage and energy production defects are probably closely related. Decreased liver glycogen levels in vitamin E-deficient rats (39) and accelerated removal from blood of injected glucose (40) both suggest impairment of carbohydrate metabolism, which can increase the level of circulating ketone bodies.

Acetone is removed from the animal body primarily via the lungs. The relatively large amounts of acetone expired by vitamin E-deficient rats probably reflects in the main altered carbohydrate metabolism. It should also be pointed out, however, that acetone has frequently been detected in peroxidizing foods, such as peas (21), corn-soy food blends (19), white rice bran (22), fresh frozen fish (23), and mayonnaise (20). Acetone was also measured in the breath of humans exposed to CCl_4 (41), and was observed in this present study to increase in rat breath following injection of a very small amount of CCl_4. Rats exposed to a high concentration (900 ppm) of *trans*-1,2-dichloroethylene and other chlorinated ethylenes also produced acetone (42), but the biochemical mechanism of its formation needs clarification by further investigation.

There are a number of volatile compounds in the breath that have been considered as potential indices of in vivo lipid peroxidation. These include hydrocarbon gases, principally pentane and ethane; carbonyl compounds, hexanal and acetone; and carbon monoxide (43). From our studies, we have concluded that among the volatile respiratory compounds, pentane and ethane are the indices of choice, Hexanal arises from both hydroperoxide decomposition and as a reaction intermediate of lipid peroxidation, and it is readily metabolized by aldehyde metabolizing enzymes. Although acetone has been indicated to be a product of lipid peroxidation, that portion in the breath deriving from peroxidation would be a very small amount of the total acetone produced, and its production as a result of ketosis due to altered carbohydrate metabolism does not appear to allow its use as an accurate index of in vivo lipid peroxidation. Lindstrom and Anders (43) also concluded that carbon monoxide is not a suitable index of in vivo lipid peroxidation because of the uncertainty of its origin.

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Analysis of the Conjugated Trienoic Acid Containing Oil from *Fevillea trilobata* by ^{13}C Nuclear Magnetic Resonance Spectroscopy[1]

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ABSTRACT

Oil of *Fevillea trilobata* has the composition palmitic acid 31%, stearic acid 12%, oleic acid 11%, linoleic acid 7%, *cis,trans,cis* 9,11,13-octadecatrienoic acid 30% and *cis,trans,trans*-9,11,13-octadecatrienoic acid 9%. The oil was analyzed and components identified by ^{13}C NMR spectroscopy; the composition was also confirmed by conventional methods. Assignment of ^{13}C NMR signals of conjugated trienoic acids is discussed and it is shown that mixtures of isomeric conjugated acids can be analyzed by this method.

INTRODUCTION

Fevillea trilobata L. is a member of the family Cucurbitaceae found in most parts of Brazil and also in some of the countries to the north. It is a climbing plant and the large capsular fruit contains 4-8 disc-shaped seeds, 4-6 cm in diameter and 1-2 cm thick. The seeds, known in Brazil as "nozes de serpente," or snake nuts, and in French Guiana as "graines de Saint Ignace," have long been used against fever and as emetics. Oil from the seeds is used for illumination and also topically against snake bite – hence, presumably, "snake nuts" (1). There does not seem to have been any modern investigation of the compoosition of this oil, although it has been suggested, from refractive index (2) and UV (3) values, that oil from two other *Fevillea* species contains conjugated unsaturated acids.

Since the oil appeared to be potentially useful, it has now been completely analyzed, mainly by ^{13}C NMR, and it is shown that ^{13}C NMR is a valuable method for determining the composition of seed oils which contain more than one conjugated trienoic acid.

MATERIALS AND METHODS

Seeds of *F. trilobata* were a gift from Professor Nuno A. Pereira, Department of Pharmacology, Federal University of Rio de Janeiro, Brazil. The shells were separated from the kernels manually and oil extracted from both parts separately with hexane by the method of Tröeng (4).

Nuclear Magnetic Resonance (NMR)

A Varian XL-100-15 spectrometer in the Fourier transform mode at 25.1603 MHz with a Nicolet 1180 data system with proton noise decoupling was used to obtain natural abundance ^{13}C NMR spectra. The number of data points was 16384; spectral width was 5 KHz and the acquisition time was 1.638 s. To determine chemical shifts of the oil or derived esters, a solution of 0.45 g in 2 ml $CDCl_3$ and 10000 acquisitions were used, and with purified fractions, weaker solutions and fewer aquisitions could be used. The solvent provided the internal deuterium lock signal and shifts are expressed in ppm downfield from TMS.

In making measurements for integration and quantitative analysis, a solution of 1 g in 2 ml $CDCl_3$ was used; 90° pulses and a pulse delay of 25 s were employed and the number of acquisitions was 2000; the proton decoupler was on only during acquisiton. Instrumental integration was verified by expansion of the relevant parts of the spectrum to 4.82 Hz/cm and triangulation. ^1H NMR spectra were obtained with a Varian HA 100 spectrometer.

GLC and Other Physical Measurements

An instrument with FID was employed with either a 1 m x 3 mm column packed with 1.5% Dexsil 300 on 80-100 mesh acid washed and silanized Chromosorb W, used isothermally at 160 C, or a 6 m x 3 mm column packed with 15% OV-275 on acid washed and silanized Chromosorb P (5), used isothermally at 195 C. Oxidation fragments were analyzed on the latter column and temperature was programmed from 60 to 190 C. UV measurements were made on isooctane solutions and IR as films on NaCl disc.

Chemical Separations

Methyl esters were prepared by refluxing the oil for 2 hr with 70 times its volume of 0.02 M

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methanolic sodium methoxide. Saturated (39%), monoenoic (12%) and dienoic (4%) esters were separated by column chromatography on silicic acid/30% silver nitrate (6). Saturated esters (40.7%) were separated by crystallization from a 1.5% solution in acetone at -60 C. Two crystallizations of the recovered mother liquors from 0.7% solution in methanol at -70 C gave a nearly pure *cis,trans,cis*-ester fraction (10% of original esters).

Conversion to the *trans,trans,trans)* isomer was carried out by treatment of a crude *c,t,c* ester concentrate with a trace of I_2 in pentane (7). The product was crystallized twice from methanol at -20 C; hydrolysis to the acid and two further crystallizations from methanol then gave pure *t,t,t* acid mp 69.5-70.5 C – literature (7) gives 72 C. Esters were oxidized with the permanganate periodate reagent in 60% t-butanol as previously described (8).

RESULTS

Analysis of the Oil

The seeds weighed ca. 6 g and contained 39% of oil; 30% of the seed weight was shell which contained 8% of oil and the kernel contained 55% of oil or almost 95% of the total seed oil. Although gas liquid chromatography (GLC) indicated that oils from the shell and from the kernel were similar in composition, only oil from the kernel was analyzed.

^{13}C NMR spectra of fresh oil and of derived methyl esters were measured, and signals observed for methyl esters are shown in Table I and Figure 1. There were no appreciable differences between spectra of esters and of oil except for the expected two C-2 signals due to the difference between acid at C-1 and C-3 of glycerol and acid at C-2 (9,10). Glyceride C-1 and C-3 signals at 62.15 ppm and C-2 signal at

TABLE I

^{13}C Chemical Shifts of Methyl Esters of Oil from *Fevillea trilobata*[a]

Signal number	Chemical shift	Assignment[b]	Integral
1	13.94	C-18 *c,t,c, c,t,t*	35
2	14.09	C-18 S,O.L	65
3	22.27	C-17 *c,t,t*	40
4	22.35	C-17 *c,t,c*	
5	22.62	C-17 L	60
6	22.72	C-17 S,O	
7	24.96	C-3 all esters	100
8	25.67	C-11 L	12
9	26.43	?	7
10	27.24	C-8, C-11 0; C-8, C-14 L	35
11	27.61	C-15 *c,t,c*	30
12	27.85	C-8 *c,t,c, c,t,t*	38
13	29.14-29.72	unassigned	---
14	31.54	C-16 L, *c,t,t*	22
15	31.92	C-16 *c,t,c*	
16	31.97	C-16 S,O	83
17	32.51	C-15 *c,t,t*	12
18	33.04	?	4
19	34.10	C-2 all esters	101
20	125.99	C-11 *c,t,t*	6
21	127.85	C-11 *c,t,c*	27
22	127.96	C-12 *c,t,c,* C-12 L	33
23	128.08	C-10 L	6
24	128.82	C-14 *c,t,c,* C-9 *c,t,t*	40
25	128.91	C-9 *c,t,c*	29
26	129.73	C-9 0	12
27	129.98	C-10 0, C-9 L	15
28	130.20	C-13 L	6
29	130.66	C-14 *c,t,t*	10
30	131.70	C-13 *c,t,t*	9
31	132.39	C-10 *c,t,c*	29
32	132.57	C-13 *c,t,c*	29
33	132.86	C-10 *c,t,t*	9
34	135.05	C-12 *c,t,t*	6

[a]Signals of OCH_3 and carboxyl carbon omitted.

[b]Abbreviations: S, saturated esters; O, oleate; L, linoleate; *c,t,c, cis,trans,cis*-9,11,13-octadecatrienoate; *c,t,t, cis,trans,trans*-9,11,13-octadecatrienoate.

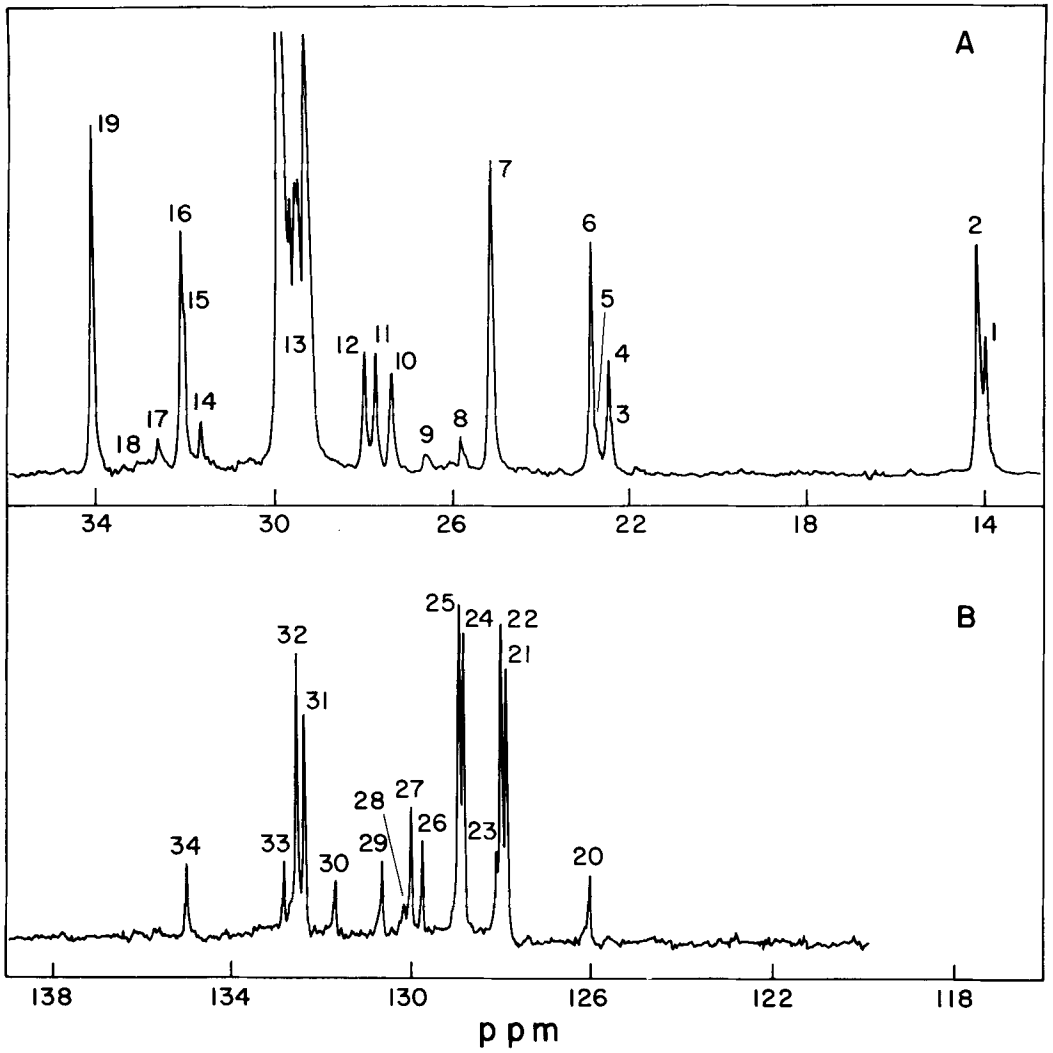

FIG. 1. ^{13}C NMR Spectrum of methyl esters from oil of *Fevillea trilobata*;A is 13 to 35 ppm region and B is 120 to 138 ppm region. Spectra were obtained with spectral width of 5 KHz and horizontally expanded 5 times; B has been amplified more than A to show small signals.

69.00 ppm showed that the oil was a normal glyceride. Signal numbers 22,32 and 34 were 0.05 to 0.08 ppm to lower field in the glyceride spectrum, but there were no signal changes to suggest formation of *trans* bonds or any shifting of double bonds. Thus, the composition of the oil fatty acids was not changed by ester formation.

The relatively highfield C-17 (numbers 3 and 4) and C-18 (number 1) signals suggested that conjugated trienoic acids were present. The six major double bond signals of approximately equal intensities (numbers 21, 22, 24, 25, 31 and 32) and the five minor double bond signals (numbers 20, 29, 30, 33 and 34), also of approximately equal intensity, indicated that there were, in fact, two conjugated acids present in the ratio 3:1.

To identify the fatty acids and to assign signals, methyl esters were fractionated. Separation on a silver nitrate-silicic acid column gave saturated, monoenoic and dienoic esters, but conjugated esters decomposed. The ^{13}C spectra of the monoene and diene fractions were indistinguishable from those of methyl oleate (11) and linoleate from sunflower oil, respectively (see Table II). Fractional crystallization of the esters gave an ester of the major conjugated acid, and the spectrum (Table II) showed that it was the *c,t,c* isomer, or punicic

acid methyl ester (methyl *cis,trans,cis*-9,11,13-octadecatrienoate).

The α effects (of the double bond system, ca. -1.80 ppm) on C-8 and C-15 showed that the 9,10 and 13,14 double bonds were *cis* bonds (9,11,12); the α effect of a *cis* bond conjugated to another bond is known to be appreciably smaller than that of an isolated *cis* bond (-2.40 ppm, Table II) (12). C-8 and C-15 could be assigned from the difference in their shifts which was that expected from the difference in the shifts of C-8 and C-15 in the spectrum of stearate (Table II). The low field signals at 132.45 and 132.63 ppm assigned to C-10 and C-13 showed that the central bond was almost certainly *trans*. The UV maxima, nm ($E^{1\%}_{1cm}$), 264 (1040), 273 (1400), 285 (1083) – literature (7) gives 262, 272 (1766), 283 (in cyclohexane) for punicic acid – confirmed this conclusion. That the double bond system occupies C-9 to C-14 followed from the shifts of C-16, C-17 and C-18, particularly the large (-0.36 ppm) γ effect of a *cis* bond on C-17 (9,11,12, and see Table II).

A crude punicic acid fraction was isomerized with iodine (7) and β-eleostearic acid, *trans,trans,trans*-9,11,13-octadecatrienoic acid, (UV nm ($E^{1\%}_{1cm}$) 258, 268 (1756), 279; literature [7] gives 259, 268 (2190), 279) isolated; chemical shifts are listed in Table II. Since signal number 17 (Table I) had the same shift, 32.52 ppm, as that of C-15 of β-eleostearic acid, the second conjugated triene in the oil contained a *trans*-13,14 double bond and the absence of a signal at 32.79 ppm showed that the 9,10 double bond was *cis;* the second triene must, therefore, be *c,t,t* or *c,c,t*. The minor component is most probably the *c,t,t* isomer, α-eleostearic or *cis,trans,trans*-9,11,13-octadecatrienoic acid, since four of the double bond signals assigned to it have chemical shifts at lower field than those of oleate or elaidate (in the *t,t,t* isomer, all double bond signals are at lower field than those of oleate); a *c,c,t* system would be expected to have more high field signals due to steric interaction. It was not possible to obtain a fraction enriched in this minor isomer or a maleic anhydride adduct, which would have confirmed the presence of tt bonds. Identification as the *c,t,t* isomer was, however, completely confirmed by comparison with the spectrum of tung oil, which contains a major proportion of α-eleostearic acid, (7) (Table II). C-8 of β-eleostearic acid appears at 32.79 ppm, and the absence of this signal from the spectrum of the esters showed that this acid had not been produced by isomerization during seed storage or ester formation.

Intensities of ^{13}C NMR signals can also be

TABLE II

Comparison of ^{13}C Chemical Shifts of Stearate, Oleate, Linoleate and Conjugated Trienoates

Carbon	Stearate	Oleate	Linoleate	*cis,trans,cis*-9,11,13-Octadecatrienoate	*trans,trans,trans*-9,11,13-Octadecatrienoic[a]	*cis,trans,trans*-9,11,13-Octadecatrienoate[b]
2	34.13	34.14	34.16	34.14	34.08	34.08, 34.25
3	25.01	25.00	25.01	24.99	24.72	24.92
4	29.21	29.19	29.18	29.15	29.13	29.18
5	29.31	29.19 δ-0.12	29.18 δ-0.13	29.15 δ-0.16	29.13 δ-0.18	29.12 δ-0.19
6	29.50	29.14 γ-0.36	29.18 γ-0.32	29.10 γ-0.40	29.05 γ-0.45	29.12 γ-0.38
7	29.64	29.73 β+0.09	29.64 β 0.00	29.65 β+0.01	29.36 β +0.01	29.71 β+0.07
8	29.69	27.20 α-2.49	27.26 α-2.43	27.88 α-1.81	32.79 α+3.10	27.87 α-1.80
9	29.72	129.77	130.08	128.92	130.60	128.84
10	29.73	130.00	128.10	132.45	130.82	132.92
11	29.77	27.26 α-2.51	25.71 αα-4.06	127.87	134.49	126.00
12	29.77	29.82 β+0.05	127.97	127.99	134.27	135.15
13	29.77	29.36 γ-0.41	130.25	132.63	130.95	131.72
14	29.71	29.57 δ-0.14	27.26 α-2.51	128.83	130.51	130.66
15	29.41	29.36	29.40 β-0.01	27.63 α-1.78	32.52 α+3.11	32.55 α+3.14
16	31.97	31.95	31.59 γ-0.38	31.92 β-0.05	31.60 β-0.37	31.56 β-0.41
17	22.73	22.72	22.62 δ-0.11	22.37 γ-0.36	22.28 γ-0.45	22.29 γ-0.46
18	14.10	14.10	14.09	13.97 δ-0.13	13.96 δ-0.14	13.97 δ-0.13

[a]Free acid. [b]Tung oil.

TABLE III

Comparison of the Composition of Oil from *Fevillea trilobata* Found by ^{13}C NMR and by GLC

Chain length	Obtained by NMR	Obtained by GLC (Carbon numbers in parentheses)[a]		
		Dexsil 300	OV-275	After hydrogenation
16:0	43	28 (16)	31 (16)	29
18:0		12 (18)	12 (18)	71
18:1	11	21 (17.6)	12 (18.3)	---
18:2	7		6 (18.9)	---
18:3 (*c,t,c*)	30	--- (19.0)	--- (23.9)	---
18:3 (*c,t,t*)	9			---

[a]Carbon numbers were obtained from the logarithmic plot of retention times against chain length, assigning 16 and 18 to the two saturated components.

employed to calculate composition of fatty acid mixtures (10,13,14) when the spectrum is measured so that integrals are proportional to percentages of different carbons. A pulse delay of 25 s was used so that nuclear magnetization had time to recover after each pulse; this is 5 times the expected longest relaxation time (a T_1 of about 5 s would be expected for C-18 [15]). Also to avoid complications due to differing nuclear Overhauser enhancements, the proton decoupler was on only during aquisition, "gated decoupling" (13,14,16,17).

Signal assignment in Table I is discussed later, but all the signals except two small ones at 26.43 and 33.04 ppm can be assigned to carbons of either saturated esters, oleate, linoleate or *c,t,c* or *c,t,t* isomers. To calculate composition, signals due to a single component were averaged; thus, the 6 signals due only to carbons of *c,t,t* the 7 signals of *c,t,c* carbons, and the 3 signals of linoleate carbons gave values for these constitutents. Signal number 26 is the only one due to oleate alone, but other values were obtained by difference from combined oleate and linoleate signals; values were also obtained for saturated esters by difference. By averaging all values, the composition shown in Table III was obtained.

GLC analysis of esters containing conjugated trienoic unsaturation is frequently unsatisfactory due to decomposition and isomerization (7,18,19) and there have been no reports of identification of particular triene isomers by GLC. In this investigation, use of a Dexsil 300 column at 160 C indicated 35% triene and an OV-275 column at 195 C indicated 31% triene, less than that found by NMR; however, the high carbon numbers (Table III) did show that conjugated esters were present. The composition after hydrogenation (Table III) showed that the unsaturated acids were all C_{18}. The GLC results in Table III have been recalculated to 39% triene and agree quite well with the NMR percentages for saturated esters, oleate and linoleate.

The UV absorption spectrum of the oil nm ($E^{1\%}_{1cm}$) 264(573, 273(665), 285(501), indicated 39% conjugated triene – calculated as punicic acid (7). Proton NMR signals in the δ 5.70-6.40 region and IR bands at 937 and 990 cm^{-1} indicated conjugated acids (7). The 9,11,13 double bond system was also confirmed by permanganate periodate oxidation of a triene fraction; azelaic acid was the only dicarboxylic acid and valeric acid was the only monocarboxylic acid produced.

Assignment of NMR Signals

Some of the signals of carbons α to the double bond system have already been discussed and the other signals will now be considered. Signals of all carbons of stearate (9,11,20), oleate (9,11,21) and linoleate (9,12,21) have been assigned previously. Those of stearate and oleate have been confirmed by ^{13}C and deuterium labeling (9,11,20,22) but, although those of linoleate have not been confirmed, the saturated carbon signals are almost certainly correct and the double bond carbon signals are probably correct, but assignments for pairs of similar signals may have to be reversed (Table II). Signals due to saturated esters, oleate and linoleate in esters from the oil were thus assigned as listed in Table I.

^{13}C NMR spectra have not been reported previously for any conjugated trienoic acids, but spectra of several *c,t* and *t,t* conjugated dienes have been discussed (12,23). From these results, signals of the saturated carbons of the *c,t,c*, *c,t,t*, and *t,t,t* isomers could be assigned as

in Tables I and II with little ambiguity. The β, γ and δ effects listed for the *c,t,c*, *c,t,t* and *t,t,t* systems may be derived from those of the *t,c* and *t,t* dienes, respectively (12). A single *trans* bond has a β effect of -0.05 and *t,t,* bonds have a β effect of -0.20 (12) so that the β effect observed for the *t,t,t* acid (-0.37, Table II) seems reasonable. In this way, signals due to C-16, C-17 and C-18 of *c,t,c*, *c,t,t* and *t,t,t* isomers were assigned.

The 6 double bond carbon signals, observed for the pure *c,t,c* ester (Table II), and 5 of the 6 signals due to double bond carbons of the *c,t,t* ester are listed in Table I. It is much less straightforward, however, to assign these signals to particular carbons because the magnitude of the electric field effect due to the ester group (9,24) on conjugated double bonds, and the effect of one or two bonds on carbons of another double bond are unknown.

The basis for tentative assignment of double bond carbons is the theory that 1:4 interaction, as between the α-carbons of a *cis* double bond, leads to steric compression (relative to the saturated analog) and an upfield displacement of the α-carbon shift of 2 to 3 ppm, whereas α-carbons of a *trans* bond are relatively less compressed and there is a downfield displacement of 2-3 ppm (9).

In the *c,t,c* isomers, signals with similar shifts can be grouped in pairs giving three pairs of signals, and molecular models indicate that C-10 and C-13 are least compressed and the two lowest field signals are assigned to these carbons. Since C-8 and C-15 both experience an upfield compression displacement of -1.80 ppm, then C-11 and C-12, which are in the γ position to these carbons, should be correspondingly compressed and are assigned to the highest field pair of signals. The remaining pair of signals are then due to C-9 and C-14.

There are also three pairs of signals in the spectrum of the *t,t,t* isomer (Table II), but two of the pairs are close. The lowest field pair are assigned to the least compressed carbons C-11 and C-12; the other four signals are assigned so that that carbon which has the highest field signal in stearate also has the highest field signal in the *t,t,t* isomer.

The *c,t,t* isomer does not have signals in pairs probably because it is not a symmetrical structure; five *c,t,t* signals appear in Table I and the sixth is obscured by the *c,t,c* signal at 128.82 (it is larger than the others). In assigning the signal at 125.99 ppm to the most compressed carbon, C-11, and the signal at 135.05 ppm to the least compressed carbon, C-12, it appears that steric interaction cannot be the only factor affecting chemical shifts because these signals differ by ca. 2 ppm (to higher to lower field, respectively) from the corresponding carbons in *c,t,c* and *t,t,t* isomers. Two conjugated dienes both had signals at 125.75 and 134.8 ppm (23) suggesting that shifts with these values characterize unsymmetrical ct systems. The signal at 128.82 ppm is assigned to C-9 by analogy with the ctc spectrum, signals at 132.86 and 131.70 ppm to C-10 and C-13, respectively, since the model indicates less steric interaction and that at 130.66 ppm to C-14 by analogy with the ttt spectrum.

These shift assignments to individual double bond carbons may be incorrect because, although shifts of simple conjugated dienes have been reported (9,23,25,26), effects of one double bond on shifts of carbons of a conjugated double bond are unclear. The problem of specific assignment, however, does not prevent general assignment of a group of six double bond carbon signals to a specific symmetrical (*c,t,c*) or unsymmetrical (*c,t,t*) structure, and shifts of the saturated α-carbons, C-8 and C-15, make it possible to distinguish *c,t,t* from *t,t,c* isomers.

DISCUSSION

Conjugated trienoic acids are fairly often found in seed oils of certain plant families and both punicic and α-eleosteric acids occur in oils from other members of the Cucurbitaceae (7). Thus, punicic acid is present in oil of *Cayaponia* and *Cyclanthera* species (3), in *Cucurbita* (27) and *Trichosanthes* and *Ecballium* species (28). α-Eleosteric acid formed over 50% of the oil of two *Momordica* species (3,28), but oil of another *Momordica* species (29) contained 58% of punicic acid. Also, besides occurring in the family Cucurbitaceae, α-eleostearic acid is well known as the major constituent of tung oil (7).

In these oils, the conjugated triene was detected by the UV spectrum and the acid was isolated by fractional crystallization and identified by the UV spectrum and comparison with authentic acid. Only the major triene acid would be detected by this procedure and, in fact, there have been no reports of more than one conjugated triene acid in a seed oil. The ^{13}C method described here has the great advantage that it is applied to the whole oil and that as many as 8 signals, those of the 6 double bond carbons and the α-carbons C-8 and C-15, characterize a particular isomer. Thus, in oil of *F. trilobata*, both the *c,t,c* and the *c,t,t* isomers are detected and estimated. The *c,t,t* and *t,t,c* isomers could also be distinguished since, although the double bond carbons would probably have similar shifts, C-8, C-15, C-16

and C-17 would have different shifts. The *t,t,t* isomer also has characteristic shifts, particularly those at 134.27 and 134.49 ppm, so that the extent of conversion of one of the *cis* bond containing isomers to β-eleostearic acid could readily be determined by ^{13}C NMR. In fact, the spectrum of the tung oil sample showed that the *t,t,t* isomer account for ca. 20% of the conjugated acids.

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Effects of Membrane Fatty Acid Composition on Sodium-Independent Phenylalanine Transport in Ehrlich Cells

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ABSTRACT

We have examined Na^+-independent phenylalanine transport in Ehrlich cells having different degrees of membrane fatty acid saturation. These differences were produced by growing the cells in mice fed a fat-free chow supplemented with either sunflower seed oil or coconut oil. Plasma membranes isolated from the cells grown on sunflower oil were enriched with polyenoic fatty acid, especially 18:2, whereas those isolated from the cells grown on coconut oil were enriched in monoenoic fatty acids, primarily 16:1 and 18:1. Arrhenius plots of phenylalanine uptake showed two transitions. The temperatures of these transitions were different in the two cell preparations; 17 C and 24 C for the cells enriched in polyenoic fatty acids, 19 C and 28 C for those enriched in monoenoic fatty acids. Therefore, this transport system is sensitive to changes in the fatty acid composition of the lipid phase in which it operates. The activation energies, however, were the same in both cell preparations; 14, 8 and 4 kcal/mol. There also was no significant effect of the lipid modifications on either the K'_m or V'_{max} of this transport process. The K'_m for phenylalanine uptake from a choline medium remained constant as the temperature was raised from 17 C to 37 C, whereas the V'_{max} showed about a two-fold increase in both cell types. Phenylalanine exodus from the cells into an amino acid-free suspending medium, analyzed using first-order kinetics, also was not influenced by these membrane fatty acid modifications. The changes in the transition temperatures probably reflect differences in the degree of fatty acid unsaturation of lipids that surround and interact with the phenylalanine carrier. Such differences, however, do not appreciably influence the catalytic activity of this transport system.

INTRODUCTION

Fatty acid composition is a major factor that influences the physical state of membrane lipids. We recently have developed a method for producing large modifications in the fatty acid composition of Ehrlich ascites cells (1). Plasma membranes derived from these modified cells showed marked differences in the transition temperatures detected with a spin-labeled stearic acid probe, suggesting that membrane fluidity is altered by these types of fatty acyl modifications (2). Moreover, we observed that the transport activity of the Na^+-independent, short chain neutral amino acid uptake system in Ehrlich cells also is influenced by these changes in the fatty acid composition of the plasma membrane phospholipids (3).

Christensen and his coworkers have shown that there are at least three distinct systems that mediate the transport of neutral amino acids in Ehrlich cells: System A, ASC and L (4,5). The sodium-dependent uptake of α-aminoisobutryic acid by Ehrlich cells, which is influenced by lipid modifications (3), is mediated by System A (4). In order to determine whether other amino acid transport systems are similarly influenced, we have extended our studies on membrane lipid modification to System L, as represented by sodium-independent phenylalanine uptake. The transition temperatures for this transport system showed the expected variations for the changes in fatty acid saturation that were produced. In contrast to System A, however, the kinetic parameters for phenylalanine transport and the activation energies for this process were not appreciably affected by these changes in membrane fatty acid saturation.

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MATERIALS AND METHODS

Cells

A fatty acid-deficient diet was provided by Teklad Test Diets (Madison, WI). Coconut oil was purchased from Ruger Chemical Co. (Irvington, NJ) and sunflower seed oil was obtained from Cargill (Minneapolis, MN). The Ehrlich ascites cells were propagated in CBA mice fed a fatty acid deficient diet supplemented with either 16% coconut oil or 16% sunflower seed oil. The fatty acid composition of these diets has been reported previously (1). Mice were placed on these diets for at least 4 weeks prior to the transplantation of the cells, and the diets were continued during the additional 2 weeks of tumor growth. The cells were transplanted by the intraperitoneal injection of 0.5 ml of a sterile solution containing 1 volume of Ehrlich ascites tumor and 9 volumes of 0.15 M NaCl.

Transport Measurements

In all solutions used for the cell transport studies, Na^+ was replaced isoosmotically with choline. We have employed the methods developed by Christensen and his coworkers for studying amino acid uptake by and efflux from Ehrlich cells (6,7). Typically, the cells were collected by centrifugation at 200 x g for 2 min and washed once with 0.15 M choline chloride solution. The cells were then suspended in Krebs Ringer-phosphate buffer, pH 7.4, supplemented with 1.5% bovine serum albumin. In this medium, the rate of phenylalanine uptake was not diminished by storage of the cells at 0 C for up to 1 hr. For the uptake measurement, a 0.2 ml aliquot of the cell suspension was added to 1.4 ml of Krebs Ringer-phosphate buffer, pH 7.4, and the reaction was initiated by addition of 0.2 ml uniformly labeled [^{14}C] phenylalanine. At the end of a 45 s incubation, 5 ml of the ice-cold buffer was added, and the cells were pelleted at 10,000 x g for 2 min. The separated cells were extracted with 1 ml of 3% sulfosalicylate for 30 min. After removal of the precipitate, the radioactivity contained in 0.2 ml aliquots of the cell extract and the supernatant fluid from the incubation medium was measured in Packard Tri-Carb 2425 liquid scintillation spectrometer with 15 ml of Tritosol scintillation fluid (8). [^{3}H] Inulin was used to estimate the volume of extracellular water in the cell pellet. Prior to the start of each incubation, both the medium and cells were equilibrated for 5 min at the temperature at which the reaction was to be performed. Without this equilibration, considerable variations in the uptake rate were observed in temperature dependence studies. The weight of intracellular water per 100 mg of the cell pellet was not appreciably affected by changes in incubation temperature.

Krebs Ringer-choline bicarbonate medium supplemented with 5 mM HEPES buffer was used for the efflux studies. The pH of this buffer was 7.6 at 20 C and 7.4 at 37 C with 95% O_2-5% CO_2 as the gas phase. Typically the cells were incubated in the buffer solution containing 20 mM [^{14}C] phenylalanine at 37 C for 10 min. The cells then were washed twice with ice-cold 0.15 M choline chloride solution and suspended in this solution. To measure exodus rates, a 0.5 ml aliquot of the cell suspension was added to 25 ml of the bicarbonate medium with 95% O_2-5% CO_2 as the gas phase. The volume ratio of suspending medium to cellular water was ca. 300. Unless specified otherwise, the decrease in levels of the radioactive amino acid in the cells was measured in the interval from 5 to 15 min after suspending the cells. The rate constant, k, was computed from the first order equation, $A_t = A_o e^{-kt}$.

For Arrhenius plots, rates of uptake were measured from 7° to 42° at intervals of 1.5°. All the data were fitted by the method of least squares using a Hewlett-Packard 98108 programmable calculator.

Lipid Analyses

Washed cells were extracted with a chloroform/methanol solution (2:1, v/v), and the chloroform phase was isolated (9). Polar lipids were isolated from neutral lipids by silicic acid column chromatography (10). Plasma membranes were isolated from Ehrlich cells as described previously (1), and the lipids contained in the membranes were extracted with the chloroform/methanol solution. The phospholipid content of the membranes was measured chemically (11), and the cholesterol content was determined by gas liquid chromatography (12). Other aliquots of the cell and membrane lipid extracts were saponified (13) and methylated (14). The fatty acid composition was determined by gas chromatography using a glass column (1.8 m x 2 mm l.d.) packed with Apolar 10C on 100/200 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA). The separations were carried out with a Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector, and relative mass amounts were determined with a Hewlett-Packard 3380A automatic integrator (12). Fatty acid methyl ester standards were obtained from Supelco, Inc (Bellefonte, PA).

RESULTS

Cells and Plasma Membrane Fatty Acid Composition

This dietary approach has been employed previously to modify the Ehrlich ascites cell plasma membrane fatty acid composition, and detailed results from both cell lipids and several subcellular membrane fractions have been reported (1,3,15-17). These analyses were repeated in the present study only to confirm that similar modifications were produced, and they were not carried out in as great detail. As shown in Table I, the fatty acid compositional changes produced in the cell lipid fractions and in the isolated plasma membranes are essentially the same as those reported earlier (1), including those produced in the cells utilized for studies of α-aminoisobutyrate transport (3). Both the polar and neutral lipids of the cells grown in the mice fed the coconut oil-supplemented diet contained more saturated

TABLE I

Fatty Acid Composition of Cell and Plasma Membrane Lipids

	Percentage composition (%)[a]					
	Cells					
	Polar lipids		Neutral lipids		Plasma membranes	
Fatty acid	Sunflower[b]	Coconut	Sunflower	Coconut	Sunflower	Coconut
Classes[c]						
Saturated	44	49	28	38	42	40
Monoenoic	20	31	21	41	19	32
Polyenoic	33	16	49	13	36	23
Major individual acids						
14:0	1.5	3.7	1.7	4.4	1.8	2.9
16:0	19.7	24.7	14.5	20.1	17.7	18.4
16:1	3.9	6.9	3.4	7.8	2.4	4.6
18:0	20.5	18.7	9.6	12.7	22.3	18.6
18:1	16.8	24.8	16.6	30.6	13.7	26.2
18:2	23.5	8.4	24.9	8.2	23.2	9.8
20:4	6.3	5.8	6.2	2.9	6.6	8.4
22:6	3.1	1.7	4.1	1.1	4.3	1.9

[a]Average of two cell and plasma membrane preparations, each analyzed in duplicate. The individual values agreed very closely in each case.

[b]Diet of the mice in which the cells were grown.

[c]These values do not add up to 100% because a small percentage of the fatty acid composition was unidentified.

and monoenoic fatty acids and considerably less polyenoic fatty acids than those from the cells grown in the mice fed the sunflower oil-supplemented diet. In the plasma membranes isolated from cells modified in this way, the differences were confined to the monoenoic and polyenoic fatty acids. Those from the coconut oil-fed animals contained more monoenoic fatty acids, both 16:1 and 18:1, whereas those from the sunflower oil fed animals contained more polyenoic fatty acid, mostly 18:2. As reported previously (1), the plasma membrane fatty acid modifications were not associated with any appreciable change in the phospholipid content of the membrane, 313 (sunflower) vs. 323 μg/mg protein (coconut), and the molar ratio of phospholipid to cholesterol was the same, 1.2, in both cases. Although not tested in the present work, we have shown previously that these fatty acid modifications are not accompanied by any changes in the phospholipid head group composition of the membrane (3).

Phenylalanine Uptake

Figure 1 shows representative time courses of phenylalanine uptake from a choline-containing medium by Ehrlich cells. The phenylalanine concentration was 0.7 mM, and the incubation temperatures were 17 C or 31 C. Values at 0 time represent the uptake that occurred during separation of the cells by

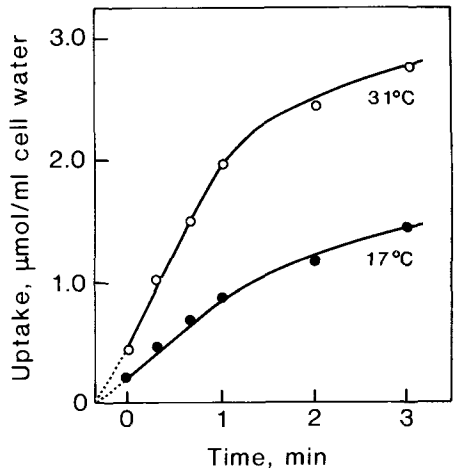

FIG. 1. Time course of phenylalanine uptake from a choline medium. The Ehrlich cells were grown in mice fed a regular diet. The phenylalanine concentration was 0.7 mM, and the incubation temperatures were 17C and 31C.

TABLE II

Kinetic Parameters for Phenylalanine Uptake at Different Temperatures[a]

Temperature	Coconut[b]		Sunflower	
	K'_m	V'_{max}	K'_m	V'_{max}
°C	mM	μmol/ml cell water•45 s	mM	μmol/ml cell water•45 s
17	0.12	0.84	0.15	0.88
23	0.14	1.14	0.13	1.14
31	0.15	1.51	0.16	1.65
37	0.12 ± 0.03[c]	1.54 ± 0.10	0.14 ± 0.03	1.77 ± 0.14

[a]Lineweaver-Burk plots to derive the constants were based on 8 different substrate concentrations ranging from 0.05 to 0.5 mM.

[b]Diet fed to the mice in which the cells were grown.

[c]Mean ± S.E. of 4 experiments.

centrifugation, and they appear to be equivalent to the uptake that occurs during 20 sec of incubation at the respective temperatures. Phenylalanine uptake deviated from linearity after 1 min of incubation at both temperatures. Because of this, we used a 45 s incubation time for all of the uptake studies.

Table II shows the effect of temperatures on the kinetic constants for phenylalanine uptake from a choline medium by Ehrlich cells grown in mice fed either the sunflower or coconut oil-supplemented diet. The nonsaturable component, estimated by the equation of Inui and Christensen (6), was found to vary from 0.04 to 0.08 min^{-1}, and it did not show any clear dependence on the lipid composition of the cells or the incubation temperatures. We used a value of 0.06 min^{-1} to correct all of the uptake data for the nonsaturable component. We did not observe any significant differences in the K'_m or the V'_{max} values for the two kinds of cells. There was also no appreciable temperature dependence of K'_m values over the range from 17C to 37C.

Temperature Dependence of Phenylalanine Uptake

A fixed substrate concentration, 0.7 mM, was used for the temperature dependence studies since the K'_m for uptake was observed to be temperature-independent over the range of interest. Figure 2 compares Arrhenius plots for phenylalanine uptake (corrected for nonsaturable uptake) in cells grown on either

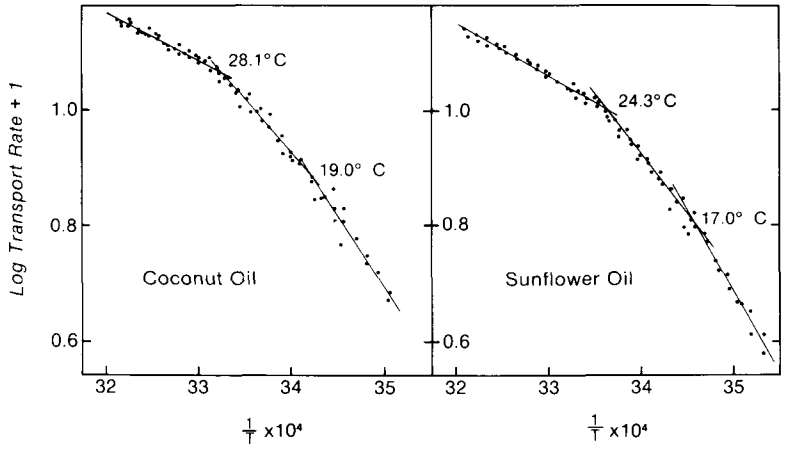

FIG. 2. Arrhenius plots for phenylalanine uptake in the Ehrlich cells grown in mice fed either the sunflower oil or coconut oil diets. Uptake was measured in a medium containing choline, and the phenylalanine concentration was 0.7 mM. Each point represents an average of duplicate values at the temperature. These data were fitted by the method of least squares analysis as described previously (3).

TABLE III

Transition Temperatures and Activation Energies Obtained from Arrhenius Plots of Phenylalanine Uptake

		Phenylalanine uptake				
		T_1	T_2	E_1[a]	E_2	E_3
Diet	Expt. no.	°C		kcal/mol		
Coconut	1	18.6	27.5	12	8	3
	2	19.8	28.5	14	9	5
	3	18.7	28.2	12	9	3
	Mean ± SE	19.0 ± 0.4	28.1 ± 0.3			
Sunflower	1	16.7	25.1	15	8	5
	2	17.5	23.0	18	9	4
	3	16.9	24.9	13	9	4
	Mean ± SE	17.0 ± 0.2[b]	24.3 ± 0.7[c]			

[a] E_1-E_3 are activation energies; E_1 is that below T_1, E_2 is that between T_1 and T_2, and E_3 is that above T_2.

[b] $0.01<P<0.02$.

[c] $P<0.01$.

coconut or sunflower oil. Table III lists the transition temperatures for the transport process and the activation energies obtained from three separate experiments of this type. In all cases, the transition temperatures observed with the cells grown on sunflower oil were from 2° to 4° lower than those for the cells grown on coconut oil. The activation energies, however, were not significantly different for the two cell preparations, being ca. 14, 8 and 4 kcal/mol in both cases.

Phenylalanine Efflux

Figure 3 shows the time course of [^{14}C]-phenylalanine exodus from the two cell preparations into an amino acid-free suspending medium. Two internal phenylalanine concentrations were tested, 4 and 17 mM. A first-order rate constant, 0.04 min^{-1}, appears to fit all of these situations. These data indicate that phenylalanine exodus at 37 C was not influenced by the types of membrane fatty acid modifications that we have produced.

DISCUSSION

Two temperature transitions for Na^+-independent phenylalanine uptake, which is mediated by System L in Ehrlich cells, were observed in the Arrhenius plots. These temperatures were influenced by the modifications in membrane fatty acid composition that were produced, and enrichments with polyenoic fatty acids was associated with a 2-3° lowering of both transition temperatures. A similar type of change was noted for Na^+-dependent, short chain neutral amino acid transport which is mediated by System A in Ehrlich cells (3). Therefore, both of these neutral amino acid transport systems are sensitive to changes in the fatty acid composition of the membrane lipids in which they are embedded. Studies with *Escherichia coli* indicate that the transition temperatures for transmembrane transport

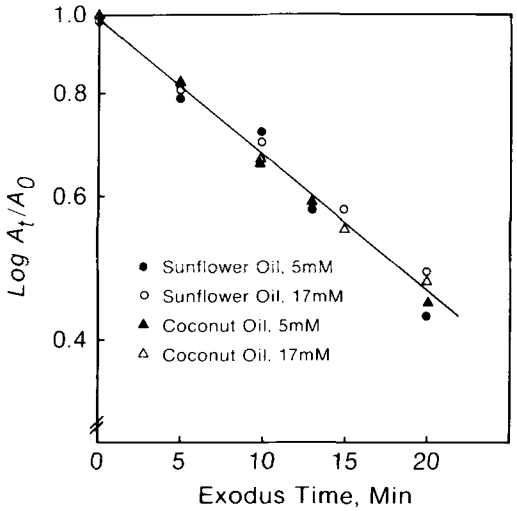

FIG. 3. Efflux of phenylalanine from the Ehrlich cells grown on either coconut oil or sunflower oil. The radioactive phenylalanine concentration inside of the cells was either 5 mM or 17 mM at the beginning of the incubation. These studies were done at 37 C, and each value is the average of two determinations.

systems are caused by phase transitions of the membrane lipids (18,19). In isolated Ehrlich cell plasma membranes, spin-labeled stearic acid probes have demonstrated the presence of two major lipid transitions (2,20). The temperatures of these transitions, however, differ by several degrees from those noted for the amino acid transport systems. Likewise, the temperatures of the activity transitions for each of the amino acid transport systems differ by several degrees (3). Therefore, unlike the *E. coli* transport systems (18,19), the neutral amino acid transport system transitions in the Ehrlich cell are not a direct reflection of the membrane bulk lipid phase transitions. A likely possibility is that the lipid microenvironments of the two transport systems differ and that the activity transitions of each transport system reflects the composition of its microenvironment. It should be noted that the activity transition of the Ehrlich cell plasma membrane (Na^++K^+)-ATPase also is not consistent with the bulk lipid transitions when the fatty acid composition of the membrane was modified by these dietary procedures (21).

Although the transport transition temperatures were altered, the fatty acid modifications that were produced had no appreciable effect on the activation energies or the K'_m or V'_{max} of phenylalanine transport. By contrast, the same dietary modifications altered the activation energy and K'_m of α-aminoisobutyrate transport in the Ehrlich cell (3). Therefore, in contrast to our previous findings with System A, the present results indicate that membrane lipid composition does not exert a modifying effect on the function of System L in the Ehrlich cell. In this regard, the K'_m for α-aminoisobutyric acid uptake in the GF-14 lymphocyte line shows a sharp transition at around 29° (22). As the temperature was raised from 20.5C to 28.9C, the K'_m decreased from 1.78 to 0.68 mM, and it then remained constant between 29C and 42C. On the other hand, the K'_m for phenylalanine uptake in Ehrlich cells showed no transition over the same temperature range. One possible explanation is that the binding site of the α-aminoisobutyric acid carrier, System A, is influenced by changes in the physical state of the surrounding lipid, whereas the binding site of the phenylalanine carrier, System L, is not affected by these kinds of membrane lipid changes. Alternatively, it is possible that the lipid dependence of the transport parameters in System A is exerted on the Na^+-dependent component of this system, perhaps on the (Na^++K^+)-ATPase (21), and therefore not directly on the amino acid carrier component. Independently of mechanism, these results demonstrate that membrane lipid modifications do not have a uniform effect on carrier mediated transport systems in a eucaryotic cell, even on those systems which transport closely related metabolites.

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Response of Free and Esterified Plasma Cholesterol Levels in the Mongolian Gerbil to the Fatty Acid Composition of Dietary Lipid[1]

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ABSTRACT

The present study was undertaken to investigate the potential suitability of the Mongolian gerbil as a useful animal model to study the effects of dietary fats on plasma cholesterol levels. Semipurified diets containing either 20% lard, 20% safflower oil, or 19.5% beef tallow + 0.5% safflower oil were equalized to contain 0.01% cholesterol and 0.05% plant sterol and were fed for a four week experimental period. The proportions of total calories contributed by fat, protein and carbohydrate (starch/sucrose ratio of 2:1) were 40, 14 and 46%, respectively, so as to approach the distribution of calories within the average North American diet. Free, esterified, and total plasma cholesterol levels of male gerbils were determined weekly by gas liquid chromatography after drawing blood via a serial sampling technique. After 1, 2, 3, and 4 weeks of feeding the experimental diets, total cholesterol levels were lowest in the safflower oil fed animals; the corresponding values were 19–64% greater in gerbils fed lard and 68–91% greater in those consuming the beef tallow diet. Cholesterol in the free form generally responded more dramatically to the type of dietary lipid than did cholesterol in the ester form. Irrespective of the type of dietary lipid or the length of the feeding trial, 18–23% of the total plasma cholesterol was in the free form and 77–82% was present as the ester. In view of the similarity to the human of the relative proportions of free versus esterified cholesterol, the type of cholesteryl esters, and their response to dietary manipulation, the gerbil appears to be a useful animal model for studying the regulatory effect of dietary lipid on plasma cholesterol levels.

INTRODUCTION

Numerous studies with human subjects and various animal models have led to the conclusion that both dietary cholesterol and the type of dietary fatty acids can have an effect on plasma cholesterol levels (1–5). As has been discussed by Reiser (6,7), most of the previous experiments dealing with the influence of dietary lipid type have involved comparisons of animal vs. vegetable fats which differ not only in their degree of fatty acid unsaturation but also in their content of both cholesterol and phytosterol (plant sterol). The hypocholesterolemic effect of dietary phytosterol has been well documented (8,9).

The present work was undertaken to determine the potential suitability of the Mongolian gerbil *(Meriones unguiculatus)* as a useful animal model for studying the effect of the type of dietary fat on the concentration of free, esterified, and total cholesterol in blood plasma. Unlike a previous report using the gerbil, which employed diets that were nutritionally inadequate and dietary fats which differed in their content of plant sterol and cholesterol (10), the diets used herein were normalized for their content of both cholesterol and phytosterol. This provided the opportunity to study the effect of a modification of the dietary fatty acid pattern on plasma cholesterol without accompanying changes in endogenous cholesterol or phytosterol levels. In addition, the level of dietary fat and protein, as well as the level and type of dietary carbohydrate in this study, were calculated to approximate levels which correspond to those consumed in the average North American diet (11–13).

EXPERIMENTAL PROCEDURES

Animals and Diets

Young male (8–10 weeks old) Mongolian gerbils (High Oak Ranch, Goodwood, Ontario), having an average body weight of 37 g, were housed two per cage in wire-bottomed, stainless steel cages with access to food and water ad libitum. The animals were maintained on a 12-hour light-dark cycle at a constant temperature of 25 C. Initially, all animals were given the same basal diet containing 20% lard (see Table I) without supplementary phytosterol for a three-week period after which they were randomly assigned to three test diet groups (as given in Table I) for a four-week period. Diet leftovers and spills were weighed every 3–4 days and weight gain and food intake were calculated weekly. The test diets contained safflower oil (SO), lard (L), and beef tallow

[1]Presented in part at the A.O.C.S. Annual Meeting, San Francisco, CA, May 1979.

(BT). The BT test diet contained a blend of beef tallow and safflower oil (19.5% and 0.5% by weight, respectively) to ensure nutritionally adequate levels of essential fatty acid in the diet. The dietary fats were added to represent 20% by weight or 38.9 calorie % of the diet. The test diets resemble the average North American diet in their level of dietary fat, and protein, and the level and type of carbohydrate (11–13).

All test diets were equalized to contain 114 mg cholesterol/kg diet which represents the endogenous cholesterol present in the 20% lard test diet and 480 mg phytosterol/kg diet which represents the endogenous phytosterol (plant sterol) present in the 20% safflower oil test diet. This equalization was accomplished by supplementing the L diet with phytosterol, the SO diet with cholesterol and the BT diet with phytosterol and cholesterol. The sterols were dissolved in the fat before mixing with the other diet constituents.

The fatty acid compositions of the fats are given in Table II and show that the P/S ratio in the SO is 22 and 60 times greater than that in L and BT, respectively.

Methods

Blood samples were obtained weekly after a 12–15 hr fast using the orbital sinus bleeding technique of Sorg and Buckner (14). Short duration anaesthesia was achieved using Metafane (methoxyfluorane, Pitman-Moore, Washington Crossing, NJ), allowing standard microhematocrit capillary tubes to be rapidly filled with blood with little trauma to the animal. Weekly hematocrit determinations on the blood samples taken showed no significant differences in packed cell volume over the seven-week course of blood sampling. Following a 5 min centrifugation (Adams autocrit desk centrifuge), 25 μl aliquots of plasma were added to screw-cap culture tubes containing 16.25 μg of tridecanoin (Nu Cheu Prep, Elysian, MN) as an internal standard. Plasma lipids and dietary sterols were extracted using a modification of the method of Folch et al. (15) which utilized 1 ml of chloroform/methanol (2:1, v/v) per sample. Lipid quantitation was effected by gas liquid chromatography (GLC) on 50 cm glass columns (2 mm I.D.) packed with 3% OV-1 on 100/120 Gas chrom Q (Supelco, Bellefonte, PA) based on procedures described elsewhere (16). In the case of the dietary samples only, the free and esterified sterol fractions were first separated by thin layer chromatography using Silica Gel G plates developed in heptane/isopropyl ether/acetic acid (60:40:3, v/v/v) before GLC analysis. The

TABLE I

Composition of Experimental Diets

Constituent	Percent by weight
Casein	16.0
Fat[1]	20.0
Carbohydrate	
Sucrose	18.2
Starch	36.4
Cellulose (alfa floc)	4.0
Salt mix[2]	4.0
Vitamin mix[3]	1.0
Choline chloride	0.3
Inositol	0.1
Chromium acetate	0.00023
Sodium selenite	0.000022
Sterol[4]	
Cholesterol	0.0114
Phytosterol	0.048

[1]The three experimental diets contained 20% by weight lard + phytosterol (L), 20% by weight safflower oil + cholesterol (SO), or 19.5% by weight beef tallow + 0.5% safflower oil + phytosterol and cholesterol (BT).

[2]Williams-Briggs, modified from Teklad Mills, Madison, WI.

[3]Amounts in mg/kg diet: DL-α-tocopherol acetate, 80; menadione, 0.2; thiamin hydrochloride, 6; riboflavin, 7; pyridoxine hydrochloride, 7; DL-calcium pantothenate, 20; nitotinic acid, 25; D-biotin, 0.2; folic acid, 0.1; cyanocobalamin, 0.012; ergocalciferol, 0.05; retinyl acetate, 3. The vitamin mix contained 97.6% alfa floc carrier so that the total amount of alfa floc in the diet represents 50 g/kg.

[4]All experimental diets were equalized to contain 114 mg cholesterol/kg diet and 480 mg phytosterol/kg diet.

TABLE II

Fatty Acid Composition of Dietary Lipids[a]

Fatty acid	Lard	Safflower oil	Beef tallow
14:0	2.4	trace	3.3
16:0	23.4	8.5	27.8
16:1	3.4	trace	3.1
18:0	15.1	2.3	23.8
18:1	40.9	11.5	35.3
18:2	13.4	77.8	6.6
20:0	1.4	---	---
Total saturated fatty acids	40.9	10.8	54.9
P/S ratio[b]	0.32	7.2	0.12

[a]Values are given as weight % of the total. Fatty acids representing less than 0.5% of the total in any dietary lipid have been omitted from the table.

[b]Calculated as total % of polyunsaturated fatty acids divided by total % of saturated fatty acids.

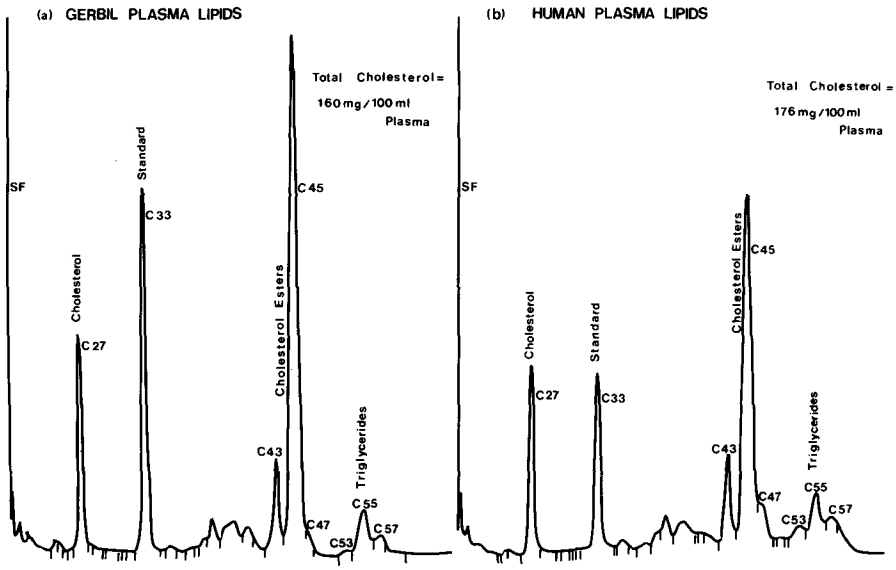

FIG. 1. Gas chromatograms of plasma lipid profiles from gerbil (a), human (b). Nitrogen carrier gas; column temperature programmed 170 to 370 C at 6 C/min; injector temp;. 350 C; detector temp., 400 C; SF, solvent front; C27, free cholesterol; C33, tridecanoyl glycerol, internal standard; C43,45, 47 represent cholesterol esters of C16, C18, and C20 fatty acids; C53, C55, C57, triacylglycerols of 53 to 57 total carbons per molecule (includes glycerol). Region between C33 and C43 represents diacylglycerols plus phospholipid degradation products.

fatty acid composition of the fats and oils fed in this study was determined by GLC using a 200 cm column (2 mm I.D.) packed with 10% SP 23–30, 100–120 Chromosorb W AW (Supelco, Bellefonte, PA) using methods similar to those reported by Andersen and Holub (17).

Statistical Analysis

Data were subjected to analysis of variance and Duncan's multiple range test (18). The level of significance was chosen at $P \leqslant 0.05$.

RESULTS

The GLC analyses of plasma lipid samples from gerbils produced chromatographic tracings such as that seen in Figure 1(a) which are similar to those for plasma from human subjects [Fig. 1(b)]. It is apparent that both the gerbil and human have ca. 70–80% of the total plasma cholesterol in the esterified form in agreement with reports in the literature (19–22). Furthermore, the relative abundance of cholesteryl esters containing fatty acids with 16(C43), 18(C45), or 20(C47) carbon atoms in gerbil and human plasma were also similar [Fig. 1(a) and 1(b)]. Cholesterol linoleate has been reported as the major species of esterified cholesterol in gerbil and human plasma with lower amounts of cholesterol palmitate and arachidonate present as well (20,21).

After completion of the initial three-week basal period when all animals were fed a 20% lard diet (without added plant sterol), the mean total plasma cholesterol level was 127 ± 7 (mean ± S.E.) per 100 ml in a random sampling of 10 of the 30 animals on the day of transfer to the three experimental diets. During the first week on the experimental diets, all animals exhibited a decrease in the total plasma cholesterol ranging from 7% for the BT group to 48% for the SO group (Fig. 2) relative to values found after three weeks on the basal diet. These decreases in total plasma cholesterol over the first week might possibly be due in part to the inclusion of plant sterol in all of the experimental diets (Table I), but not in the basal diet which was fed to all animals during the initial three-week period. However, it is readily apparent from the data in Figure 2 that the influence of the type of dietary lipid on plasma cholesterol levels manifests itself by one week. At this time, the mean plasma level of total cholesterol in the gerbils fed diets containing SO was 66% and 56% of that found in the L and BT groups, respectively. At all sampling times (1,2,3, and 4 weeks), total cholesterol levels were lowest in the SO animals; the corresponding values were 19–64% greater in gerbils fed L and 68–91% greater in those consuming the BT diet. Plasma cholesterol

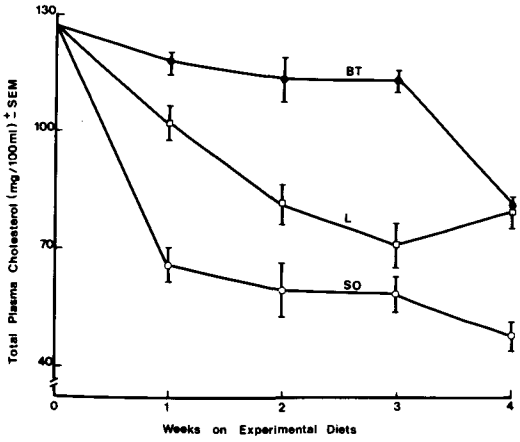

FIG. 2. Total plasma cholesterol levels of gerbils fed three different experimental diets. □, lard (L); ○, safflower (SO); ●, beef tallow (BT). Bars represent means ± SEM.

levels in the animals fed BT decreased during the fourth week and approached those in the L group.

Table III presents results on the effect of feeding the three experimental diets for 1, 2, 3 and 4 weeks on the total, free and esterified cholesterol levels in gerbil plasma. At all times, both the mean free and esterified cholesterol levels were lowest in the SO groups and highest in the animals fed BT. However, cholesterol in the free form generally responded more dramatically to the type of dietary lipid than did cholesterol in the ester form. Specifically, the free cholesterol levels in the SO-fed animals ranged from 40–50% of those for the BT group, whereas the esterified cholesterol levels were 56–63% of the value for animals given BT. Regardless of the dietary lipid or the length of the feeding trial, 18–23% of the total plasma cholesterol was in the free form and 77–82% was present as the ester. At all times, the ratio of free/esterified cholesterol was lowest for the gerbils fed SO. Okey et al. (23) feeding human subjects diets containing safflower oil as the major dietary lipid, have reported that the linoleic acid content of plasma cholesterol esters increased while the percentage of total and free plasma cholesterol decreased. The mean ratio of C43:C45 cholesteryl esters was 0.15 or 0.16 in the SO group and 0.20–0.23 in the other two groups at all times.

DISCUSSION

The purpose of the present investigation was two-fold: first, to determine if the Mongolian gerbil might possibly serve as a useful animal model for studying the effect of dietary lipids on the concentration of plasma cholesterol; second, to examine the effects on free, esterified, and total plasma cholesterol levels of dietary fats having widely different P/S ratios. The diets as constituted were balanced so as to contain equal amounts of cholesterol and phytosterol. BT with a P/S ratio of 0.12 and safflower oil with a P/S ratio of 7.2 were chosen as the two extremes with lard serving as the control. The 20% BT diet was blended to contain 19.5% beef tallow + 0.5% safflower oil to ensure adequate linoleic acid in the diet. The initial study of Hegsted and Gallagher (10) did not provide any dietary supplement to polyunsaturated fatty acids (PUFA) to the saturated fat tested so that a minimum level of essential fatty acid (EFA) was not ensured. In subsequent work, Seiler et al. (24) showed a reduced level of 18:2 in liver and adipose tissue in gerbils fed coconut oil for 8–10 weeks. More recent studies by Hegsted et al. (25) have shown that, in addition to the need for adequate linoleic acid in the diet, the gerbil also has a dietary requirement for inositol which can be increased when diets containing saturated fats or cholesterol are fed. Therefore, our diets were constituted to contain 0.1% inositol.

Comparative nutrition has, as a major interest, the discovery of species and dietary conditions that have relevance to human nutrition. When GLC tracings of human and gerbil plasma lipids are compared [Fig. 1(a), 1(b)], the similarity of the gerbil pattern with the human is apparent. In contrast to the rat, which has as much as 50% of total plasma cholesteryl esters as cholesterol arachidonate (20), the gerbil, like the human (19,20), has only 4–8% cholesterol arachidonate present in plasma. The major cholesteryl ester in the gerbil, as in the human (19), is cholesterol linoleate (75–80%). The relative amounts of free to esterified cholesterol in the human are reported as 70–75% (19–22), and we find that the gerbil has ca. 75% of total plasma cholesterol as cholesteryl esters.

The present experiments indicate that plasma cholesterol levels in the gerbil can be significantly influenced by the type of dietary lipid. The substitution of SO for L produced a significant decrease in the absolute amounts of free, esterified and total plasma cholesterol (Fig. 2, Table III). Since the cholesterol and plant sterol levels were equalized in the diets fed to these animals, it appears that the fatty acid composition of the dietary fat has an effect on the level of plasma cholesterol. Gerbils fed SO also maintained lower plasma cholesterol levels throughout the experimental

TABLE III

Effects of Experimental Diets on Free, Esterified, and Total Plasma Cholesterol Levels[1]

Dietary Lipid	Total cholesterol (mg/100 ml)	Free cholesterol (mg/100 ml)	Esterified[b] cholesterol (mg/100 ml)	Free cholesterol / Esterified cholesterol
		Week 1		
Lard	101.0 ± 4.7[a]	22.5 ± 1.4[a]	78.5 ± 3.5[a]	0.28 ± 0.01[a]
Safflower oil	66.6 ± 4.1[b]	12.3 ± 0.9[b]	54.3 ± 3.2[b]	0.23 ± 0.01[b]
Beef tallow	117.9 ± 2.6[c]	27.6 ± 1.0[c]	90.2 ± 2.2[c]	0.31 ± 0.01[c]
		Week 2		
Lard	81.8 ± 5.2[a]	16.6 ± 1.6[a]	65.1 ± 3.8[a]	0.25 ± 0.01[a]
Safflower oil	60.1 ± 6.5[b]	10.6 ± 1.1[b]	49.5 ± 5.6[b]	0.22 ± 0.02[a]
Beef tallow	113.8 ± 5.4[c]	25.6 ± 1.6[c]	88.1 ± 4.2[c]	0.29 ± 0.01[b]
		Week 3		
Lard	71.3 ± 5.4[a]	16.2 ± 2.4[a]	55.1 ± 3.4[a]	0.28 ± 0.02[a]
Safflower oil	59.7 ± 4.7[b]	10.6 ± 1.4[b]	49.1 ± 3.3[a]	0.21 ± 0.02[b]
Beef tallow	113.9 ± 2.7[c]	26.6 ± 1.1[c]	87.3 ± 2.1[b]	0.30 ± 0.01[a]
		Week 4		
Lard	80.8 ± 4.1[a]	17.2 ± 0.9[a]	63.5 ± 3.8[a]	0.27 ± 0.02[a]
Safflower oil	49.2 ± 3.6[b]	9.3 ± 0.8[b]	39.9 ± 3.0[b]	0.24 ± 0.02[b]
Beef tallow	82.2 ± 2.4[a]	18.5 ± 1.1[a]	63.7 ± 1.5[a]	0.29 ± 0.02[a]

[1]All values are given as means ± SEM for 10 animals in each group. Values in a column not sharing a common letter are significantly different at $P<0.05$.

[2]Represents cholesterol portion of cholesteryl ester only.

period than did the BT-fed animals. These data further support the suitability of the Mongolian gerbil as an appropriate animal model for studying the effects of dietary modification on plasma cholesterol levels since, in human subjects, animal fats tend to be hypercholesterolemic relative to vegetable oils rich in linoleic acid (2,26).

Reiser (6) has reviewed the evidence that the hypocholesterolemic effect of certain vegetable oils is due solely to their PUFA content and concluded that certain other dietary constituents such as the plant sterols in the vegetable oils, and the endogenous cholesterol content of the animal fats, must also be considered when assessing the effect of diet on palsma cholesterol levels. The hypocholesterolemic effect of plant sterols has been well documented by Beveridge et al. (8). In this regard, Hegsted and Gallagher (10) noted that with diets containing no added cholesterol the plasma cholesterol response of gerbils was in the same order of magnitude as those seen in human subjects (26). However, no attempt was made to equalize the plant sterol content of these diets.

The fact that the diets used in the present experiments were constituted to contain proportions of fat, protein, and carbohydrate (type as well as amount) that resemble those consumed by the average North American (11–13) lends support to the applicability of the present animal model to the human situation. The responsiveness of the gerbil to diets with levels of fat typical of North American consumption further strengthens its potential usefulness as an animal model for studying the effects of diet on the regulation of plasma cholesterol.

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Effect of Estrogen on Fatty Acid Synthetase in the Chicken Oviduct and Liver

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ABSTRACT

Estrogen administered to one-month-old female chickens resulted in a 180-fold increase in the amount of fatty acid synthetase, a seven-fold increase in the enzyme activity per gram of tissue and a 25-fold increase in the weight of the oviduct. In contrast, the fatty acid synthetase content in liver increased three-fold; activity per gram of tissue increased two-fold and the weight increased two-fold. The large increase in the fatty acid synthetase activity in the oviduct was due to a corresponding increase in the amount of the fatty acid synthetase protein since the specific activities of highly purified preparations of oviduct and liver fatty acid synthetases were the same and the two enzymes had the same end point as determined by immunoprecipitation. That the increase in activity of the oviduct enzyme is not due to a modification was further supported by physicochemical comparison of the oviduct enzyme with the chicken liver enzyme. Thus, the synthetase complexes have similar size, their subunit composition and size appear to be the same, and both are multifunctional enzymes. Finally, kinetic studies and product analyses indicated no catalytic difference between the enzyme induced by estrogen in the oviduct and the liver enzyme.

INTRODUCTION

Little is known about the effects of estrogens on the metabolism of fatty acids and on the fatty acid synthetase. Baquer and McLean (1) reported a two-fold increase per uterus in the fatty acid synthetase of the estrogen-treated rat. The weights of immature fowl (2) and adult rooster (3) livers doubled on estrogen treatment; however, the effect on the fatty acid synthetase was not reported.

Since the rate of protein synthesis in the avian oviduct is regulated by steroid hormones (4-6) and since ovalbumin synthesis in the chicken oviduct is induced by estrogen (7), it was of interest to determine the effect of estrogen on fatty acid synthetase in the chicken oviduct and liver. Specifically, we have determined that estrogen results in a large alteration in the fatty acid synthetase activity of the oviduct and, therefore, we have investigated the nature of this change.

To facilitate this investigation, we have purified the fatty acid synthetase from laying hen oviduct and characterized its enzymic, physicochemical, and immunological properties. We have also compared it to similar enzymes from other tissues (8-10). The results of these studies are presented in this communication.

MATERIALS AND METHODS

Reagents

The [1-^{14}C]acetyl- and [1,3-^{14}C]malonyl-CoA were obtained from New England Nuclear (Boston, MA). Acetyl- and malonyl-CoA and NADPH were purchased from P.L. Biochemicals (Milwaukee, WI), while the β-estradiol-3-benzoate was obtained from Sigma Chemicals, St. Louis, MO. Since the commercial preparation of malonyl-CoA was found to contain 10% acetyl-CoA, malonyl-CoA was prepared by the method of Eggerer and Lynen (11). This preparation was free of acetyl-CoA as determined by paper chromatography; butanol/glacial acetic acid/H_2O (5:2:3).

All buffers were prepared from reagent grade chemicals. Sodium dodecyl sulfate was a product of BDH Chemicals, England.

Enzyme Assay

Fatty acid synthetase activity was measured by incubating the enzyme in 0.5 ml of a reaction mixture containing the following components at the indicated concentrations: acetyl-CoA (20 μM), NADPH (112 μM), dithiothreitol (4 mM) and potassium phosphate 60 mM (pH 6.5). After 2 min of incubation at room temperature (23-26C), the malonyl-CoA (final concentration 60 μM) was added to initiate the reaction. The decrease in absorbance at 340 nm was measured with an Aminco DW-2 recording spectrophotometer. Protein concentration was estimated by measuring the absorbance at 280 nm. A unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 nmole of NADPH/min/mg (A_{280}). The enzyme activity was also measured by the incorporation of ^{14}C from [1,3-^{14}C]malonyl-CoA (2 Ci/mole) in palmitate in the presence of the above

[1]To whom inquiries and requests for reprints should be sent.

reaction mixture containing 2.6 mM NADPH. The reaction was terminated after 20 min by the addition of 0.2 ml of 2.5 N NaOH. The mixture was then acidified and the radioactive long chain fatty acids were extracted with pentane and the radioactivity was measured as described previously (8).

The radiolabeled products of fatty acid synthesis were identified as the methyl esters after methylation with diazomethane (12). The methyl esters were identified with a Varian 2700 gas chromatograph equipped with 6 ft x 1/8 in. id stainless steel column packed with 10% APOLAR-10C on 100/120 mesh GAS-CHROM Q at 180 C and flame ionization detector. Radioactivity was monitored with a Nuclear Chicago counting system.

Animals

One-month-old DeKalb Leghorn chickens were given intraperitoneal injections of 4 mg estrogen/hen/day for 6 days and then sacrificed. The adipose tissue (from the Pelvic region), the liver, and the oviduct tissues were removed. For purification purposes, 15 laying hens were decapitated and their oviducts removed and all tissues were treated as described under enzyme purification.

Enzyme Purification

Tissues were immediately placed in 0.9% NaCl solution. All manipulations were performed at 0-4 C. Fat and connective tissues were removed and the oviducts were rinsed with the salt solution. The tissue was homogenized for 1 min in 2 volumes of a solution containing sucrose (0.25 M), dithiothreitol (1 mM), EDTA 1 mM and potassium phosphate 0.05M, (pH 7.4). The homogenate was centrifuged for 75 min at 100,000 x g, and the resulting supernatant was passed through glass wool.

The supernatant solution was diluted two-fold with 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol and potassium phosphate 0.05 M, pH 7.4 and applied to a DEAE-cellulose (Brown Paper Co., Berlin, NH) column 5.3 x 87 cm equilibrated with the same buffer. The column was washed and the enzyme eluted with a linear gradient of 2 ℓ each of the starting buffer and a solution containing in addition potassium phosphate 0.2M (pH 7.4). Fractions containing specific activity of 1 or more were pooled, and the enzyme was precipitated with an equal volume of saturated ammonium sulfate (Schwarz-Mann, Orangeburg, NY, ultra pure) pH 7.4. After centrifugation, the precipitate was redissolved in a minimum volume of 0.25 M potassium phosphate, 5% glycerol, 5 mM dithiothreitol and 1 mM EDTA (pH 7.4). The solution was then applied to a 5.2 x 43 cm Sepharose 6B (Phamacia Fine Chemicals, Piscataway, NJ), column equilibrated with same buffer. The fractions containing synthetase activity were pooled, and the enzyme was precipitated with ammonium sulfate as described above and dissolved in a minimum volume of 10 mM potassium phosphate, 10% glycerol, 5 mM dithiothreitol, and 1 mM EDTA (pH 7.4) and dialyzed against this buffer overnight.

The dialyzed enzyme was applied to a DEAE-Biogel (Bio-Rad) 1.2 x 10 cm column equilibrated with the dialysis buffer above. The column was washed with the same buffer and the enzyme was eluted with a linear gradient of 150 ml each of the starting buffer and buffer containing in addition potassium phosphate 0.08 M. The fractions containing synthetase activity were pooled and the enzyme was precipitated with ammonium sulfate as described previously. The enzyme was dissolved in a minimum volume of 0.25 M potassium phosphate, 10% glycerol, 5 mM dithiothreitol, and 1 mM EDTA (pH 7.4) and applied to a Sepharose 6B column 1.2 x 90 cm equilibrated with this buffer and eluted as described in the previous Sepharose 6B step.

The final step of this purification was a repetition of the DEAE-Biogel step except that the column was 1.2 x 5 cm and the gradient was 75 ml in each solution. The protein was precipitated in 50% ammonium sulfate and preserved by dissolving in a solution of 0.8 M potassium phosphate, 20% glycerol, 10 mM dithiothreitol, and 1 mM EDTA (pH 7.4) and storing at -80 C or in liquid nitrogen. Under these conditions, the enzyme could be stored for at least six months without loss of activity.

Immunological Procedure

Chicken liver fatty acid synthetase was prepared according to the procedure of Arslanian and Wakil (13). Rat liver and yeast enzymes were prepared according to the procedures of Stoops et al. (9,14).

Goat antiserum prepared against chicken liver fatty acid synthetase (sp. act. 1200) was partially purified by DEAE cellulose chromatography (15). Ouchterlony double diffusion analysis was performed as described by Stollar and Levine (16) using 1% agar plates. Equivalence point determinations were performed as described by Kabat and Mayer (17).

Sodium Dodecyl Sulfate Gel Electrophoresis

The sodium dodecyl sulfate gel electrophoresis was performed as described by Stoops

et al. (8). The gels were destained using a solution of acetic acid/methanol/water, 10:40:50. Sodium dodecyl sulfate gel electrophoresis was performed on enzyme labeled with [1-^{14}C] acetyl or [1,3-^{14}C] malonyl groups and the gels were sliced and counted as described previously (8).

Amino Acid Analyses

The procedure for determining the amino acid analysis has been described (18). Protein was hydrolyzed in sealed evacuated tubes heated at 105 C for 24, 48 and 72 hr using constant boiling HCl (Pierce, Rockford, IL). Performic acid oxidation was carried out at 0 C for 2 hr in a solution of 15% H_2O_2 and 84% formic acid. The protein was freeze dried and subjected to HCl hydrolysis for 24 hr.

Analytical Ultracentrifugation

The measurements were performed using a Beckman Model E Analytical ultracentrifuge equipped with a RTIC temperature control and an electronic speed control. A Kel-F coated aluminium double sector centerpiece and sapphire windows were employed, and interference optics were utilized to determine the concentration distribution. The sample was brought to equilibrium at 9000 rpm. The data were analyzed as described previously (19,20).

RESULTS

The effect of estrogen (β-estradiol-3-benzoate) on fatty acid synthetase activity and mass of the liver, oviduct and adipose tissues from chickens is summarized in Table I. There is a 25-fold increase in the mass of the oviduct obtained from estrogen treatment as opposed to a two-fold increase in the mass of the liver. Total enzyme units per organ increased 180-fold in the oviduct while in the liver it only tripled. Enzyme activity per gram wet tissue rose seven times in the oviduct compared to three times in the liver. The specific activity of the fatty acid synthetases increased two-fold in these tissues and adipose on treatment with estrogen. The reason for the lower specific activity than expected for the oviduct enzyme is that soluble protein per gram tissue increased over three-fold in the estrogen treated oviduct as opposed to the liver and adipose tissues.

Enzyme Purification

The results of the purification of the oviduct fatty acid synthetase are summarized in Table II. The purification procedure gives a respectable yield of enzyme. It is clear, however, from the results of Table I and II that the oviduct

TABLE I

Effect of Estrogen on Fatty Acid Synthetase Activity in Chicken Oviduct, Liver and Adipose Tissues[a]

	Control hens					Estrogen-treated hens				
Tissue	Mean weight (g)	mg Soluble protein/g tissue	Units/ organ	Specific activity	Units/g tissue	Mean weight (g)	mg Soluble protein/g tissue	Units/ organ	Specific activity	Units/g tissue
Liver	23.2	113	13350	5.1	575	38.4	107	40550	9.9	1056
Oviduct	0.3	15	1.5	0.4	5.8	7.0	49	270	0.8	40
Adipose	--	4.7	--	1.4	--	--	5.1	--	2.8	--

[a]There were five hens each in the control and in the estrogen-treated groups. The enzyme activities are all measured on the high speed supernatant (See Materials and Methods). The specific activity of the fatty acid synthetase is expressed as nmoles NADPH oxidized $min^{-1}mg^{-1}$.

TABLE II

Purification of Fatty Acid Synthetase from Laying Hen Oviduct

Purification step	Total protein[a] mg	Spec. act. (units/mg protein)	Total activity[b] (units)	Yield (%)
High speed Supernatant[c]	56548	0.14	7917	100
DEAE-cellulose	1272	5.50	7000	88
50% Ammonium sulfate	419	16.7	7000	88
Sepharose-6B	127	45.7	5800	73
50% Ammonium sulfate	79	70.8	5600	71
DEAE Biogel	15	240	3500	44
Sepharose-6B	6	500	3000	38
DEAE Biogel	2	950	1900	24

[a]Protein was measured spectrophotometrically at 280 nm.

[b]Synthetase activity was measured by the spectrophotometric method except in the crude extract where the radioactive assay was used.

[c]Extract from 700 g of tissue.

contains less than 1/100 the fatty acid synthetase found in chicken liver. The specific activity of the purified preparations varied between 900 to 1100. Based on the sodium dodecyl sulfate gel electrophoresis results (Fig. 1), it appears that the procedure yields an essentially homogeneous preparation of the enzyme.

Molecular Weight and Subunit Weight of the Oviduct Fatty Acid Synthetase

Sedimentation equilibrium determination of the molecular weight of the oviduct enzyme in 0.25 M potassium phosphate, 1 mM EDTA, and 1 mM dithiothreitol (pH 7.4) at 10C yielded a molecular weight value of 488,000 using a value for the specific volume of 0.74 calculated from the amino acid analysis (Table III). A linear plot of the interference fringe displacement vs. r^2 was obtained over the protein concentration range of 0.1-0.6 mg/ml.

Sodium dodecyl sulfate gel electrophoresis estimate of the subunit molecular weight was 220,000. To further insure the dissociation of the enzyme complex, the protein was incubated at 100 C for 1 min in sodium dodecyl sulfate and 2% 2-mercaptoethanol. The same gel pattern was obtained after the protein was incubated in the sodium dodecyl sulfate solution above or in 0.1 M iodoacetamide (instead of 2% 2-mercaptoethanol) at room temperature for 30 min before gel electrophoresis was performed (8). This indicates that the two chains are not linked by disulfide bonds.

Acetate and Malonate Binding to the Fatty Acid Synthetase

Since previous studies of the animal (8,9,18) and yeast (14) synthetases have demonstrated that acetyl and malonyl moieties are covalently bound to the enzyme presumably at the transacylases, condensing and 4′-phosphopantetheine sites, the binding of acetyl and malonyl groups to the oviduct synthetase was investigated. The fatty acid synthetase of the oviduct was treated separately with ^{14}C-labeled acetyl-CoA and malonyl-CoA; the radioactive enzyme was isolated and subjected to sodium dodecyl sulfate gel electrophoresis as described by Stoops et al. (8).

Figure 1 shows that more than 95% of the label of [^{14}C] acetyl or [^{14}C] malonyl-labeled enzyme was associated with the protein with a molecular weight of 220,000. These results suggest that the acetyl- and malonyl-transacylases, the condensing and 4′-phosphopantetheine sites are associated with the 220,000 peptide (8). These results and those above, are, therefore, consistent with the conclusion that the oviduct fatty acid synthetase is a multifunctional enzyme composed of two polypeptide chains.

Immunological Properties

The results of double diffusion analysis of the oviduct and liver synthetases against antiserum to the liver enzyme are shown in Figure 2. The smooth continuum of the precipitin lines and the absence of spurs indicate the cross-reaction of homologous antigens. Furthermore, identical equivalence points for the synthetases from both tissues were obtained on addition of increasing amounts of enzyme to a constant amount of antiserum against the liver enzyme (data not shown).

Kinetic Studies

As in the case of the liver enzyme, the fatty acid synthetase of the oviduct has an absolute requirement for both acetyl-CoA and malonyl-CoA. No oxidation of NADPH occurs in the absence of either substrates. The apparent Km values for malonyl- and acetyl-CoA and NADPH for the oviduct synthetase were 8.5, 14, and 6.4 μM, respectively. The Km values were determined with the concentration of the fixed substrates at 60 μM malonyl-CoA, 20 μM acetyl-CoA and 112 μM NADPH. The reaction obeyed Michaelis-Menten kinetics over the 2-20 μM concentration range in acetyl-CoA, 2-60 μM concentration range in malonyl-CoA and 6-100 μM in NADPH. Above 20 μM acetyl-CoA or 60 μM malonyl-CoA, substrate inhibition was observed. The order of addition of substrates had no effect on the rate. The apparent Km values for the chicken liver enzyme were 8, 14 and 6 μM for malonyl- and acetyl-CoA and NADPH, respectively, determined under similar conditions.

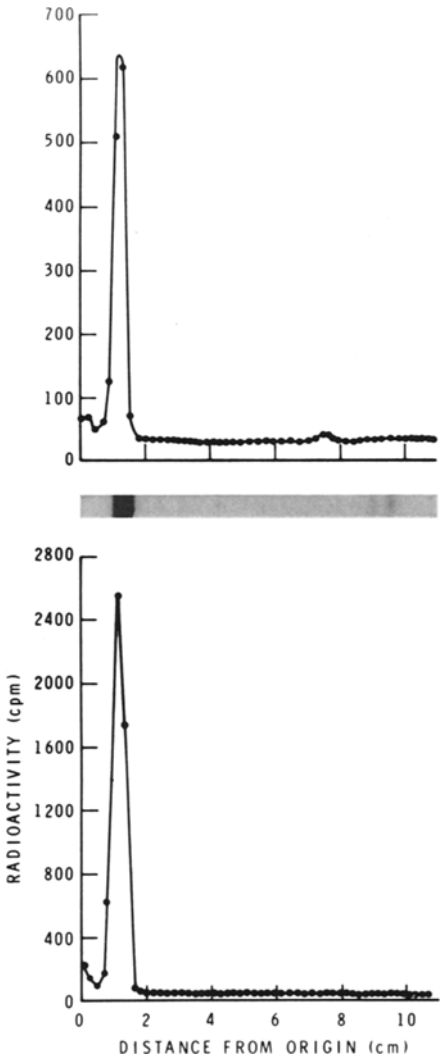

FIG. 1. Sodium dodecyl sulfate gels of acetyl or malonyl-labeled oviduct fatty acid synthetase. The gels were loaded with 30 μg of protein after incubating the enzyme (0.6 mg/ml) with [1-^{14}C]acetyl- (0.15 mM, 51 Ci/mole or [1,3-^{14}C]malonyl-(0.22 mM, 20 Ci/mole) for 1 min before treatment with sodium dodecyl sulfate.

Chromatographic analysis of the products of fatty acid synthesis catalyzed by the oviduct enzyme using [1,3-^{14}C]malonyl-CoA showed that the label in the products was distributed between palmitic (65%) and stearic acids (35%). The products of the synthetase were the free fatty acids, since the extraction of the acids could be achieved independent of the hydrolysis employed above.

DISCUSSION

The 180-fold increase in the amount of the fatty acid synthetase activity per oviduct organ corresponds to a seven-fold induction of the enzyme as measured by the activity per gram wet tissue. This is consistent with the report of Harris et al. (7) who found an even higher induction of the specific egg white protein, ovalbumin. Since administering estrogen to the chickens resulted in a large increase in the fatty acid synthetase activity, we determined some of its physical characteristics. The molecular weight of the emzyme was found to be 488,000 and since its molecular weight determined under denaturing conditions was found to be 220,000, the enzyme consists of two subunits. These characteristics are similar to the fatty acid synthetase isolated from chicken liver. Moreover, they have similar amino acid contents (8) and catalytic properties. Highly purified preparations have the same specific activities (1000) and similar Km values for acetyl-CoA, malonyl-CoA and NADPH as well as the same product distribution between palmitic and stearic acids. Finally, there is antigenic homology as was shown by double diffusion analysis (Fig. 2) and both enzymes had the same equivalence points.

As a result of the similarities between the estrogen-induced oviduct enzyme and the chicken liver enzyme, we conclude that the 180-fold increase in the fatty acid synthetase induced in the oviduct results from an increase in the amount of this enzyme. As these studies indicate, it is not due to an alteration in the enzyme structure which affects its activity.

TABLE III

Amino Acid Analysis of Oviduct Fatty Acid Synthetase[a]

Residue	Number of residues	Residue	Number of residues
Lys	251	Half cystine[b]	85
His	127	Val	287
Arg	200	Met	100
Asp + Asn	463	Ile	184
Thr	241	Leu	467
Ser	419	Tyr	124
Glu + Gln	582	Phe	141
Gly	432		
Ala	396		

[a]Number of residues per mole of enzyme based on 488,000 molecular weight. Tryptophan and proline contents were not determined.

[b]Determined as cysteic acid after performic acid oxidation.

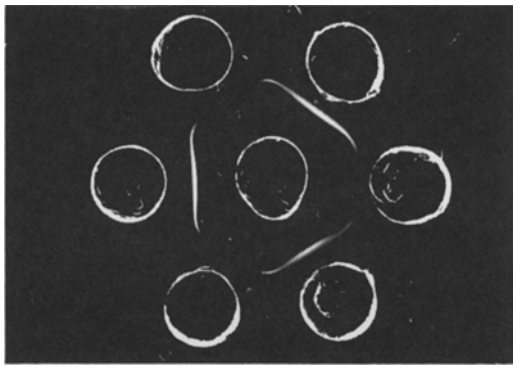

FIG. 2. Ouchterlony double diffusion analysis of hen oviduct and liver fatty acid synthetases. The center well contained 120 μg of goat antiserum to the liver enzyme. The wells resulting in the more or less intense precipitin lines contained 4 μg of the liver and 2 μg of the oviduct enzymes, respectively.

It was interesting to note that neither the amount of liver fatty acid synthetase nor the activity per gram wet tissue increased to the degree of the oviduct enzyme after estrogen administration. Thus, the activity per organ increased three-fold while the activity per gram of tissue increased only two-fold. The larger increase in the activity of the oviduct enzyme after administering estrogen indicates that this hormone plays an important role in its regulation and further suggests that fatty acid synthetase makes an important contribution to the large lipid requirement of the developing egg.

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Lipid Binding Properties of a Factor Necessary for Linoleic Acid Desaturation

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ABSTRACT

Suspension and centrifugation of crude microsomes of rat liver in low ionic strength solution separated a soluble protein fraction that is necessary for the full activity of the linoleic acid desaturase. The fraction partially purified through Sephadex G-150 still retains lipids which are mainly constituted by phosphatidylcholine. Linoleic acid predominates in the fatty acid composition. By NaCl gradient centrifugation and electrophoresis in gelatinized cellulose acetate, the factor behaves like a lipoprotein. The factor binds linoleic acid and linoleyl-CoA that are desaturated to γ-linolenic acid when incubated with washed microsomes. Albumin does not replace the factor.

INTRODUCTION

The enzymatic system involved in stearyl-CoA desaturation has three components embedded in the lipid medium of the microsomes: the NADH cytochrome b_5 reductase, the cytochrome b_5 and the desaturase (1-3). All of them have been purified (4) and the system reconstituted artificially on liposomes (5). Linoleic acid is considered to be desaturated by a similar system (6).

In our laboratory it has been possible to obtain a "soluble factor" either from unwashed microsomes or cytosol which is necessary to obtain maximal desaturation of fatty acid (7-9). Washed microsomes lose most of their desaturation capacity which is recovered when the microsomal washing or cytosol separated at 105,000 x g is re-added into the microsomal suspension. This factor or factors would be involved specially in the Δ6 desaturation of fatty acids (7).

The factor has been partially purified (9), and it has been proved that a protein is involved in its activity (7). It also contains lipids (7). We have also shown that catalase can reactivate washed microsomes (9). However, there was no correlation between the catalase activity of the factor and the activating effect (9). This result was confirmed by Jeffcoat et al. (10).

In this work, we have shown that the factor behaves like a lipoprotein and it is able to bind linoleyl-CoA or linoleic acid. The linoleic acid bound to the "soluble factor" can be desaturated by the desaturase of the microsomal membrane, being converted to γ-linolenic acid.

MATERIALS AND METHODS

[1-^{14}C] Linoleic acid (61 μCi/μmol) was provided by the Radiochemical Center, Amersham, England. [^{14}C] Labeled and unlabeled linoleyl-CoA were prepared according to Kornberg and Pricer (11). Sephadex G-100, G-150 and bovine serum albumin fraction V were provided by Sigma Chem. Co., St. Louis, MO. ATP, CoA, NADH disodium salt and glutathion were obtained from Boehringer, Argentina, Buenos Aires.

Microsomes

Wistar rats of 150-250 g were used. Liver microsomes were obtained by differential centrifugation as mentioned elsewhere (7). Two main fractions were obtained at 105,000 x g: the whole microsomes (M) and the cytosol (C).

Soluble Factor

It was obtained from microsomes by extraction with low ionic strength solution (7) and by centrifugation at 105,000 x g for 60 min. The extracted microsomes (Me) and a supernatant (Sp) containing the soluble factor were separated.

Desaturation Assay

It was performed using free fatty acids or acyl-CoA. Proteins of whole microsomes (M), 2.5 mg, were incubated with 66 μM [1-^{14}C]-18:2 in a total volume of 1.6 ml with the necessary cofactors (9) at 35 C for 20 min. When the activity of the extracted microsomes (Me) was investigated, the incubated amount corresponded to the remainder after the extraction of the "soluble factor" of 2.5 mg of whole microsomal protein (M).

The desaturated amount of labeled acid was measured by gas liquid radiochromatography as described previously (12). Proteins were determined either by a micro-biuret method (13) or by the procedure of Lowry et al. (14).

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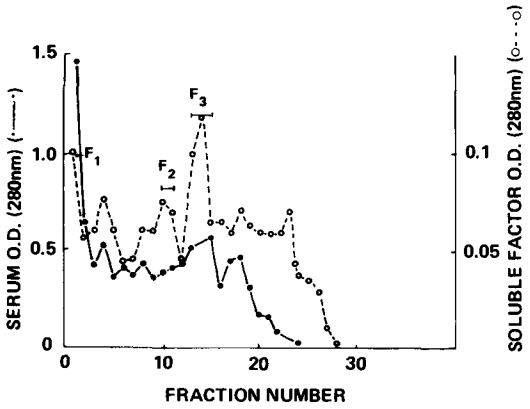

FIG. 1. Spectrophotometric scanning of fractions obtained by gradient centrifugation of crude factor (Sp) and human serum. Fractions 0 to 10 (d > 1.070) and 10 to 24 (D 1,020-1,070) correspond to α and β lipoproteins, respectively.

Lipid analysis

Lipids from different fractions were extracted by the method of Folch et al. (15) and estimated by weighing and phosphorus determination (16). Qualitative and quantitative analysis were performed by thin layer chromatography (TLC) and photodensitometry (17). Complex lipids were separated with $Cl_3CH/CH_3OH/H_2O$ (65:25:4, v/v). Simple lipids were separated with petroleum ether (BP 30-40 C) ethyl ether/acetic acid (90:10:1, v/v).

The soluble factor was delipidated by Albutt's method (18) with ethanol/ether (3:1, v/v) at 18 C.

Fatty acid composition was determined by gas liquid chromatography on a Carlo Erba instrument (Fractovap Mod. GT) with flame detector. The column was filled with 10% ethylene-glycol succinate in Chromosorb W (80-100 mesh).

Lipoprotein Fractionation

Lipoproteins were fractionated by different procedures. In one case, the crude factor (Sp) was centrifuged in a sodium chloride gradient according to Albutt (18) at 105,000 x g for 16 hr. Besides, the fraction obtained with Sephadex G-150 was separated by electrophoresis in 3.5 x 16 cm gelatinized cellulose acetate strips at 200 volts for 55 min. The buffer was 0.04 M sodium diethyl barbiturate (pH 8.6). Lipoproteins were stained overnight with 0.9% fat red 7B. Proteins were stained with 0.5% Amidoblack for 7 min.

Fatty Acid Binding

[1-^{14}C] Linoleic acid and [1-^{14}C] linoleyl-CoA were bound to Sp or to albumin by incubation for 15 min at 25 C. 370 nmol of the free acid (8.59 mCi/mmol) or 290 nmol of the linoleyl-CoA (8.59 mCi/mmol) were incubated with 5 mg of Sp while 20.8 nmol of linoleyl-CoA (0.09 mCi/mmol) were incubated with 10 mg of albumin. The bound material was separated through a Sephadex G-100 column. The protein content was monitored by spectrophotometry at 280 nm, and the radioactivity was counted in a Packard scintillation counter.

RESULTS AND DISCUSSION

Lipoprotein Structure

Previous results have shown that a soluble

TABLE I

Capacity of Fractions Obtained by a Gradient Centrifugation of a Crude Protein Factor and Human Serum Protein to Reactivate the Δ6 Desaturation Capacity of Me[a]

Fractions	Relative % desaturation	Specific activity (nmol/min. mg protein)
M	100	0.16
Me	17	0.03
Me + Sp	75	0.12
Me + Serum proteins	16	0.03
Me + F_1	46	0.08
Me + F_2	86	0.15
Me + F_3	64	0.11

[a]The conversion of linoleic acid to γ-linolenic acid was measured. The incubation conditions are described in the experiment part. F_1, F_2 and F_3 correspond to the fractions obtained after ClNa gradient (see Fig. 1). 0.2 mg protein of each of this fraction and serum were added to the incubation system. Sp = Soluble fractor obtained by washing the microsomes.

protein factor, present in the cytosol and loosely bound to the microsomes, is necessary to obtain full activity of the Δ6 desaturase of fatty acids (7-9). The crude factor contains lipids (7). After partial purification through Sephadex G-150 gel filtration or DEAE-cellulose chromatography (9), lipids extractable by the procedure of Folch et al. (15) were still found fixed to the factor. In this experiment it was shown that whereas the lipid/protein ratio (w/w) of cytosol was 0.017:0.038, the ratios found for Sp and the partially purified factor through Sephadex G-150 were 0.95:1.1 and 0.17:0.33, respectively. These results suggest a lipoprotein structure. Therefore, it was decided to compare the properties of the factor to those of the serum lipoproteins. Both the crude factor (Sp) separated from rat liver microsomes and human serum were centrifuged in a NaCl gradient at 10 C for 16 hr at 105,000 x g (18). The UV scanning at 280 nm is shown in Figure 1. It was found that the fractions F_1, F_2 and F_3 of the factor that correlated α, the boundary between α and β and β serum lipoproteins activated microsomal desaturation of linoleic acid to γ-linoleic acid (Table I), but the highest activity was found in fraction F_2. Therefore, this fraction would have a hydrated density between α and β serum lipoproteins. However, serum components were inactive.

The lipoproteic behavior of the factor was also investigated by gelatinized cellulose acetate electrophoresis. The results compared to human serum are shown in Figure 2. The partially purified factor (G-150) obtained by chromatography of Sp through Sephadex G-150, according to Figure 3, showed several protein bands, but only one band was stained by the lipid reagents. The lipoprotein band ran between β- and α-lipoproteins of serum.

The composition of the lipids bound to the factor was also investigated. A factor partially

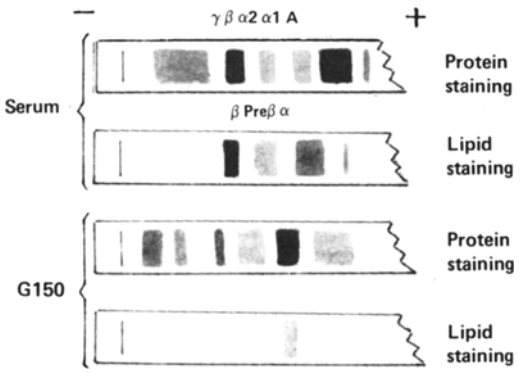

FIG. 2. Electrophoresis is gelatinized cellulose acetate. 10 μl of human serum and 108 μg of the factor purified with Sephadex G-150 were applied. Experimental conditions are described in Materials and Methods.

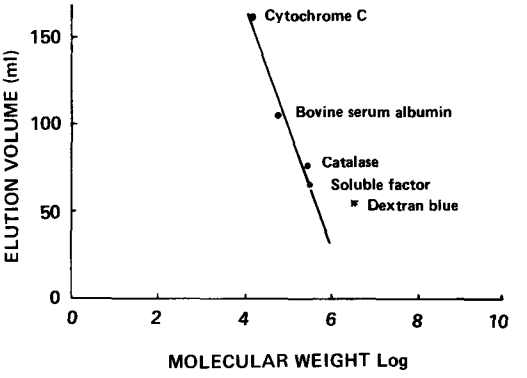

FIG. 3. Tentative molecular weight of "soluble factor" determined by filtration through a Sephadex G-150 (1.5 x 20 cm) equilibrated with 0.02 M Phosphate buffer (pH 7.4) and 0.1 mM EDTA, standards of *Cytochrome C* (14,000 dtns), *bovine serum albumin* (68,000 dtns), catalase (244,000 dtns) and dextran blue (2 x 10^6 dtns) were used.

TABLE II

Lipid Composition of Cytosol, Me, Sp and Sp Partially Purified through Sephadex G-150 (G 150)

Lipids[a]	Cytosol (%)	Me (%)	Sp (%)	G-150 (%)
Free fatty acids	13.4 - 13.9	3.3 - 7.8	12.7 - 22.1	15.2 - 15.9
Phosphatidylethanolamine	6.7 - 9.6	16.1 - 16.2	1.8 - 6.9	7.7 - 9.4
Phosphatidylcholine	19.5 - 20.6	28.1 - 30.4	5.4 - 6.4	28.0 - 42.5
Sphingomyelin	---	---	0.0 - 1.5	0.0 - 1.1
Lysophospholipids	14.6 - 38.1	9.3 - 22.3	37.5 - 64.0	6.4 - 1.3
Triacylglycerols	9.8 - 32.6	11.3 - 15.5	10.3 - 25.4	7.9 - 15.5
Cholesterol	7.0 - 12.2	18.7 - 20.9	1.6 - 4.5	4.2 - 7.6
Diacylglycerols				8.2 - 16.9

[a]Cholesteryl esters were minor components in all the fractions.

TABLE III

Fatty Acid Composition of Cytosol, Me, Sp and Partially Purified Factor through Sephadex G-150[a]

Acids	Cytosol (%)	Me (%)	Sp (%)	G-150 Fraction (%)
16:0	10.3 - 21.5	13.8 - 18.0	3.8 - 13.6	9.3 - 14.8
16:1	1.0 - 3.2	0.64 - 2.7	0.3 - 3.3	0.9 - 1.1
18:0	14.8 - 17.2	32.0 - 33.1	2.1 - 11.9	10.1 - 16.7
18:1	4.9 - 10.8	5.8 - 11.1	1.2 - 6.8	5.4 - 5.6
18:2	25.8 - 51.7	20.6 - 21.6	41.5 - 90.0	59.0 - 63.0
20:4	11.5 - 19.4	18.6 - 21.0	1.5 - 2.8	3.0 - 4.4

[a]Results are the extreme values of three experiments. Other minor components make for 100%.

purified through Sephadex G-150 was extracted by the procedure of Folch et al. (15). The lipids analyzed by TLC showed the composition stated in Table II. They were compared to the lipid composition of cytosol, Sp (crude factor) and washed microsomes (Me). The composition of the Sephadex G-150 fraction varied between certain limits from experiment to experiment, and both polar and nonpolar lipids were found. Phosphatidylcholine, phosphatidylethanolamine, lysophospholipids and free fatty acids amounted to 60 to 75.5% of total lipids. Nonpolar lipids constituted by triacylglycerols, diacylglycerols, cholesterol and cholesteryl esters completed the lipid composition. It is remarkable that phosphatidylcholine largely predominated and in some experiments constituted 42.5% of all the lipids. On the other hand, phosphatidylethanolamine was a minor component. Free fatty acids were also present in a rather constant proportion of the order of 15%. The lipid composition of the partially purified factor was undoubtedly related to the compositions of the cytosol and Sp, but some differences were found. Sp was remarkably rich in lysophospholipids, but the purification of the factor evoked an outstanding enrichment in phosphatidylcholine at the expense of other fractions, mainly lysophospholipids and triacylglycerols. The lipid composition was also different from the washed microsomes (Me). It contained less phosphatidylethanolamine and more free acids. Therefore, it is possible to consider that the protein of the factor is mainly and specifically bound to phosphatidylcholine, but is also carries other minor lipids, specially free fatty acids (Table II).

The fatty acid compositions of cytosol, washed microsomes (Me), Sp and partially purified factor through Sephadex G-150 are shown in Table III. The fractions showed

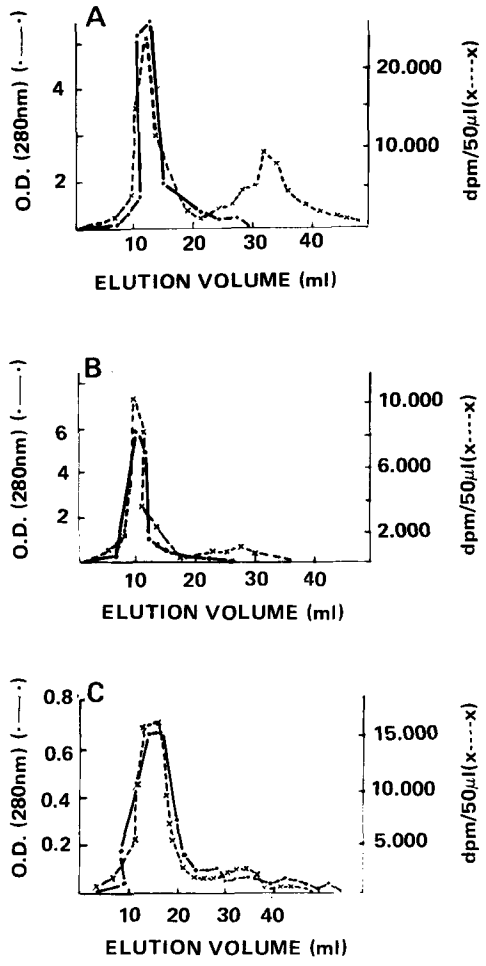

FIG. 4. Sephadex G-100 column profiles of (a) [1-^{14}]linoleyl-CoA binding to crude factor (Sp); (b) [1-^{14}]linoleic acid binding to crude factor (Sp) and (c) linoleyl-CoA binding to albumin. Experimental conditions are described in Materials and Methods.

TABLE IV

Reconstitution of the Δ6 Desaturation Activity by Addition of the Complexes: Sp-Linoleyl-CoA, the Sp-Linoleic Acid and Albumin-Linoleyl-CoA to Extracted Microsomes (Me)[a]

Fractions	Specific activities (n mol γ 18:3/min. mg prot.)		
	Sp-linoleyl-CoA	Sp-linoleic acid	Albumin-linoleyl CoA
M	0.16	0.13	0.14
Me	NM[e]	0.02	0.03
Me + Sp	0.16	0.10	0.13
Me+(Sp-linoleyl-CoA)[b]	0.22	---	---
Me+(Sp-linoleic acid)[c]	---	0.14	---
Me+(Albumin-linoleyl-CoA)[d]	---	---	0.01

[a]The incubation conditions are described in the experimental part.
[b]1.2 mg of Sp protein; 35 nmol [1-^{14}C]linoleyl-CoA.
[c]1.2 mg of Sp protein; 59 nmol [1-^{14}C]linoleic acid.
[d]1.1 mg of albumin; 28 nmol [1-^{14}C]linoleyl-CoA.
[e]NM not measurable.

different compositions, but in cytosol, Sp, and G-150, linoleic acid predominated. The highest concentration of arachidonic acid was only found in Me and cytosol. The purification of Sp through Sephadex G-150 concentrated a lipoprotein with a less variable fatty acid composition in which linoleic acid constituted ca. 61% of all the fatty acids, whereas the saturated acids, palmitic and stearic, contributed 25%.

This rather typical composition of the lipids would suggest that the protein structure of the factor would determine a selective binding of lipids specifically rich in linoleic acid. This specificity for linoleic acid might also be related with the preference of the factor to activate the Δ6 desaturation of linoleic acid and similar acids (7).

To investigate the contribution of lipids to the activity of the factor, delipidation experiments were performed. In earlier experiments diethylic ether was employed. The loss of 70% of the lipids did not inactivate the soluble factor and lipids "per se" did not activate the desaturation (7). Different procedures were tested using sodium deoxycholate, phospholipase A_2 and solvent extraction (ethanol/ether; 3:1, v/v). However, little success was obtained and deactivation was shown together with protein precipitation. Therefore, it was impossible to discriminate if the effect was due to the lipid extraction or to protein deactivation and precipitation. Anyhow, it was found that the lipids were tightly bound to the protein of the factor, and it was difficult to obtain a totally delipidated protein. It could be possible that lipids were necessary to stabilize the proteins and a protein/lipid structure would be involved in the active form of the factor. The insolubility of the protein of the factor in an aqueous medium when it was deprived of the lipids would suggest a lipoprotein structure, similar in some way to the β type of serum, since the proteins of this last class have also poor solubility in aqueous buffers in the lipid-free form.

The molecular weight of the factor tentatively estimated by filtration through a Sephadex G-150 column was a bit higher than 260,000 (Fig. 3) and, therefore, lower than the molecular weight of β-lipoproteins of serum.

Binding of Linoleic Acid and Linoleyl-CoA

The capacity of the factor partially purified through Sephadex G-100 filtration to bind linoleic acid and linoleyl-CoA was also investigated by incubation with the substrates at 25 C for 15 min. After incubation, the material was fractionated in Sephadex G-100. In Figure 4a and b, it is shown that the label of either [1-^{14}C]linoleyl-CoA or [1-^{14}C]linoleic acid was found in the peak of the factors. The incubation of albumin with [1-^{14}C]linoleyl-CoA showed a similar curve (Fig. 4c). However, the albumin bound linoleyl-CoA, was not desaturated in the same conditions (Table IV). Only the linoleyl-CoA or linoleic acids bound to the factor were desaturated to γ-linolenic acid when they were incubated with Me and the suitable cofactors (Table IV). Therefore, these

results show that the factor did not accomplish the mere function of binding the substrate, but it would be involved very probably in some direct interaction between the substrate and the desaturating system of the microsomes. Besides, the present experiments also show that the partially purified factor has some properties that are different from other proteins, such as albumin and catalase, in spite of the possibility that some of the catalase activity found in the factor (9) may contribute to its activating effect.

If we consider that the factor contains ca. 15% (Table II) of free acids, and it is remarkably rich in linoleic acid (Table III), the addition of Sp evokes an increase of unlabeled substrate. Therefore, it would be necessary to correct the values shown in Table IV with Sp by a positive coefficient. This concentration has not been calculated, since it would not modify the meaning of the experiment and it would only demonstrate higher potency of the factor than that one calculated.

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COMMUNICATIONS

The Effect of Hibernation on the Positional Distribution of Ethanolamineglycerophospholipid Fatty Acids in Hamster Brain Membranes

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ABSTRACT

Microsomal and myelin membrane fractions were prepared from the brains of warm-adapted (room temperature) and hibernating Syrian Hamsters (*Mesocricetus auratus*). Ethanolamineglycerophospholipids were isolated and subjected to a fractionation scheme to separate the fatty acids of the plasmenylethanolamine and the phosphatidylethanolamine 1 and 2 positions. The major changes in microsomal phosphatidylethanolamine with hibernation were relative increases in 18:1 at the 1 position and 20:4(n-6) in the 2 position. In myelin, 18:1 increased and 18:0 decreased at the 1 position while the 2 position showed an increase in 16:0 and a decrease in 22:6(n-3). Plasmenylethanolamine fatty acids also changed in microsomes and myelin although the magnitudes were not as great and confined to longer chain fatty acids. In both membranes, fatty acid alterations were position-specific, and no new types of fatty acids were introduced at any position.

INTRODUCTION

It has been shown previously that the phospholipid and fatty acid compositions of brain membranes are altered in the hamster with hibernation (1,2). Such alterations are believed to aid in the maintenance of membrane function and hence cell viability at otherwise lethal temperatures. Ethanolamineglycerophospholipids may play an important role in cold adaptation because a decrease in plasmalogen content and modifications in fatty acid composition of this lipid class are found in brains of both the hibernating hamster and cold-acclimated goldfish (1,3,4). The fatty acid changes consist of an increase in unsaturation among the C_{16} and C_{18} species and a decrease in the average chain length of polyunsaturates.

Considerably less information is available regarding possible regulatory mechanisms responsible for such brain lipid changes. Goldman (5) has found an increase in stearoyl CoA desaturase in the liver but not in the brain of the hibernating hamsters. We have proposed that decreases in plasmalogen content may be due to a net conversion of plasmalogen to the diacyl form during the cold adaptive process (2). Somewhat more is known about lipid metabolic alterations in fish liver with cold acclimation, but the applicability of such results to the brain is undetermined (6-8).

The present study was undertaken to better characterize the ethanolamineglycerophospholipid fatty acid changes in brain myelin and microsomes with hibernation in the hamster. This major lipid class (comprising 40% of the phospholipid of brain myelin and microsomes) possesses a wide variety of acyl chains in both its phosphatidyl and plasmalogen subclasses. Hence, position-specific analyses of fatty acid alterations with hibernation may be expected to yield considerably new information on modes of lipid metabolic adaptation.

MATERIALS AND METHODS

Adult male Syrian hamsters (*Mesocricetus auratus*) between the ages of 36-44 weeks were induced to hibernate after which brain myelin and microsomes were isolated from hibernating and warm-adapted (room temperature) control animals (1). Lipids were extracted from the membrane fractions (1) and further treated as described below.

Ethanolamineglycerophospholipids were preparatively isolated by thin layer chromatography (TLC). Aliquots of the lipid extracts were applied to 0.5 mm thickness Silica Gel G (Supelco, Belletone, PA) plates in a N_2 atmosphere. The plates were developed, also in a N_2 atmosphere, with chloroform/methanol/water (140:60:8, v/v) containing 0.05% BHT. Bands were visualized by spraying with distilled water (9), the ethanolamineglycerophospholipid band was scraped off, and the lipid was extracted

from the silica gel by adding 2 ml chloroform/methanol (2:1, v/v) followed by 0.5 ml water.

The lower phase, lipid solution was evaporated to dryness under N_2 and the plasmenylethanolamine (ethanolamine plasmalogen) alkenylether groups cleaved by the mild acid treatment outlined by Renkonen (10). The lysophosphatidylethanolamine (derived from the plasmalogens) and the phosphatidylethanolamine were separated and isolated by the TLC and extraction systems described above. Tests showed that ca. 98% of the alkenylether groups and negligible amounts of fatty esters were cleaved by this treatment.

The plasmalogen-depleted phosphatidylethanolamine was treated with phospholipase A_2-containing snake venom *(Crotalus adamanteus,* Sigma Chemical Co., St. Louis, MO) according to the ether solution method of Brockerhoff (11), except that the concentration of venom was doubled and the reaction allowed to proceed for 4 hr. Lysophosphatidylethanolamine and free fatty acids were separated by the above TLC procedure. Under the conditions used, ca. 90% of the phospholipid was hydrolyzed. Efforts to achieve 100% hydrolysis were unsuccessful, possibly due to the presence of residual plasmenylethanolamine, which is only slowly hydrolyzed by the enzyme (12).

Lysophosphatidylethanolamine recovered from the mild acid and phospholipase A_2 treatments were transmethylated with methanolic KOH while the free fatty acids were methylated with BF_3-methanol. Both methods are described by Brockerhoff (11). Immediately prior to gas liquid chromatographic analyses, described previously (1), hexane extracts of the reaction mixtures were evaporated to dryness under N_2 and the fatty acid methyl esters dissolved in 0.1-0.3 ml chloroform. Results were statistically analyzed by Students' t-test.

RESULTS AND DISCUSSION

Fatty acid alterations at all three acylated positions of the ethanolamineglycerophospholipids (phosphatidylethanolamine 1 and 2 positions, plasmenylethanolamine 2 position) were found in hibernator microsomes (Table I). The 1 position fatty acids showed an increase in the proportion of 18:1 and the 2 position exhibited relative increases in 18:2 and 20:4-(n-6), while the plasmalogen fatty acid distribution revealed an increase in 20:3(n-6) and decreases in 20:1 and 22:4(n-6). Also, the 2 position showed a trend toward decreased 22:6(n-22:6(n-3), although an unusually large standard error resulted in the difference being below the level of significance. Thus, in the microsomal ethanolamineglycerophospholipids, changes in the relative amount of a specific fatty acid occurred at the position where that fatty acid is most abundant.

Analysis of myelin ethanolaminglycerophospholipids also revealed compositional alterations at all three fatty acyl positions (Table II). However, the changes were more numerous than in the microsomes, and alterations in particular fatty acids were often observed at more than one position. The 1 position showed relative increases in 16:0, 16:1, 18:1 and 18:2, with decreases in 18:0, 20:1 and 20:2(n-6). The 2 position exhibited relative increases in 16:0, 18:0, 18:2 and 20:0, and decreases in 20:1 and 22:6(n-3). The plasmenylethanolamine fatty acids increased in the proportion of 20:4(n-6) while decreasing in 20:2 and 22:6(n-3).

In general, C_{16} and C_{18} fatty acid changes were found at the 1 and 2 positions of phosphatidylethanolamine, while polyunsaturate changes were found in the phosphatidyl 2 positions and in plasmenylethanolamine.

The fatty acid alterations observed in specific ethanolamineglycerophospholipid positions are similar to those found the previous winter hibernating season when the unfractionated lipid was analyzed (1), although minor discrepancies are noted. The magnitude of the fatty acid alterations in the total ethanolamineglycerophospholipid isolated in the present study (data not shown) was somewhat smaller than that noted previously. Thus, for example, whereas microsomal 22:6(n-3) significantly decreased before, the decrease is now suggested at the phosphatidyl 2 position but is not statistically significant. Nevertheless, most of the major fatty acid changes found previously can now be assigned to specific positions. Any small differences between the ethanolamineglycerophospholipid fatty acid compositions determined previously and those calculated from the present positional data may be due to the less than 100% yields obtainable from the fractionation procedures, since both the unfractionated fatty acid compositions and plasmalogen-to-phosphatidyl ratios were virtually identical to those found before (data not shown). The reproducibility of the positional compositions (Tables I and II), however, demonstrates the utility of this methodology for present purposes.

In some instances fractionation revealed details which could not have been seen with the unfractionated lipid. For example, 16:2, which is enriched and certainly quantifiable at the phosphatidyl 2 positions, is only a trace component of the fatty acids as a whole. Similarly,

TABLE I

Positional Composition of Brain Microsomal Ethanolamineglycerophospholipid Fatty Acids from Warm-Adapted and Hibernating Hamsters

Fatty acid	PE 1 FA		PE 2 FA		PEpl Fa	
	Warm-adapted (n=3)[a]	Hibernating (n=3)	Warm-adapted (n=3)	Hibernating (n=3)	Warm-adapted (n=3)	Hibernating (n=3)
16:0	30.4 ± 1.4[b]	30.5 ± .7	6.51 ± .73	5.63 ± .33	4.0 ± .52	4.53 ± .54
16:1	1.65 ± .17	1.45 ± .08	1.22 ± .21	1.24 ± .05	0.46 ± .09	0.62 ± .13
16:2	Tr[c]	Tr	1.04 ± .09	0.80 ± .06	Tr	Tr
17:0	0.92 ± .03	0.85 ± .03	Tr	Tr	Tr	Tr
18:0	41.6 ± .5	43.2 ± .9	3.53 ± .35	3.41 ± .35	1.91 ±.26	2.30 ± .33
18:1	18.8 ± .3	21.1 ± .5[d‡]	9.94 ± .41	9.57 ± .22	11.5 ± .3	12.0 ± .4
18:2	0.57 ± .08	0.75 ± .04	0.53 ± .06	1.19 ± .02[§]	Tr	Tr
20:0	Tr	Tr	Tr	Tr	Tr	Tr
20:1	Tr	Tr	0.59 ± .07	0.46 ± .03	4.81 ±.25	4.05 ± .06[+]
20:2(n-6)[e*]	ND	ND	ND	ND	Tr	Tr
20:3(n-6)	ND	ND	Tr	Tr	0.40 ±.04	0.58 ± .04[+]
20:4(n-6)	Tr	Tr	24.4 ± .4	28.9 ± .7‡	25.6 ± .3	25.7 ± .5
20:5(	ND	ND	Tr	Tr	Tr	Tr
22:4(n-3)*	ND	ND	Tr	Tr	1.04 ±.13	0.70 ± .14
22:4(n-6)	ND	ND	2.67 ± 0.08	3.05 ± .17	10.2 ±.1	9.3 ± .2‡
22:5(n-3)	ND	ND	Tr	Tr	0.96 ±.06	1.16 ± .09
22:6(n-3)	1.27 ± .32	0.82 ± .18	44.9 ± 1.3	41.7 ± .7	36.7 ±.6	36.0 ± .5
Total unknowns	2.51	1.32	1.10	0.91	1.49	1.52

[a]Values of n indicate number of independent lipid isolations from the membrane lipid extracts.

[b]Results are expressed as area percent ± S.E.M.

[c]Tr - trace (<0.05) ND = not detectable.

[d]+p<0.05 ‡p<0.01 §p<0.001.

[e]*Tentative identification.

TABLE II

Positional Composition of Brain Myelin Ethanolamineglycerophospholipid Fatty Acids from Warm-Adapted and Hibernating Hamsters

	PE 1 FA		PE 2 FA		PEpl FA	
Fatty acid	Warm-adapted (n=3)	Hibernating (n=3)	Warm-adapted (n=3)	Hibernating (n=3)	Warm-adapted (n=3)	Hibernating (n=3)
16:0	22.1 ± .5	23.9 ± .2+	13.6 ± .4	16.5 ± .9+	2.4 ± .2	2.7 ± .1
16:1	2.9 ± .2	4.9 ± .4‡	4.5 ± .4	5.3 ± .3	1.0 ± .1	0.8 ± .1
16:2	0.75 ± .08	0.80 ± .01	2.0 ± .2	1.8 ± .1	ND	ND
17:0	0.98 ± .04	1.04 ± .26	0.50 ± .04	0.54 ± .04	ND	ND
18:0	16.5 ± .4	11.0 ± .4§	6.4 ± .4	8.7 ± .4‡	0.9 ± .1	1.2 ± .2
18:1	45.2 ± 1.8	50.1 ± .6+	27.2 ± .2	27.5 ± .6	29.8 ± .4	30.1 ± .3
18:2	0.78 ± .05	1.65 ± .11§	1.5 ± .1	2.0 ± .1+	Tr	Tr
20:0	0.70 ± .15	0.60 ± .06	0.63 ± .05	0.79 ± .03+	0.73 ± .03	0.70 ± .01
20:1	2.16 ± 2.8	1.42 ± .04+	5.1 ± .1	3.8 ± .3‡	20.6 ± .3	19.4 ± .2+
20:2(n-6)*	1.29 ± .10	0.64 ± .06‡	ND	ND	0.9 ± .2	0.4 ± .1
20:3(n-6)	0.51 ± .15	0.47 ± .07	0.79 ± .04	1.15 ± .15	1.06 ± .07	1.28 ± .07
20:4(n-6)	Tr	Tr	15.2 ± .4	15.5 ±4	16.7 ± .5	18.1 ± .2+
20:5(n-3)	0.95 ± .09	0.43 ± .21	ND	ND	ND	ND
22:4(n-3)*	ND	ND	Tr	Tr	ND	ND
22:4(n-6)	ND	ND	3.1 ± .2	2.8 ± .2	10.8 ± .2	11.0 ± .5
22:5(n-3)	ND	ND	Tr	Tr	0.60 ± .11	0.68 ± .03
22:6(n-3)	ND	ND	15.0 ± .6	11.4 ± .6§	6.8 ± .2	5.9 ± .3+
Total unknowns	3.52	2.60	2.09	1.84	7.40	7.27

the increase in 18:1 in the myelin lipid occurs only at the phosphatidyl 1 position and was not detected previously. In the present study, myelin 22:6(n-3) decreased consistently while before it increased slightly. Since that increase was the least significant change noted in the ethanolamineglycerophospholipid fatty acids of either myelin or microsomes, it may have been artifactual.

Earlier results from this laboratory have shown that, in both myelin and microsomes, plasmenylethanolamine decreases with hibernation, while total ethanolamineglycerophospholipid levels are constant (1). It was hypothesized that a net conversion of plasmenyl- to phosphatidylethanolamine takes place through a 2-acylphosphatidyl intermediate during the cold-adaptive process. Such a conversion could be effected by the sequential action of plasmalogenase, which cleaves the alkenylether group (13), and an acyltransferase which would acylate the empty 1 position. This direct conversion would result in a transfer of plasmenyl fatty acids to the 2 position of phosphatidylethanolamine, leading to perceptible increases in 20:1 and 22:4(n-6) at the microsomal and myelin phosphatidyl 2 positions and a decrease in 16:0 at that of myelin. In fact, no such changes occurred indicating no direct conversion of plasmenyl- to phosphatidylethanolamine via the postulated pathway.

Goldman (5) has demonstrated that stearoyl CoA desturase activity of liver is increased with hibernation in the hamster, while that of brain is unchanged. It was postulated that the increased oleic acid synthesized in the liver and carried in the blood acts as the source, but not the cause, of the increased amounts of oleic acid seen in the brain phospholipids. Our results are consistent with this conclusion. It would seem unlikely, however, that our observed fatty acid changes are due solely to altered patterns of fatty acid synthesis or availability, because this would most likely lead to generalized compositional changes rather than the position-specific alterations presently reported. Also, since the nutritional deprivation of the hibernating hamster is not pronounced due to the periodic nature of its hibernation (14), and since the fatty acid alterations observed do not resemble those produced by fat-free diets in adult animals (15), it is improbable that the fatty acid changes noted here have a nutritional origin.

Consideration of the positional fatty acid data does lead to some general conclusions regarding the nature of the fatty acid adaptive mechanisms involved. Both in microsomes and myelin, alterations occur at all three fatty acyl positions, and thus mechanisms capable of producing changes at each position must be operative. In addition, unlike the temperature-adaptive changes in the intestinal mucosal lipids of goldfish, where significant levels of polyunsaturates begin to be found at the 1 position of phosphatidylethanolamine with low acclimation temperature (16), no new types of fatty acids are introduced at any position. Apparently, the overall fatty acid specificity of the phospholipid synthetic or degradative enzymes which are presumably responsible for the fatty acyl alterations need not change drastically. The lower magnitude of the fatty acid modifications seen in the plasmenyl- as opposed to the phosphatidylethanolamine, probably reflects the greater metabolic stability of the former (17,18). Nevertheless, at least in myelin, the plasmenylethanolamines contribute significantly to the absolute amounts of fatty acid change due to their high concentration (ca. 75% of the ethanolamineglycerophospholipids).

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Kidney Lipids: Changes Caused by Dietary 9-*Trans*,12-*Trans*-Octadecadienoate

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ABSTRACT

Trans,trans-linoleate at 50 and 100% of dietary fat decreased kidney size and altered its composition. *Trans,trans*-linoleate as the sole source of dietary fat imparied growth and caused more severe symptoms of essential fatty acid deficiency than was observed with hydrogenated coconut oil (HCO). The concentration of renal cholesterol, phospholipids (PL), triglycerides (TG) and cholesteryl esters (CE) were also decreased. Linoleic (18:2), homo-γ-linolenic acid (20:3n6) and arachidonic acid (20:4n6) were significantly depressed in lipid classes, especially in PL and CE, by dietary *trans,trans*-linoleate. The increase in eicosatrienoate (20:3n9), especially in PL and CE of kidneys of rats fed HCO (essential fatty acid deficient), was slight in rats fed 100% *trans,trans*-linoleate, indicating that the *trans,trans* acid probably inhibited acyl elongation and desaturation.

Commercial vegetable oil-based shortenings and margarines contain varying amounts of *trans* fatty acids, composed mostly of elaidic acid, though measurable levels of linolelaidate may also occur in plastic shortenings (1,2). Studies with rats have demonstrated that relatively high dietary levels of *trans,trans*-linoleate retarded body and organ growth, aggravated symptoms of essential fatty acid deficiency, impaired the conversion of linoleate to eicosatrienoate (20:3n6) and arachidonate (20:4n6) and of oleate (18:1) to eicosatrienoate (20:3n9), suppressed cholesterol esterase activities, and caused the accumulation of palmitoleate and oleate in tissue lipids (3-7). High levels of dietary *trans,trans*-linoleate (18:2) can also impair prostaglandin production by platelets (7), and this may reflect the depression of PG precursors, 20:3n6 and 20:4n6 by *trans,trans*-18:2.

The concentration of prostaglandins (PG) and associated enzymes is comparatively high in kidney (8-10) and PG and their endoperoxides are actively involved in renal functions (10-15). PG production by the kidney is apparently affected by the availability of precursor fatty acids because in essential fatty acid deficiency PG synthesis is significantly depressed (14).

In conjunction with studies of the effects of dietary *trans,trans*-linoleate on tissue prostaglandins, we examined their effects on the composition of kidney with particular emphasis on unsaturated fatty acids.

MATERIALS AND METHODS

Animals and Diet

Weanling male Sprague-Dawley rats weighing 40 to 50 g were individually housed in stainless steel cages in a room maintained at constant temperature (23 ± 2C). The composition of the diet was described previously (7). Highly purified (>99%) methyl esters of 9-*cis*,12-*cis*-octadecadienoate and 9-*trans*,12-*trans*-octadecadienoate (Nu-Chek Prep., Elysian, MN) were used as dietary sources of fat. The hydrogenated coconut fat (HCO) (Nutritional Biochemicals, Cleveland, OH) contained 0.3% of *cis,cis*-linoleic acid. The rats were randomly divided into 4 groups of 15 each. Each group was fed the balanced diet containing HCO (treatment A); *trans,trans*-linoleate (treatment B), a mixture of equal amounts of *cis,cis*-linoleate and the *trans,trans* isomer (treatment C) or *cis,cis*-linoleate (treatment D) as sole sources of dietary fats, respectively. The content of fat was 5% by weight of the diet, equivalent to ca. 11% of calories. All diets except the HCO diet were mixed every 3 to 4 days and stored in a refrigerated room (5 C). Fresh diet was provided daily to each group.

After 12 weeks, rats from each group were lightly anesthetized with ether. The kidneys were removed from the rats, weighed, frozen in liquid nitrogen and stored at -20 C until analysis.

Lipid Analysis

The lipids were extracted from kidney tissue which was homogenized in a polytron with chloroform/methanol (2:1, v/v) according to the method of Folch et al. (16). The lipids were dried under nitrogen and dissolved in chloroform containing 0.01% BHT.

Triglycerides (TG), cholesteryl esters (CE) and phospholipids (PL) were separated by thin layer chromatography (TLC) on activated Silica Gel G plates with standard reference lipids on both edges (17). The plates were developed once in petroleum ether/ethyl ether/acetic acid

(70:30:1), dried and sprayed with 0.2% of 2.7-dichlorofluorescein. The TG, CE and the total PL bands were scraped off, extracted [TG and CE extracted with diethyl ether containing 1% acetic acid; PL with chloroform/methanol (2:1)], filtered through Whatman No. 1 paper, and concentrated under nitrogen. The isolated PL were quantified colorimetrically (18).

Known concentrations of internal standards, i.e., pentadecanoic acid, tripentadecanoin and diplamityl-phosphatidylcholine, were added to the lipid samples prior to saponification and methylation for quantification of CE, triacylglycerides and PL, respectively. Fatty acid methyl esters and cholesterol were quantified by gas liquid chromatography as previously described (19,20).

Protein Determination

The fat-free kidney tissues were dried in a vacuum dessicator to a constant weight, and ground finely to a powder form. Proteins were determined by the method of Lowry et al. (21) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The rats on diets A and B showed the classical symptoms of essential fatty acid (EFA) deficiency (22,23). The effects of the four types of dietary fats on body and organ weights, total lipids, PL and proteins of rat kidneys are shown in Table I. Compared to the rats fed *cis,cis*-18:2 as sole source of fats, body weights kidney sizes, renal lipids and proteins of the EFA-deficient rats on diets A and B were reduced, particularly in the case of the rats fed *trans,trans*-18:2. However, relative to body weights, the kidneys from the EFAD rats were somewhat enlarged.

These data corroborated earlier findings showing that high levels of dietary *trans,trans*-18:2 retarded growth (3-6) and exacerbated EFA deficiency symptoms (6). This was reflected in depressed organ weights and lipid components. Similar changes were observed with cardiac tissue (24). The *trans,trans*-18:2 in the presence of *cis,cis*-18:2 also measurably affected growth and organ weights. The reduction in protein could adversely affect cell structure and function and the activities of various enzymes (4-6, 25,26).

The PL were slightly depressed in kidneys from rats on EFA deficient diets, particularly in diet B. The TG and CE levels were lowest in kidneys from rat receiving 100% *trans,trans*-18:2. Small differences in composition were observed between renal tissue from rats on diets C and D, indicating that dietary *trans,trans*-

TABLE I

The Variations in Body and Kidney Weights, Renal Proteins, Lipids and Lipid Classes of Rats Following 12 Weeks on Four Different Sources of Dietary Fatty Acids

Group[a]	Body weight, g	Kidney, g	Kidney/body weight (mg/g)	Components (mg/g tissue)					
				Lipids	Proteins	Phospholipids	Triglycerides	Cholesterol	Cholesteryl esters
A	308 ± 29 (12)	2.47 ± 0.28	8.0	38.87 ± 2.39	162.90 ± 2.02	20.38 ± 3.24	17.60	3.5	0.2
B	228 ± 26 (12)	2.03 ± 0.18	8.9	35.15 ± 1.99	154.70 ± 2.27	18.64 ± 1.90	15.70	2.0	0.1
C	334 ± 54 (12)	2.50 ± 0.59	7.5	40.92 ± 2.71	172.70 ± 0.61	19.48 ± 1.27	17.31	4.4	0.2
D	373 ± 26 (12)	2.52 ± 0.39	6.7	42.59 ± 1.67	216.30 ± 0.86	20.48 ± 2.06	18.54	4.4	0.4

[a]A to D denote groups of rats receiving hydrogenated coconut oil, *trans,trans*-linoleate, a 50:50 mixture of linoleate and *trans,trans*-linoleate, and linoleate, respectively. (Mean ± SD)

TABLE II

Fatty Acid Composition of Total Lipids, Cholesteryl Ester, Triglyceride and Phospholipids of Kidneys from Rats on Specific Fat Diets (Weight Percent)[a]

Fatty acids Methyl esters	Total lipids				Phospholipids				Triglycerides				Cholesteryl esters			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
12:0	1.5 ± 0.2	—	—	—	—	—	—	—	2.5 ± 0.3	—	—	—	4.4	0.8	0.8	0.4
14:0	2.0 ± 0.3	0.1 ± 0	0.1 ± 0	0.6 ± 0	—	—	—	—	3.6 ± 0.2	1.4 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	2.8	2.7	1.0	0.8
14:1	—	—	—	—	—	—	—	—	—	—	—	—	2.7	0.6	0.4	—
16:0	27.6 ± 1.9	22.2 ± 2.3	27.5 ± 1.2	27.8 ± 2.3	14.7 ± 0	15.3 ± 0.6	17.1 ± 2.7	18.2 ± 0.4	29.5 ± 1.1	28.7 ± 0.4	29.0 ± 2.8	29.7 ± 0.9	26.4	31.9	32.1	31.2
16:1	7.6 ± 0.4	7.4 ± 0.8	6.1 ± 0.7	5.4 ± 0.9	3.5 ± 0.4	6.1 ± 0.5	1.6 ± 0	1.6 ± 0.3	9.4 ± 0.5	9.1 ± 0.9	8.6 ± 0.2	7.8 ± 0.6	3.2	3.6	2.2	2.7
18:0	9.6 ± 2.1	8.9 ± 2.3	9.4 ± 0.6	11.1 ± 1.1	22.5 ± 2.0	18.1 ± 1.3	23.6 ± 0.2	23.3 ± 2.6	4.8 ± 1.0	4.9 ± 1.2	4.8 ± 0.2	6.4 ± 0.8	10.5	9.5	9.5	10.8
18:1	43.5 ± 5.2	51.6 ± 7.2	38.4 ± 0.6	25.1 ± 3.3	25.8 ± 4.3	39.1 ± 7.1	16.5 ± 2.9	14.3 ± 3.9	45.9 ± 5.1	49.0 ± 1.3	41.5 ± 0.4	32.1 ± 0.6	20.6	22.8	25.2	23.6
18:2tt	—	4.8 ± 1.3	3.4 ± 0.6	—	—	3.1 ± 0.7	1.1 ± 0.3	—	—	5.2 ± 0.4	5.1 ± 0.8	—	—	4.9	2.5	—
18:2cc	0.5 ± 0.4	1.2 ± 1.7	8.8 ± 0.1	17.3 ± 0.2	3.4 ± 1.2	4.3 ± 0.2	14.1 ± 0.6	11.6 ± 1.6	0.3 ± 0	0.1 ± 0.1	8.4 ± 0.7	19.9 ± 0.4	0.6	2.9	5.6	10.7
18:3w6	—	—	—	—	—	—	—	0.1 ± 0	—	—	0.1 ± 0.1	0.3 ± 0.1	0.2	—	0.1	0.2
20:2w6	—	—	—	—	—	—	—	—	—	—	—	—	0.3	0.3	0.1	0.1
20:3w9	4.0 ± 2.4	1.4 ± 2.0	—	—	14.2 ± 0.3	5.4 ± 3.1	—	—	1.05 ± 1.32	—	—	—	7.7	—	—	—
20:3w6	—	—	—	t	—	—	—	0.7 ± 0.3	—	—	—	—	0.1	—	0.2	0.4
20:4w6	3.6 ± 3.1	2.3 ± 3.0	6.5 ± 2.3	13.0 ± 5.2	13.9 ± 2.4	7.5 ± 1.9	24.3 ± 1.0	27.0 ± 3.9	0.6 ± 0.8	0.1 ± 0.1	0.5 ± 0.7	1.7 ± 1.1	2.8	1.4	11.0	12.9
20:5w3	—	—	—	—	0.8 ± 0.2	0.3 ± 0.4	0.1 ± 0	0.2 ± 0.2	—	—	—	—	0.1	0.3	—	0.3
22:4w6	—	—	—	—	—	—	0.6 ± 0.4	1.0 ± 0.4	—	—	—	—	0.7	0.5	0.3	0.4
22:5w6	—	—	—	—	—	—	—	1.3 ± 0.7	—	—	—	—	0.1	—	0.2	0.5
22:5w3	—	—	—	—	—	—	—	—	1.6 ± 2.2	0.3 ± 0.4	—	—	13.0	6.2	7.5	4.3
22:6w3	—	—	—	—	0.3 ± 0.3	0.1 ± 0.2	0.4 ± 0.4	—	0.5 ± 0.5	0.6 ± 0.4	0.1 ± 0	0.1 ± 0	3.8	1.5	1.5	0.8

[a]Rats were fed hydrogenated coconut oil (A), *trans,trans*-linoleate (B) a 50:50 mixture of *trans,trans*-linoleate and *cis,cis*-linoleate (C), or linoleate (D) as sole source of fat (Means of triplicate analyses ± SD).

18:2, in the presence of *cis,cis*-18:2, was exerting measureable effects. Thus, in contrast to other data (4), which revealed no difference between renal lipids of rats fed hydrogenated coconut fat or *trans* fatty acids (21% elaidic, 48% linolelaidate), the present study showed that both hydrogenated coconut fat and *trans,trans*-18:2 depressed all the lipid classes analyzed.

The fatty acid composition of the various renal lipid classes was altered by the dietary fat (Table II). Palmitoleic (16:1), oleic (18:1) and 20:3n9 were increased in all the lipid classes from rats on EFA deficient diets A and B. Significantly the increase in 18:1 was higher in B, whereas 20:3n9, which is peculiar to the EFA deficiency state, was consistently higher in group A. This was particularly pronounced in the case of the PL and CE and may reflect the possible inhibition of desaturation and elongation of 18:1 to 20:3n9 by *trans,trans*-18:2. Dietary *trans,trans*-18:2 did not accumulate to any great extent in any of the lipid classes from rats on diets B and C. No elongated products of *trans,trans*-18:2 were detected. The concentrations of *cis,cis*-18:2 in all lipid classes varied directly with dietary levels of this acid, but it was not as depleted in rats from group B compared to those fed hydrogenated coconut oil. The presence of *cis,cis*-18:2 and 20:4n6, even though in small amounts, after twelve weeks on diets A and B was notable, confirming the ability of renal tissue to conserve these essential fatty acids (15). The reduction of n6 fatty acids in kidneys from rats on hydrogenated coconut fat was consistent with other observations (6,15,27).

Eicosatrienoic acids, 20:3n6, the precursor of PGE_1, was detected only in PL of rats on diet D and in CE of rats on diets A, C and D. Arachidonic acid, 20:4n6, which was most concentrated in PL, was markedly affected by dietary fatty acids being particularly depressed in rats receiving *trans,trans*-18:2 and was also noticeably lower in PL and CE of fats from group B compared to A suggesting that *trans,trans*-18:2 impaired its synthesis. Similar effects have been observed after feeding of dietary *cis,trans*-18:2 (28).

Eicosatrienoate 20:3n9 accumulated in the lipids, particularly in PL and CE of rats on hydrogenated coconut fat, but it did not accumulate to nearly the same extent in rats on *trans,trans*-18:2 (and was absent from CE of group B) though these rats showed severe EFA deficiency symptoms indicating that the *trans,trans*-18:2 inhibited the conversion of 18:1 to 20:3n9, consistent with the higher concentrations of 18:1 in the kidneys from these rats. In this case, the 20:3n9 to 20:4n6 ratio would not validly reflect the severity of EFA deficiency indicating that this may not be used as a universal index. Incidentally, 20:3n9 may impair PG synthesis in the kidney (15).

Measurable amounts of long chain polyunsaturated fatty acids were present in the PL and CE. The n-6 fatty acids, 22:4n6 and 22:5n6, were detected in PL of rats receiving *cis,cis*-18:2n6. In EFA sufficiency, 20:4n6 rather than 18:2 accumulated in the PL and CE, whereas 18:2 increased in the TG class. The presence of 22:4n6 and 22:5n6 in PL of EFA sufficient rats may represent reserve forms of 20:4n6 which can be made from these acids by retroconversion (29). The n-3 series, i.e., 22:5n3 and 22:6n3, occurred in PL, TG and CE, being significantly concentrated in the CE of EFA deficient rats.

This study showed that renal lipid composition is altered differently in EFA deficiency induced by dietary hydrogenated coconut fat or *trans,trans*-18:2.

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