Uptake and Interconversion of Cholesterol and Cholesteryl Esters by *Phytophthora cactorum*

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ABSTRACT

When cholesterol, cholesteryl palmitate and cholesteryl acetate were added individually to sterol-free cultures of *Phytophthora cactorum*, the free sterol was at first taken up more rapidly. By 24 hr, the uptake of esters and free sterol was similar. The 2 esters apparently are taken up by different mechanisms, since much acetate was found in extracts of the mycelium at early harvests, but very little palmitate. In cultures supplemented with a mixture of cholesterol and cholesteryl palmitate, the palmitate-derived cholesterol was preferentially incorporated into the free sterol fraction of mycelial extracts. Cholesteryl palmitate and acetate were both hydrolyzed, and free cholesterol esterified by filtrates of cultures grown on sterol-free medium. Reverse-phase chromatography on hydroxyalkoxypropyl-Sephadex resolved the sterol esters of mycelial extracts into 3 zones, the most polar comprising mainly the linolenate ester, the next linoleate, and the least polar mainly oleate. Linoleate was predominant among the first sterol esters synthesized by the mycelium whether the supplement was free sterol, palmitate or acetate. Later, oleate became predominant.

INTRODUCTION

Fungi of the genera *Phythium* and *Phytophthora* do not synthesize sterols (1-3). They will grow on a defined, sterol-free medium, but such cultures remain purely vegetative.Addition of sterols brings about dramatic changes in growth and development. There is an increase in dry weight, and both asexual and sexual reproduction are induced. Both acetate and long-chain fatty acyl steryl esters can serve as sources of sterol for promoting growth and development.

The sterol taken up from the medium is recovered in extracts of the mycelium as free sterol, steryl esters and metabolites more polar than the free sterol (4-8). We showed previously (5) that cholesteryl oleate was initially taken up at a slower rate than free cholesterol, but after a few hours the rate of its uptake increased. We suggested that the ester required prior hydrolysis to free sterol before it could be taken up by the fungus. In this paper, we compare the uptake and metabolism of free cholesterol, cholesteryl palmitate and cholesteryl acetate.

In our earlier work (5), the sterols and esters in mycelial extracts were analyzed by direct-phase chromatography on columns of lipophilic Sephadex (9). In this system, in which a non-polar mobile phase of toluene or benzene is used, and in thin layer chromatography (TLC), the esters migrated as a single zone. However, when we attempted to isolate the polar metabolites by reverse-phase chromatography on lipophilic Sephadex using a polar mobile phase of acetone-water, the esters appeared in 3 separate zones (Fig. 1). We report the characterization of these zones and the changes in their occurrence with time which has provided new insight into the metabolism of these esters.

METHODS

Culture Medium

Phytophthora cactorum was grown in 100-ml conical flasks containing 10 ml of the following medium: 10.0 g sucrose, 1.0 g L-asparagine, 0.5 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2$, 1 ml trace elements solution, 1 mg thiamine hydrochloride and 1 ℓ water. The trace elements solution contained 88 mg $Na_2B_4O_7.10H_2O$, 393 mg $CuSO_4.5H_2O$, 910 mg $Fe_2(SO_4)_3.9H_2O$, 72 mg $MnCl_2.4H_2O$, 50 mg $Na_2MoO_4.2H_2O$, 4403 mg $ZnSO_4.7H_2O$ and 5 g ethylenediamine tetraacetic acid disodium salt in 1 ℓ water.

Sterols

4-[^{14}C]Cholesterol and 1,2-[^{3}H]$_2$cholesterol were obtained from the Radiochemical Centre Amersham. [^{14}C]Cholesterol palmitate and [^{14}C]cholesteryl acetate were synthesized with palmitoyl chloride and acetic anhydride, respectively, in pyridine. Excess reagent was destroyed by careful addition of methanol, and the derivatives isolated by removal of solvent and purification by TLC. The high specific activity (sp act) materials were diluted with unlabeled sterol or steryl ester to give sp act of the order of 1 μCi mg^{-1} for ^{14}C and 10 μCi mg^{-1} for ^{3}H. Sterols and esters were purified by TLC immediately before each experiment.

Sterol Uptake Experiments

Flasks were inoculated with a disc cut from

a culture growing on the medium just described solidified with agar (10 g l^{-1}). After 5-8 days' incubation at 25 C, labeled sterol or steryl ester, dissolved in 1 ml 1% aqueous Tween 80, was added to each flask. After selected periods of time, mycelium from several flasks was combined, washed with water and freeze-dried. Radioactivity in the filtrate was estimated by drying aliquots on a hot plate or in a freeze-drier and adding toluene-PPO scintillant (0.4% PPO in toluene), or by adding aliquots to toluene-Triton-PPO scintillant (0.4% PPO toluene-Triton X100, 2:1). A Packard Tricarb scintillation spectrometer was used.

The culture filtrates were extracted with chloroform and the extract analyzed by TLC on Silica Gel G; chromatograms were developed with chloroform/petroleum ether (40-60 C) (3:7).

Extraction of Mycelium and Analysis of Extracts

The freeze-dried mycelium was extracted in a Soxhlet apparatus with acetone (7 hr) followed by chloroform/methanol (2:1, 7 hr). In the earlier experiments, mycelium was extracted with acetone only, and in one experiment with chloroform/methanol (14 hr) only.

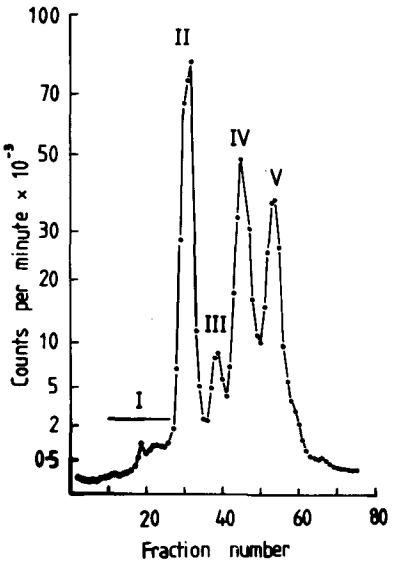

FIG. 1. Chromatogram on reverse-phase hydroxyalkoxypropyl-Sephadex of combined acetone and chloroform/methanol extracts of *P. cactorum* mycelium grown with [^{14}C] cholesterol. Sterol supplement (300 μg/culture flask of 10 ml medium) added to 7-day-old cultures; mycelium harvested 24 hr later. Zone: I, polar material; II, free cholesterol; III-V, cholesteryl esters; III, linolenate; IV, linoleate; V, oleate. The ordinate is plotted on a square root scale.

Radioactivity remaining in the extracted mycelium was estimated either by placing portions of the extracted mycelium directly into scintillation cocktail, or by their combustion in an Intertechnique IN 4101 liquid scintillation sample oxidiser.

The extracts of mycelium were evaporated using a rotary evaporator and redissolved in acetone/heptane/water (220:23:5, v/v) (3 ml) for reverse-phase chromatography on hydroxyalkoxypropyl-Sephadex LH-20/1114 (made for us by Dr. R. Anderson, Chemistry Department, University of Glasgow, by the Ellingboe et al. method [9]) (27 x 1.5 cm column, bed volume 52 ml). Eighty fractions (3 ml each) were collected. Fractions which contained esters (zones III-V, Fig. 1) were analyzed by TLC on $AgNO_3$-impregnated Silica Gel G (20% w/w, 0.5 mm thickness) using 1% diethyl ether in petroleum ether as developing solvent and overrunning in a continuous development tank (10). The fatty acid moieties of the esters were analyzed by gas liquid chromatography (GLC) of their methyl esters following transesterification with Methelute (Pierce Chemical Co.) for 2 hr and then removal of excess reagent on a water bath. Two columns were used: (a) a 1.5-m column packed with 15% w/w diethyleneglycol succinate coated on 100-120 mesh Universal Support (Phase separations Ltd.) and operated at 180 C in a Pye 104 gas chromatograph; (b) a 50-m SCOT glass capillary coated with SE-30 (Scientific Glass Engineering Pty Ltd.) operated at 200 C in a Pye GCD gas chromatograph. Peaks were identified by comparison of retention data with those for authentic compounds.

Where extracts of mycelium contained both cholesteryl acetate and free cholesterol, these chromatographed together as zone II. The proportions of free sterol and acetate were determined by TLC on silica gel, developing with chloroform/petroleum ether (3:7).

TLC zones were located by the following methods: (a) spraying with dichlorofluorescein and viewing under an ultraviolet lamp; (b) scanning with a Panax radio TLC scanner; and (c) by contact autoradiography using X-ray film. The relative amounts of the resolved substances were estimated from the areas of the peaks on the radio scans.

Hydrolytic Activity of Culture Filtrates

Filtrate (10 ml) was incubated at 25 C with 1 ml of steryl ester solution in 1% aqueous Tween 80. After various time intervals, the reaction mixture was extracted with chloroform and analyzed by TLC.

TABLE I

Growth and Sterol Uptake of Cultures of *Phytophthora cactorum* Supplemented with either Cholesterol, Cholesteryl Palmitate or Cholesteryl Acetate: Experiment 1[a]

Time after addn. of sterol (hr)	Replicate	Cholesterol			Cholesteryl palmitate			Cholesteryl acetate		
		Flasks per replicate	Mycelium wt/flask (mg)	Radioactivity in extract (%)	Flasks per replicate	Mycelium wt/flask (mg)	Radioactivity in extract (%)	Flasks per replicate	Mycelium wt/flask (mg)	Radioactivity in extract (%)
3	1	12	17.2	28.7	–	–	–	12	16.9	8.6
	2	12	20.5	–	–	–	–	12	16.8	9.8
6	1	9	20.6	40.4	12	19.4	16.4	9	19.2	17.0
	2	9	19.4	44.5	12	21.4	17.5	9	18.9	16.4
14	1	5	23.8	62.0	8	24.3	30.5	5	19.2	33.8
	2	5	26.0	58.5	8	21.9	35.6	5	17.8	31.1
24	1	4	31.3	58.0	4	34.3	55.5	4	25.5	65.9
	2	4	25.3	53.5	4	25.0	58.3	4	26.3	65.3
48	2	3	39.3	75.2	3	42.3	84.1	3	43.7	98.4
	2	3	41.3	84.4	3	43.7	86.8	4	42.5	99.2
72	1	2	50.5	59.5[b]	2	53.0	77.0	–	–	–
	2	2	53.5	69.1	2	51.0	69.1	–	–	–

[a]Cultures (8 days old) were supplemented with 100 μg [^{14}C]cholesterol (49,000 cpm) or 169 μg [^{14}C]cholesteryl palmitate (141,000 cpm) or 111 μg [^{14}C]cholesteryl acetate (261,000 cpm). Harvests made at stated intervals; mycelium from several flasks (numbers are indicated) combined to make one replicate. Mycelium extracted 14 hr with chloroform/methanol (2:1). Radioactivity in extract shown as percentage of that added.

[b]Radioactivity in 72-hr extracts less than in 48-hr extracts. In most experiments, recovery of radioactivity similarly declined with increasing age of the myeclium.

TABLE II

Growth and Sterol Uptake of Cultures of *Phytophthora cactorum* Supplemented with Cholesteryl Palmitate or with a Mixture of Cholesterol and Cholesteryl Palmitate: Experiment 2[a]

Time after addn. of sterol (hr)	Replicate	[^{3}H] Cholesterol + [^{14}C] cholesteryl palmitate				Cholesteryl palmitate		
		Flasks per replicate	Mycelium wt/flask (mg)	Radioactivity in extract (%)		Flasks per replicate	Mycelium wt/flask (mg)	Radioactivity in extract (%)
				^{3}H	^{14}C			
3	1	12	19.6	18.4	2.2	12	20.1	5.0
	2	12	19.7	18.2	1.9	12	18.2	5.1
6	1	9	20.4	26.7	11.0	12	18.8	10.6
	2	9	20.8	30.6	10.7	12	20.4	11.4
12	1	5	24.6	42.9	28.6	9	19.9	27.9
	2	5	21.0	45.6	31.3	9	23.8	27.5
24	1	4	29.5	50.9	50.5	5	24.0	52.2
	2	4	28.8	52.8	52.8	5	22.0	58.0
48	1	3	42.0	85.3	89.1	3	36.0	86.8
	2	3	38.7	88.0	92.9	3	36.3	87.4
72	1	4	51.5	88.9	91.5	4	49.5	85.0

[a]Cultures (8 days old) were supplemented with 50 μg [^{3}H]cholesterol (75,000 cpm) + 84 μg [^{14}C]cholesteryl palmitate (13,000 cpm) or with 169 μg [^{14}C]cholesteryl palmitate (37,000 cpm). Harvests made at stated intervals; mycelium from several flasks (numbers are indicated) combined to make one replicate. Mycelium extracted 7 hr with acetone and then for 7 hr with chloroform/methanol (2:1); extracts combined. Radioactivity in extract shown as percentage of that added.

RESULTS

Uptake of Cholesterol and Cholesteryl Esters

Experiment 1 (Table I) showed that when 8-day-old cultures of *P. cactorum* were supplemented with cholesterol (100 μg/flask) or with the equivalent amount of cholesteryl palmitate or acetate, cholesterol was rapidly taken up by the mycelium, but the uptake of palmitate and acetate was initially slower. By 24 hr, however, the uptake of the 3 supplements was comparable.

In experiment 2 (Table II), cultures were supplemented with a mixture of [^{3}H]cholesterol and [^{14}C]cholesteryl palmitate or with cholesteryl palmitate alone. The [^{3}H]labeled cholesterol was initially taken up from the mixture much more rapidly than the ^{14}C label of the cholesteryl palmitate, but by 24 hr the uptake of the 2 sources was again similar.

Analysis of Culture Filtrates

TLC of chloroform extracts of the culture filtrates showed that appreciable amounts of long chain fatty acyl esters were in the cultures supplemented with [^{14}C]cholesterol (Fig. 2a). When cultures were supplemented with cholesteryl palmitate, small amounts of free cholesterol were found in the filtrates. Figure 2b shows the changes with time in composition of the filtrates of cultures supplied with cholesteryl acetate.

The filtrate from cultures grown without sterol hydrolyzed steryl esters; boiled filtrate was inactive. The experiments were carried out with volumes of solution and amounts of steryl ester similar to those used in the experiments on sterol uptake. When cholesteryl palmitate was incubated with culture filtrate, free cholesterol was found in the solution after 3 hr. With cholesteryl acetate in a different experiment, free cholesterol was detectable after 40 min and long chain fatty acyl esters after 1.5 hr; by 4 hr the solution contained ca. 13% free cholesterol, 76% cholesteryl acetate and 11%

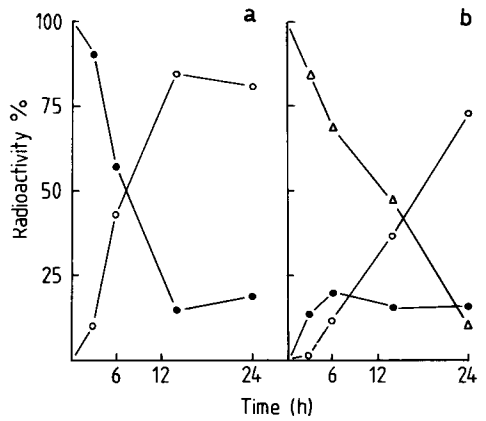

FIG. 2. Analysis of culture filtrates; experiment 1 (Table I), (a) cultures supplemented with [^{14}C]cholesterol, (b) with [^{14}C]cholesteryl acetate. The distribution percentage between fractions, free cholesterol (●—●), long chain fatty acyl esters (○--○), and cholesteryl acetate (△--△), is plotted against time after addition of supplement.

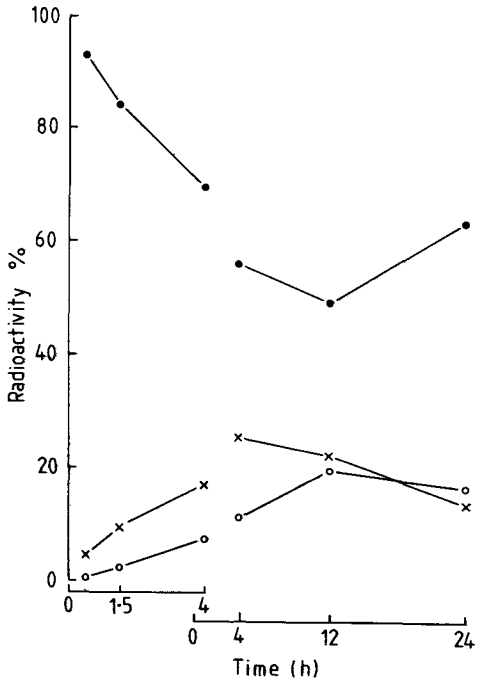

Fig. 3. Analysis of extracts of mycelium; cultures supplemented with [^{14}C] cholesterol. Two experiments: in the first (harvests 0.5, 1.5 and 4 hr after addition of supplement) each flask received ca. 150 μg cholesterol 6 days after inoculation; in the second (harvests 4, 12, 24 hr), each flask received 50 μg 5 days after inoculation. The percentage distribution of radioactivity between zones II (•--•), IV (X—X) and V (○—○) is plotted against time after supplementation.

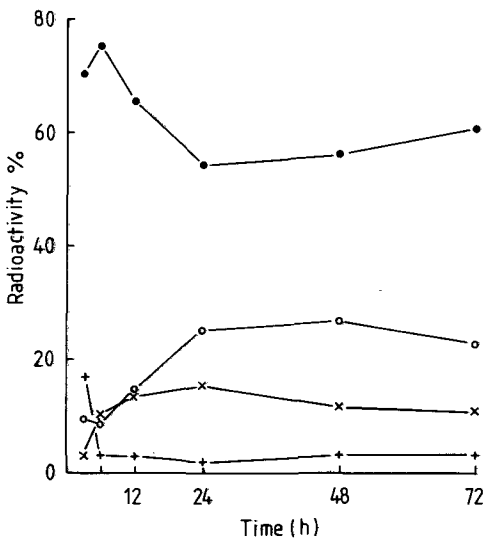

FIG. 4. Analysis of extracts of mycelium; experiment 2 (Table II), cultures supplemented with [^{14}C]-cholesteryl palmitate. The percentage distribution of radioactivity between zones I (+—+), II (•—•), IV (X—X) and V (○-—○) is plotted against time after addition of supplement. Note that at 3 hr the zone V peak was at fraction 58-59 (palmitate), at 6 hr and subsequently at fraction 52 (oleate) (cf Fig. 1).

long chain esters.

Characterization of Zones Obtained by Reverse-Phase Chromatography of Extracts of Mycelium

The zones recognized in reverse-phase chromatograms of extracts of mycelium supplemented with cholesterol are shown in Figure 1. Zone I contained compounds more polar than cholesterol, zone II free cholesterol and zones III-V were steryl esters. Using $AgNO_3$-silica gel TLC and GLC, the main component of zone III was shown to be cholesteryl octadecatrienoate, zone IV cholesteryl octadecadienoate and zone V cholesteryl octadecenoate, or cholesteryl linolenate, linoleate and oleate, respectively. Zone V also contained small amounts of material migrating to the top of the plate (no double bonds) and this was accentuated in the tail following zone V.

Changes in the Ester Zones with Time

Figure 3 shows the distribution of radioactivity between zones in extracts of mycelium supplemented with cholesterol. At the earliest harvests, zone IV (linoleate) formed the predominant steryl ester, but with the passage of time the proportion in zone IV decreased and zone V (oleate) increased.

When the cultures were supplemented with cholesteryl palmitate, a small amount of palmitate often was in the mycelial extract at the earliest harvest (3 hr, Fig. 4, zone V). Its identity as palmitate was shown by its later retention on the reverse-phase column compared to the usual zone V peak and by $AgNO_3$-silica gel chromatography of the relevant fraction. Very little oleate was present at 3 hr. Six hr after addition of the palmitate supplement, the main component of zone V was oleate. The changes with time in the relative amounts of linoleate (zone IV) and oleate (zone V) were as with cultures supplemented with cholesterol.

Analysis of extracts of mycelium that had been supplemented with an equimolar mixture of cholesterol and cholesteryl palmitate (Table III) revealed that at the 3 hr harvest virtually no steryl esters were derived from the cholesteryl palmitate; the small amount of palmitate which had been taken up was converted to free sterol and polar material. Later, esters were derived from both the free cholesterol and the palmi-

TABLE III

Analysis of Extracts of Mycelium of *P. cactorum* Supplemented with a Mixture of [^{3}H]Cholesterol plus [^{14}C]Cholesteryl Palmitate: Experiment 2

Time after addition of sterol (hr)		Relative amounts of radioactivity in zones					
		I	II	III	IV	V	III + IV + V
3	^{3}H	9.7	65.2	3.9	14.6	6.6	25.1
	^{14}C	22.2	73.0	0.6	1.6	2.5	4.7
6	^{3}H	9.7	61.8	1.6	11.6	15.4	28.6
	^{14}C	5.1	71.9	1.2	8.7	13.1	23.0
12	^{3}H	6.1	56.7	4.2	20.0	13.0	37.2
	^{14}C	3.3	67.9	3.0	14.0	11.8	28.8
24	^{3}H	6.7	51.9	4.1	17.6	19.6	41.3
	^{14}C	2.6	64.8	2.8	12.5	17.2	32.5
48	^{3}H	10.5	55.5	2.1	12.2	19.6	33.9
	^{14}H	4.9	67.9	1.4	8.7	17.1	27.2
72	^{3}H	10.5	54.9	2.5	15.7	16.5	34.7
	^{14}C	4.0	67.3	1.9	12.4	14.4	28.7

tate. However, at every harvest, the proportion of esters from cholesterol (^{3}H-labeled) was greater than that derived from cholesteryl palmitate (^{14}C-labeled); more of the ^{14}C-label ended in the free sterol fraction. There is evidently a bias in the metabolism of sterol from the 2 sources. From the uptake figures (Table II) and the distribution between chromatographic zones (Table III), we calculate the figures in Table IV. The interval 12-24 hr had the greatest disparity in the fates of the 2 labels. At this time, the uptake of palmitate was catching up with that of the free cholesterol. There was little disparity in the fates of the 2 labels between 24 and 48 hr when the rate of uptake was the same for each; however, by this time, it is unlikely that there was much ^{3}H cholesterol in the medium as free sterol: there would have been more ^{3}H sterol ester (cf. Fig. 2).

With cultures supplemented with cholesteryl acetate, a substantial amount of acetate was found in the extracts of the mycelium. As shown in Figure 5, at the 3-hr harvest nearly half the radioactive material was acetate, but the proportion of acetate gradually declined. Of the long chain fatty acyl esters, zone IV was predominant at the earliest harvest, but with time, zone V became relatively more important.

No sterol acetate was found in zone II when the cultures had been supplemented with cholesterol or cholesteryl palmitate.

DISCUSSION

The resolution of the steryl esters into 3 zones on the reverse-phase hydroxyalkoxypropyl-Sephadex column is the result of differential affinity of the mobile phase (aqueous acetone) for unsaturated fatty acid chains and of the stationary phase (heptane) for longer, saturated chains (11). This resolution has enabled us to show how changes in composition of the ester fractions occur with time. The first esters to be formed are predominantly linoleate. After this, the marked increase in amount of ester up to 24 hr is associated mainly with formation of cholesteryl oleate. The polyunsaturated esters may have different roles from the less unsaturated.

The rise in steryl ester associated with rapid sterol uptake, and the fall in the amount of ester in the mycelium associated with depletion of sterol in the medium and continuing growth of the fungus (Figs. 3 and 4; [5]) are analogous to the Aries and Kirsop observations (12) in yeast, where there is a rapid increase in sterol ester associated with aerobic sterol synthesis and a decrease in esters during the subsequent period of reduced oxygen concentration. Interconversion of steryl ester and free sterol in yeast was described by Taylor and Parks (13) and by Taketani et al. (14).

Although the rates of uptake of both palmitate and acetate are initially slower than that of free cholesterol, the mechanisms by which these 2 esters enter the cell apparently differ. Small amounts of palmitate are found in the mycelial extracts at the earliest harvests following addition of cholesteryl palmitate, but it is uncertain whether this palmitate is really within the cellular structure or merely superficial and not removed during the washing at harvest. However, the amounts of acetate in the early mycelial extracts seem far too great to be merely adherent. Acetate itself must be taken into the cells. This is possible because its

TABLE IV

Distribution into the Free Sterol and Ester Fractions of Mycelial Extracts of the Sterol Taken up at Different Times by *P. Cactorum* after Supplementation with a Mixture of [^{3}H]Cholesterol and [^{14}C]Cholesteryl Palmitate: Experiment 2

Time interval (hr)		Sterol taken up at each time interval (μg)	Distribution of sterol taken up (%)[a]	
			Free sterol	Esters
0- 3	^{3}H	9.2	65	25
	^{14}C	1.0	78	5
3- 6	^{3}H	5.2	56	35
	^{14}C	4.4	70	27
6-12	^{3}H	7.8	48	53
	^{14}C	9.6	66	32
12-24	^{3}H	3.8	24	65
	^{14}C	10.9	61	38
24-28	^{3}H	17.4	61	23
	^{14}C	19.7	72	20

[a]Calculated from the data in Tables II and III.

molecular size is not so much greater than that of cholesterol. Cholesteryl palmitate is a much larger and differently shaped molecule. It is illuminating that when cholesterol and cholesteryl palmitate are presented together to the cell, the cholesterol is preferentially converted to ester and the cholesteryl palmitate to free sterol. This must be a consequence of the way they enter the cell and implies hydrolysis of the palmitate in the entry process. *P. cactorum* evidently produces extracellular sterol hydrolase(s) constitutively, but hydrolysis in the medium may be different from hydrolysis at the cell membrane, or, for that matter, within the cell. The sterol hydrolase of yeast has a much higher affinity for oleate and palmitate than for acetate (15). Sterol acetates are not normal metabolites of *Phytophthora*. The proportion of cholesteryl acetate in the mycelial extracts decreases slowly with time, but we have not measured the rates of hydrolysis of acetate and palmitate under comparable conditions.

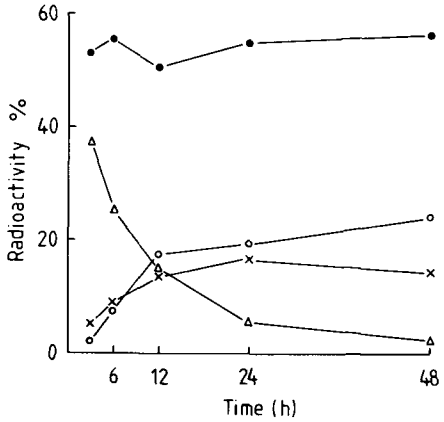

FIG. 5. Analysis of extracts of mycelium; experment 1 (Table I), cultures supplemented with [^{14}C]-cholesteryl acetate. The percentage distribution of radioactivity between free cholesterol (●-–●), acetate (△—△), zone IV (X—X) and zone V (○-–○) is plotted against time after addition of supplement. Free cholesterol and acetate elute from column together (zone II), and their proportions are determined from TLC.

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Lymphatic Absorption of Nonvolatile Oxidation Products of Heated Oils in the Rat

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ABSTRACT

The lymphatic absorption of nonvolatile oxidation products (NVOP) formed during heating of fats was studied. Heated colza or soybean oils or synthetic triglycerides containing a definite aromatic or alicyclic fatty acid were fed to thoracic duct-cannulated rats. Tritium-labeled triolein was added to each dietary fat, as an internal standard, in order to calculate the percentage of lymphatic absorption of the ingested NVOP. Results show that 4% of the total polymeric acids, 53% of the total oxidized monomeric acids and 96% of the total cyclic monomeric acids were recovered in the lymphatic lipids. Gas liquid and quantitative thin layer chromatography of these 3 classes indicated that, within a NVOP class, the various constituents did not present the same absorption rate. The lymphatic absorptions of individual oxidized monomers were between 25 and 93%. Concerning the polymer fraction, the lymphatic recoveries were 1% (nonpolar dimers), 6.8% (polar dimers) and 12% (polar oligomers). Aromatic acids were absorbed to a lesser degree (50-60%) than cyclohexenic acids (91-98%).

INTRODUCTION

The heating of oils under the conditions used for domestic frying leads to the formation of volatile breakdown derivatives and nonvolatile oxidation products (1-8). Nonvolatile oxidation products (NVOP) have triggered numerous nutritional studies. Because they accumulate in the heated oil, they are subsequently ingested with the fried foods. Furthermore, their analysis has shown the presence of potentially toxic compounds (5). However, though the toxic effects are dependent on their intestinal absorption, their uptake into the lymph has only been determined in a limited number of cases (9-12). In these instances, they were generally fed to the animals in the form of methyl esters, whereas in heated oils they are essentially present as glycerides (13). Insofar as results obtained with natural fatty acids (14) can be extrapolated to their thermal oxidation derivatives, the possibility that the lymphatic recovery of NVOP is lower when they are ingested as methyl esters rather than as glycerides cannot be excluded. As a matter of fact, the natural fatty acid methyl esters are not absorbed intact by the intestinal mucosa, even when they are fed with triglycerides to favor their micellar solubilization in the intestinal lumen (14). On the other hand, the hydrolysis of normal fatty acid methyl esters by pancreatic lipase is considerably slower than that of the corresponding triglycerides (15-17). In NVOP, the hydrolysis rate of methyl esters probably is even lower. Indeed, heated oil itself is not as rapidly hydrolyzed as the corresponding fresh oil (18).

In this study, thoracic duct-cannulated rats were fed either heated oils of a known nonvolatile oxidative product (NVOP) composition or triglycerides containing a synthetic model NVOP. The lymphatic recovery of ingested NVOP was estimated. However, an exact determination of the absorption rate must take into account the dilution of lymphatic lipids of dietary origin, such as NVOP, by those of endogenous origin (19-21). Consequently, a radioactive internal standard (glycerol tri[9,10-^{3}H] oleate), of which the intestinal absorption is complete (22), was added to each studied dietary fat. The endogenous dilution was calculated by comparison of the specific radioactivities of the total fatty acids from dietary and lymphatic lipids, respectively.

MATERIALS AND METHODS

Dietary Fats

Heated oils. Refined soybean oil was heated 14 times for 30 min at 220 C in a stainless steel commercial-type deep fat fryer. Between the different heating periods, which were separated by a one-day interval, the oil was allowed to cool to room temperature in the same receptacle. A Primor colza oil was continuously heated for 12 hr at 275 C in a nitrogen atmosphere in order to obtain a higher content in cyclic monomeric derivatives than is usually formed in household fryings. These heated oils were fed to the animals without any previous fractionation.

Synthetic triglycerides containing a cyclic monomeric acid. All syntheses were done by Dr. J. Graille and Mrs. P. Perfetti. 9-(2′-Propyl benzene) nonanoic acid was synthesized accord-

ing to Friedrich (23). Its purity (> 98%) was verified by gas liquid chromatography (GLC) and its structure confirmed by infrared (IR) spectroscopy and nuclear magnetic resonance (NMR). A mixture containing 10% of this acid and 90% of pure olive oil total fatty acids was used to esterify the glycerol directly at 130 C, under nitrogen and in the presence of *p*-toluene sulfonic acid (0.2% by weight of the fatty acids). The triglyceride obtained in this way was then purified by chromatography on a Florisil column. The GLC analysis showed that the aromatic acid was unmodified during esterification and represented 9.5% of the triglyceride total fatty acids.

9-(2′-Propyl cyclohex-4′-en-1′-yl) nonanoic acid was synthesized as previously described (24). Analysis demonstrated the presence of a mixture containing the expected cyclohex-4′-en isomer (70%) and 2 positional isomers (20 and 10%) (24). Pure olive oil total fatty acids were added to this isomer mixture. The corresponding acyl chlorides were prepared and condensed with glycerol in the presence of pyridine (25). The triglyceride was finally purified on a Florisil column.

Procedures for Lymph Cannulation and Lipid Extraction

A permanent thoracic duct cannulation was performed, as indicated by Bollman et al. (26) on male Wistar rats weighing 200-250 g. The rats were placed in restraining cages and allowed free access to a 0.9% NaCl solution and a fat-free diet of boiled rice. A few hours after the surgical operation, when a regular flow of lymph was achieved, the fat (0.7 ml of test fat + 0.53 nmol of [^{3}H]triolein, specific radioactivity = 0.414 μCi/nmol) followed by 1 ml of 0.9% NaCl solution were given to each rat by stomach tube under slight ether anesthesia. The animals were then replaced in their restraining cages, with free access to the saline solution and boiled rice. During the 48 hr following the fatty meal, the lymph was collected without fractionation in a tube maintained in an ice bath. At least 7 cannulated rats were used for each studied fat. The corresponding lymphs were combined and the lipids directly extracted using the Folch et al. (27) method. No measurable NVOP quantities were found in the discarded aqueous solutions.

Total NVOP Assay

The procedure described by Naudet and Biasini (28) was essentially followed except that, after the saponification step, an ether extraction according to the IUPAC method (29) was preferred. This was, in fact, the only method which allowed the total elimination of unsaponifiable material from the lymphatic lipids without any NVOP loss, as verified by chromatography. The total fatty acids (normal and NVOP) were separated by thin layer chromatography (TLC) on Silica Gel G (Merck) and the NVOP were directly determined on the plate, by photodensitometry after charring of compounds (28). The total NVOP were conventionally expressed in ricinoleic acid weight, by reference to a standard curve (28).

Cyclic Monomeric Acid Assay

Total fatty acids, free from unsaponifiable material, were prepared as previously described and then methylated with 4% anhydrous HCl in methanol (30). In the heated oils and corresponding lymphatic lipids, the fraction containing all of the normal and cyclic monomeric esters was isolated from the total methyl esters by alumina column chromatography (31). This fraction was then hydrogenated before GLC analysis (32). In this way, the cyclic derivatives were eluted between methyl stearate and methyl arachidate, without any overlapping with the normal fatty acid methyl esters (32). In the experiments with the 2 model cyclic acids, the total methyl esters were directly analyzed by GLC. Fractionation on alumina and hydrogenation are unnecessary in this case because of the GLC retention times of the model compounds (see Results).

The methyl esters were separated on a 10 ft. x 1/8 in. stainless steel column packed with 7% butanediol succinate (BDS) on gas chrom Q (100-120 mesh). The carrier gas (nitrogen) flow was maintained at 30 ml/min and the column temperature at 185 C. The chromatograms were quantified using a Varian model CDS 111 integrator.

Polymeric and Oxidized Acids Assay

All the methods for these assays have been published. The dietary or lymphatic triglycerides were completely hydrolyzed by the nonspecific *Candida cylindracea* lipase (33). After removal of the unsaponifiable material (29), the acids were isolated and methylated using methyl sulfate at room temperature. These conditions do not modify the NVOP structure (33). The methyl esters were then separated into 2 fractions by a reversed-phase partition chromatography on a polyethylene powder column (33). The first fraction contained esters of the oxidized monomeric acids which were quantified after TLC, charring and photodensitometry (28,34). The second fraction eluted

from the polyethylene column was subjected to TLC (30) in order to separate the polymeric acid esters from the other constituents (esters of cyclic monomeric acids and of normal fatty acids). The polymers were determined, as before, by photodensitometry after charring.

Radioactivity Measurements

Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer, using a POPOP (100 mg/ℓ)-PPO (3 g/ℓ) toluene solution as scintillation mixture.

Calculation of Recovered NVOP

The lymphatic recovery of NVOP was calculated as follows. First, from the specific radioactivities of the total fatty acids respectively isolated from the dietary (χ, CPM/g) and lymphatic (x, CPM/g) lipids, an "isotopic dilution factor" ($f = \frac{\chi}{x}$) was calculated. Because of the dilution of lymphatic lipids of dietary origin by endogenous lipids (19-21), this factor is higher than one. Secondly, from the quantitative analysis of the 2 already mentioned fatty acid fractions, the weight percentage of each studied NVOP present in the dietary (Y) and lymphatic (y) total fatty acids was calculated.

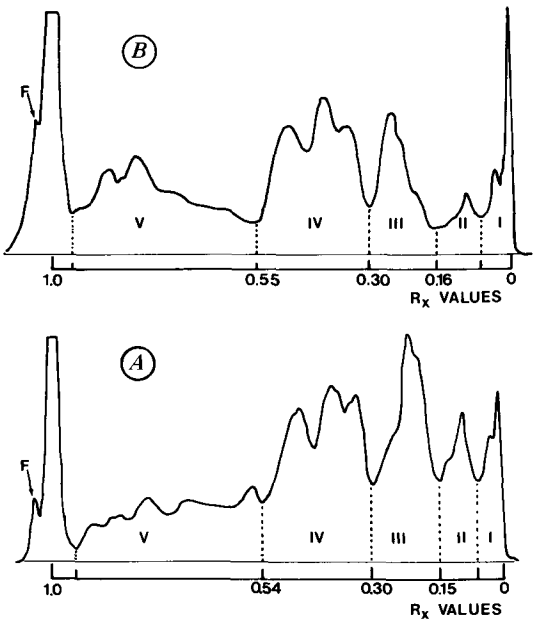

FIG. 1. Photodensitograms of the oxidized monomeric acid fraction in heated soybean oil (A) and in lymphatic lipids (B). The oxidized acids were separated by TLC as methyl esters (see Materials and Methods). R_X = distance of the oxidized ester from the starting point/distance of methyl stearate from the starting point. F - solvent front.

The term $\frac{y}{Y} \cdot 100$ corresponds to the apparent lymphatic absorption which does not take into account the endogenous dilution. The true percentage of ingested NVOP recovered in the lymph (R) was given by $R = f \cdot \frac{y}{Y} \cdot 100$.

RESULTS AND DISCUSSION

In a first experiment, 9 thoracic duct-cannulated rats were given the heated soybean oil containing tritium-labeled triolein. The lymph was collected for 48 hr in order to take into account the delay in absorption of the NVOP (10). During this short test-period, the rats never suffered diarrhea. The total fatty acids isolated from the dietary and lymphatic lipids had specific radioactivities equal to 415,000 CPM/g and 300,000 CPM/g, respectively. From these values, an isotopic dilution factor equal to 1.38 was calculated. Moreover, these 2 fatty acid fractions contained 9.8% and 2.9% total NVOP, respectively. These data were used to calculate that 41% of the total ingested NVOP were absorbed through the lymph. A value of 31% has been obtained for the same lymph collection time (10). However, in the 31% results, the total ^{14}C-labeled methyl esters from heated oil, and not the oil itself, were fed to the rats. The difference between these two results suggests that the intestinal absorption of NVOP is better when they are ingested as glycerides rather than as methyl esters. From this point of view, NVOP would behave like natural fatty acids (14).

Intestinal Absorption of Oxidized Monomeric Acids

The oxidized monomer fraction, isolated by polyethylene column chromatography, represents 4.7 and 1.8%, respectively, of the total fatty acids in the heated soybean oil and the corresponding lymphatic lipids. Taking into account the previously determined isotopic dilution factor, 1.38, it can be calculated that 53% of the oxidized monomeric dietary derivatives, considered as a whole, were recovered in the lymph within 48 hr after administration of the fat.

Given the structural diversity of the oxidized compounds likely to be present in a heated oil (5), a comparison of the composition of the oxidized acid fraction in the dietary and lymphatic lipids seemed interesting in order to see if all the constituents were equally absorbed through the lymph. Such a detailed study of this NVOP class does not seem, as yet, to have been done. Typical photodensitograms obtained after TLC of these 2 fractions are shown in Figure 1. On the whole, the 2 photodensito-

TABLE I

Relative Composition of the Dietary and Lymphatic Monomeric Oxidized Acids and Lymphatic Recovery

	Total fatty acids (%)		Total monomeric oxidized acids (%)		
	Heated soybean oil	Lymphatic lipids	Heated soybean oil	Lymphatic lipids	Lymphatic absorption[a] (%)
Total oxidized acids	4.70	1.80	100	100	53
Fraction 1[b]	0.24[c]	0.16[c]	5.0	8.7	93
Fraction 2	0.50[c]	0.09[c]	10.7	5.1	25
Fraction 3	1.15[c]	0.30[c]	23.9	16.3	36
Fraction 4	1.71[c]	0.68[c]	36.5	38.1	55
Fraction 5	1.12[c]	0.57[c]	23.9	31.8	70

[a]The isotopic dilution factor 1.38 was used to calculate these values (see Materials and Methods).
[b]The numbers correspond to the fraction numbers indicated on the photodensitograms in Fig. 1.
[c]Calculated compositions. The other compositions were obtained from chromatographic data.

grams are qualitatively similar, suggesting that the various oxidized monomers from heated soybean oil are recovered in the lymphatic lipids. However, the multiplicity of the compounds, added to their incomplete TLC separation, did not allow an accurate determination of the relative proportions of each oxidized monomer. We have, therefore, preferred to regroup these oxidized monomers into 5 groups, as shown in Figure 1, according to their R_X values and, consequently, their polarity. The respective proportions of the 5 groups were then calculated (28) from the corresponding areas determined by planimetry on the photodensitograms. No attempt was made to determine if the same charring intensity/μg of carbon was given by each of these groups. In these conditions, the oxidized monomer composition of the lymphatic lipids in Table I must be considered as relative to that of the dietary lipids. On the contrary, the calculated values of lymphatic recovery certainly were not influenced by such eventual differences on charring. Indeed, for each studied oxidized monomer group, the absorption rate was calculated from the quantities of carbon under the corresponding 2 homolog peaks in the photodensitograms of the lymphatic and dietary lipids.

From the results shown in Table I, a selection seemed to exist between the different oxidized monomers, the lymphatic absorptions of which were between 25 and 93%. Furthermore, with the exception of group I constituents, which only account for 5% of total oxidized monomeric acids in heated soybean oil, the absorption through the lymph seemed to increase with the apolarity of the oxidized derivative. A lymphatic recovery of 16% has been reported (9) for ricinoleic acid. In our TLC conditions, this hydroxy acid would correspond to a group IV compound.

Intestinal Absorption of Polymeric Acids

Figure 2 is a comparison of the photodensitograms obtained after TLC separation of the polymeric derivatives present in heated soybean oil and lymphatic lipids. The chromatographic conditions separate these polymers

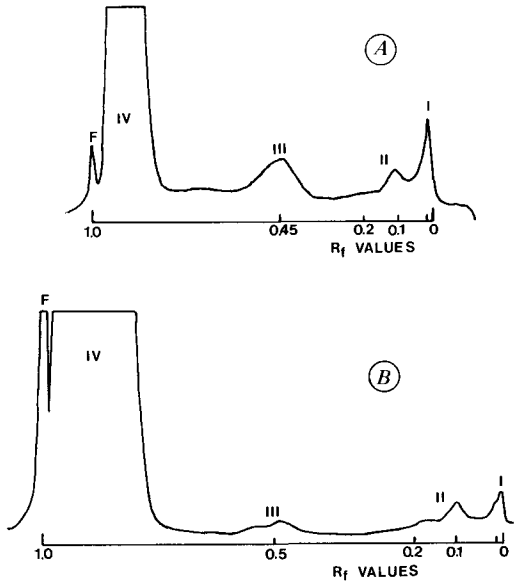

FIG. 2. Photodensitograms of the methyl esters corresponding to polymeric acids (peaks I, II, III) in heated soybean oil (A) and in lymphatic lipids (B). The band IV corresponds to normal and cyclic fatty acid esters present in the sample (see Materials and Methods). F = solvent front.

into 3 classes. Their relative proportions and lymphatic recoveries are shown in Table II. It is necessary to underscore that, to obtain satisfactory photodensitograms for quantitative determinations, the lymphatic lipid sample to be chromatographed was considerably larger than the heated oil sample in order to compensate for the low polymer content in the lymphatic lipid sample. Moreover, the consequences of possible differences in charring densities of these 3 polymer classes were the same as those previously discussed.

In the photodensitograms, peak III certainly corresponded to nonpolar dimeric compounds. First, the R_F value (0.45) was comparable to that of a model thermal dimer in the same solvent (8,30). Second, compound III represented 2% of the total fatty acids in heated soybean oil (Table II). This percentage corresponds to the average nonpolar dimer content present in fats which have been heated in conditions similar to ours (8,35). Finally, the fraction of compound III absorbed through the lymph (1%) was of the same order as that measured (0.2%) with purified nonpolar dimer (12), especially if the fact is taken into account that the purified nonpolar dimer was administered to the rats as methyl ester. The double peak II (R_F values: 0.10 and 0.20) and peak I (R_F: 0.02) in the Figure 2 photodensitograms are components previously identified in the heated oils as dimers and oligomers, both of which are polar (36).

The toxicity observed in certain nutritional studies with heated oils has, in a number of cases, been attributed to the presence of polymeric acids and, more precisely, to their polar constituents (37). Our results show that, in fact, these polar polymers are the most readily absorbed: 6.8 and 12% for polar dimers and polar oligomers, respectively, against 1% for the nonpolar dimers (Table II). On the other hand, however, this higher intestinal absorption is, to a certain degree, compensated by the fact that polar polymers are less abundant in the heated oil than their nonpolar homologs (Table II). This is in agreement with the observation that polar dimers present in a heated oil are only dangerous when the oil is fed to the rats in a sufficiently large quantity, or if the animals ingest the purified polar fraction of the dimeric acids (37). Diarrhea often was described in these cases (12,38). In our study, such a symptom was never observed during the 48-hr test period.

The Intestinal Absorption of Cyclic Monomeric Acids

The cyclic monomers generated during the heating of oils have also been cited as potentially toxic compounds (37). The only available information on their lymphatic absorption shows that 61% of a labeled cyclohexadienyl methyl ester ingested by rats are recovered into the lymph (11). This study compares the absorption of the total cyclic monomers contained in a heated oil and the absorption of 2 model acids, one aromatic, the other cyclohexenic. The results are shown in Table III.

When rats were given heated Primor colza oil, 96% of the total cyclic monomeric acids were absorbed through the lymph. Under our GLC conditions, the cyclic derivatives in the heated oil eluted as 4 major peaks, in agreement with previously published results (32). The absorption percentages calculated for each of these 4 fractions were between 93 and 98%. These particularly high values are in no way

TABLE II

Relative Composition of the Dietary and Lymphatic Polymeric Fatty Acids and Lymphatic Absorption of the Various Polymers

	Total fatty acids (%)		Total polymers (%)		
	Heated soybean oil	Lymphatic lipids	Heated soybean oil	Lymphatic lipids	Lymphatic absorption[a] (%)
Total polymers	4.60	0.14	100	100	4.2
Fraction 1[b] (polar oligomers)	0.46[c]	0.04[c]	10	29	12.0
Fraction 2[b] (polar dimers)	1.60[c]	0.08[c]	35	57	6.8
Fraction 3[b] (nonpolar dimers)	2.54[c]	0.02[c]	55	14	1.0

[a]Calculated using the isotopic dilution factor 1.38 (see Materials and Methods).

[b]The fraction numbers correspond to those indicated on the photodensitograms in Fig. 2.

[c]Calculated compositions. The other compositions were obtained from chromatographic data.

TABLE III

Lymphatic Recovery of Various Dietary Cyclic Monomeric Fatty Acids

Experiment number	Dietary lipid[a]	GLC retention ratio[b]	Total fatty acids (%)		Lymphatic absorption (%)[c]
			Dietary lipids	Lymphatic lipids	
II (9 rats)	Total cyclic monomers	1.06-1.35	3.1	2.0	96
	Component 1	1.06	0.22	0.14	95
	Component 2	1.12	0.64	0.42	98
	Component 3	1.24	1.06	0.69	97
	Component 4	1.35	1.17	0.73	93
III (7 rats)	9-(2′-propylbenzene) nonanoic acid	3.47	0.50	0.18	51
IV (7 rats)	9-(2′-propylbenzene) nonanoic acid	3.47	9.50	3.30	60
V (7 rats)	9-(2′-propyl cyclohexen-1′-yl) nonanoic acid	1.60[d]	0.24	0.14	96
		1.72[d]	1.10	0.61	91
		1.81[d]	2.70	1.60	98
VI (7 rats)	9-(2′-propyl cyclohexen-1′-yl) nonanoic acid	1.81	0.22	0.13	95

[a]Heated Primor colza oil (experiment II) or synthetic triglycerides (experiments III-VI) were fed to the rats. Within each experiment, the lymphs obtained from the rats fed the same fat were combined.

[b]Retention time of component retention:time of methyl stearate; GLC were performed after (exp. II) or without (exp. III-VI) hydrogenation of methyl esters (see Materials and Methods).

[c]Calculated using the following isotopic dilution factors: 1.50 (exp. II), 1.43 (exp. III), 1.74 (exp. IV), 1.65 (exp. V) and 1.61 (exp. VI) (see Materials and Methods).

[d]Respective retention ratios of the 3 cyclohexenyl isomers (see Materials and Methods).

related to the fact that heated colza oil contained large quantities of cyclic monomeric acids (3% of the total fatty acids). As a matter of fact, an identical absorption was measured for 9-(2′-propyl cyclohex-4′-en-1′-yl) nonanoic acid whether it represented 0.3 or 4% of the triglyceride total fatty acids fed to the rats. This result is important insofar as this synthetic acid is an excellent model for alicyclic structures which compose the majority of the cyclic monomeric fraction of heated oils (5). The aromatic acids are absorbed to a lesser degree, judging from the results obtained with the disubstituted benzene model.

In the course of this study, the NVOP lymphatic recoveries were calculated considering that the absorption of [^{3}H] triolein, known to be complete (22), was not reduced by the presence of NVOP in the intestinal lumen. This certainly was true, as suggested by the fact that isotopic dilution factors comparable to our own values may be calculated from experiments in which cannulated rats were fed only triolein (39). Moreover, it must be remembered that in the present work, the NVOP quantities ingested by the animals were low (< 10% of total dietary fatty acids) compared to those of normal and well absorbed fatty acids also present in the dietary heated oils or synthetic triglycerides. Finally, the lymph was collected over 48 hr, whereas absorption of oleic acid was achieved within 24 hr (21). All these conditions are favorable to a complete recovery of the added [^{3}H] triolein.

The results obtained during this study suggested a discrimination exists between the various NVOP at the intestinal mucosa level. This differential absorption could result from several factors. The triglyceride hydrolysis in the intestinal lumen is a prerequisite step in the absorption of fatty acids, either free or as monoglycerides. The pancreatic lipase activity on triglycerides is known to be influenced by the structure of the fatty acids and by their position on the triglyceride skeleton (40). Among the NVOP esterified in the 1- or 3-position of the *sn*-glycerol, it seems likely that the preferential hydrolysis of certain of these compounds could favor their transfer into the lymph as free fatty acids. In this connection, it is not inconceivable that all the dietary NVOP may be recovered in the lymph as monoglycerides, independently of their structure, if they were in the 2-position of ingested triglycerides, provided that pancreatic lipase hydrolyzes the fatty acids in 1- and 3-positions. The distribution of the NVOP on the *sn*-glycerol was, however, unknown in the heated oils and synthetic glycerides used in this work. From another point of view, because of their large structural diversity, certain NVOP, or their corresponding monoglycerides, could be more

soluble than others in bile salt micelles (41,42), thus favoring their intestinal absorption (41). The observed differences in the NVOP lymphatic recovery as a function of their polarity therefore could be significant. Of course, these are hypotheses for which validity studies must be done.

ACKNOWLEDGMENTS

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Competitive Deposition of *trans*-12- and *cis*-9-Octadecenoates into Egg Yolk Lipids

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ABSTRACT

The deposition of *trans*-12-octadecenoate-12(13)-^{3}H (12*t*-18:1-^{3}H) was compared to *cis*-9-octadecenoate-10-^{14}C (9*c*-18:1-^{14}C) in the major egg yolk neutral lipids and phospholipids. *trans*-12-Octadecenoate was preferentially incorporated into cholesteryl esters (CE), phosphatidylcholines (PC), and phosphatidylethanolamines (PE) but was discriminated against in triglycerides (TG). Isotopic ratios indicate that 5.9 and 5.6 times more 12*t*-18:1-^{3}H than 9*c*-18:1-^{14}C was esterified at the 1-acyl position of PE and PC, respectively. The combined 1- and 3-acyl positions of TG and the 2-acyl position of TG, PE and PC were each preferentially esterified with 9*c*-18:1-^{14}C.

INTRODUCTION

Partially hydrogenated fats used in commercially available margarines, cooking oils and shortenings contain both geometric and positional isomers of the *cis*-unsaturated fatty acids normally found in vegetable oils (1-4). Despite the fact that the American public consumes over 6 billion pounds of partially hydrogenated vegetable oil per year (5), our knowledge of the metabolic fate of the positional fatty acid isomers is not extensive. Most research has focused on the effects of hydrogenated fats and oils as a whole, with little attention given to individual isomeric fatty acids contained in processed oils.

Recently, the distribution of positional *cis*- and *trans*-octadecenoate isomers was determined in several lipid classes from rats fed partially hydrogenated fat (6). In vitro studies have examined the use of individual *cis*-positional octadecenoate isomers from Δ3 to Δ13 in rat liver mitochondria (7) and in bacteria and yeast cells (8). The metabolism of a series of *cis*-octadecenoic acids has been compared to *cis*-9-octadecenoic acid (oleic) in the laying hen (9).

trans Fatty acids comprise up to 47% (2,3,10) of the monoenoic fat content in products containing processed vegetable oils. Although *trans*-12-octadecenoic acid accounts for only 4-9% of the octadecenoic acids in some margarines, this represents 12-20% of the *trans* acid content of these products (2,10).

This study compares the metabolism of *trans*-12- and *cis*-9-octadecenoic acids (12*t*- and 9*c*-18:1) in the laying hen by examining the deposition of these fatty acids into the major egg yolk lipids.

MATERIALS AND METHODS

Oleic acid-10-^{14}C, specific activity of 41.5 Ci/mol, was purchased from Schwarz-Mann (Orangeburg, NY). The methyl ester was prepared by refluxing the labeled acid with 5% HCl-methanol (11). Previously prepared 12-octadecynoic acid (12) was methylated with HCl-methanol and reduced using tritiated water and Lindlar catalyst (13). *cis*-12-Octadecenoate-12(13)-^{3}H was isomerized to the *trans*-12-isomer with nitrous acid catalyst (14). The labeled esters were purified by preparative argentation thin layer chromatography (TLC) (Brinkmann 20 × 20 × 2 mm plates, Silica Gel 60 dipped in 20% $AgNO_3$ in ethanol; developing solvent was 20% petroleum ether in benzene). The double bond position in the esters was confirmed by ozonolysis (15). Specific activities of the 9*c*-18:1-^{14}C and 12*t*-18:1-^{3}H were 33.8 mCi/mmol and 10.5 mCi/mmol, respectively.

Radiochemical purity of the 9*c*- and 12*t*-octadecenoates was determined to be greater than 98% by radiochromatogram scan (Packard 7201 Radiochromatogram Scanner) after argentation-TLC and by radioisotope gas liquid chromatography (GLC) (Aerograph gas chromatograph; 6 ft. × 1/4 in. aluminum column packed with 15% EGSS-X in conjunction with a Cary 5010 ion chamber).

Three hens were fed ad libitum a standard laying ration (Purina CF 6501, Ralston Purina, St. Louis, MO) which contained 2.6% hexane extractable fat. Fatty acid composition of the feed was 14:0, 0.6%; 16:0, 17.2%; 16:1, 0.8%; 18:0, 5.7%; 18:1, 27.9%; 18:2, 44.5%; 18:3, 2.9%. No detectable *trans* acids (<0.1%) were found.

Methods used for administration of radioactive methyl esters to 3 laying hens, for collection of eggs after feeding and for lipid extraction have been described previously (16). Lipid analyses were performed on individual egg yolks collected from each hen during peak incorporation of the radioactive labels (fourth

and fifth eggs after feeding labeled esters) and also on combined yolk samples from the 3 hens both before (third egg) and after (sixth egg) peak incorporation. Neutral lipid components (cholesteryl esters and triglycerides) were isolated by preparative TLC (prep-TLC) using benzene/petroleum ether/ethyl ether/acetic acid (8:2:1:0.25); major phospholipids (phosphatidylethanolamine and phosphatidylcholine) were isolated with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1).

Enzymatic hydrolysis of egg yolk triglycerides was done with pancreatic lipase (Calbiochem, San Diego, CA) (17). Hydrolysis products were separated by prep-TLC using ethyl ether/petroleum ether/acetic acid (1:1:0.02). Phospholipase A_2 (*O. Hannah*, Ross Allen Reptile Inst., Inc., Silver Springs, FL) was used for fatty acid positional analysis of phospholipids (18). Products were isolated by prep-TLC using the phospholipid solvent system just described.

Radiochemical assays were made using a Beckman 3-channel LS-250 liquid scintillation counter with previously described parameters (16). Output from the counter was interfaced to a Mod-Comp computer (Modular Computer System, Inc., Ft. Lauderdale, FL) programmed to calculate disintegrations/min (dpm) and the $^3H/^{14}C$ ratios. All dpm were corrected by external standard ratios and previously established quench correction curves.

RESULTS

Selectivity values were calculated as the logarithm of the quotient of the $^3H/^{14}C$ ratio found in the yolk lipids divided by the $^3H/^{14}C$ ratio in the feed mixture. These values (Table I) in the major yolk lipid components decreased in the order phosphatidylethanolamine (PE), phosphatidylcholine (PC), cholesteryl ester (CE), and triglyceride (TG), indicating that 12*t*-18:1-^3H was incorporated most preferentially into PE and discriminated against only in TG when compared to 9*c*-18:1-^{14}C. Enzymatic hydrolysis of PE and PC showed that 12*t*-18:1 was selectively esterified at the 1-acyl position and that it was discriminated against at the 2-position. *cis*-9-Octadecenoate-^{14}C was concentrated at the 2-position of yolk TG, whereas only a slight preference for the *cis* isomer was seen in the 1- and 3-positions.

DISCUSSION

Competitive deposition of 12*t*-18:1-^3H into yolk lipids relative to 9*c*-18:1-^{14}C is plotted as selectivities in Figure 1. Similar values determined after feeding 12*c*-18:1 to laying hens (9) and both 12-18:1 isomers to a human subject (19) are also plotted for comparison. Because 9*c*-18:1 is common to this and previous dual-isotopic-labeled feeding studies of isomers with laying hens, it serves as an internal standard and should permit direct comparison with previously published data (9).

Triglycerides

Triglycerides were the only lipid fraction to incorporate more 9*c*-18:1-^{14}C than 12*t*-18:1-^3H. This selection for the 9*c*- rather than the 12*t*-isomer seen in egg yolk triglyceride also

TABLE I

Radiochemical Analysis of Egg Yolk Lipids after Feeding 12*t*-18:1-^3H and 9*c*-18:1-^{14}C ($^3H/^{14}C$ = 0.82)

Lipid component[a]	Lipid acyl positions	Specific activity[b]	$^3H/^{14}C$ in yolk lipids	Selectivity
CE		2,041	1.53 ± 0.16 (6)[c]	0.27
TG		4,567	0.64 ± 0.02 (7)	-0.11
	TG 1+3	4,801	0.71 ± 0.02 (6)	-0.06
	TG 2	4,951	0.50 ± 0.02 (6)	-0.21
PE		5,179	3.97 ± 0.17 (6)	0.68
	PE 1	7,301	5.98 ± 0.35 (6)	0.86
	PE 2	2,586	0.50 ± 0.05 (6)	-0.21
PC		5,294	2.15 ± 0.12 (7)	0.42
	PC 1	7,032	5.64 ± 0.23 (7)	0.84
	PC 2	4,284	0.30 ± 0.03 (7)	-0.43

[a]Abbreviations: CE, cholesteryl esters; TG, triglycerides; PE, phosphatidylethanolamines; PC, phosphatidylcholines.

[b]Total ^3H + ^{14}C dpm/mg.

[c]Average ± SE. The number of egg yolk samples analyzed is given in parentheses and includes at least one egg yolk from each hen collected during peak incorporation of radioactivity.

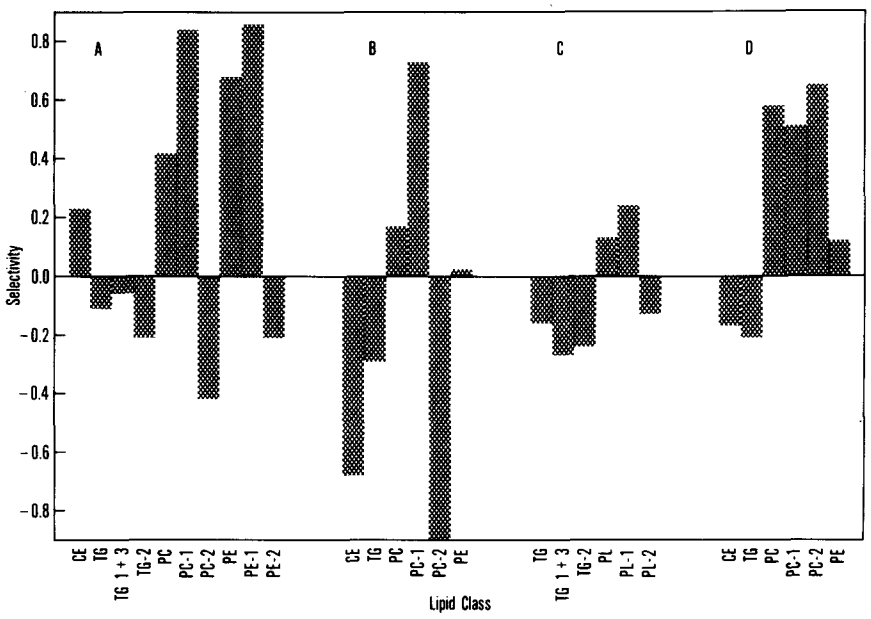

FIG. 1. Relative incorporation of *trans*-12- and *cis*-12-octadecenoates vs *cis*-9-octadecenoate into egg yolk lipids (A, 12*t*/9*c*; C, 12*c*/9*c* [ref. 9]) and human plasma lipids (B, 12*t*/9*c* [ref. 19]); D, 12*c*/9*c* [ref. 19]). Abbreviations: see Table I; PL, phospholipid.

occurs in chicken liver, rat plasma and human plasma. Bickerstaffe and Annison (20) have shown that *cis* acids are more readily incorporated into triglycerides than phospholipids in perfused chicken liver. Rats fed partially hydrogenated safflower oil fatty acids (PHSOFA) had a *cis*/*trans* ratio of 2:1 in plasma triglycerides even though the dietary octadecenoate content was 2/3 *trans* (6). Human plasma triglycerides had a selectivity of -0.29 when 12*t*-18:1 was fed with 9*c*-18:1 (19).

The small negative selectivity value determined for yolk TG-1+3 indicates almost equal amounts of 9*c*-18:1-^{14}C and 12*t*-18:1-^{3}H at these positions, whereas incorporation at the 2-position is more selective for the 9*c*-18:1. These values agree with previous acyl positional analyses of triglycerides which show that, although 89% of the *cis*-9-octadecenoic acid in yolk triglyceride is almost equally esterified at positions 2 and 3 (21), *trans* octadecenoates are preferentially esterified at the 1- and 3-positions and discriminated against at position 2 (20,22-24). This preference for 9*c*-18:1 in triglycerides appears to be not only a geometric selectivity, but also a positional one. Data given for the 12*c*-18:1 feeding to laying hens (9) also yielded negative selectivities for egg yolk TG (-0.21) and its acyl positions (TG-1+3, -0.27; TG-2, -0.24). Human plasma triglycerides (19) show the same preference for 9*c*-18:1 over the 12*c*-18:1 (-0.17). Either enzymatic selection is widespread for an 18-carbon fatty acid with a *cis* double bond at the 9-position for use in triglycerides, or the geometrical and positional isomers are selectively channeled to the phospholipids.

Phospholipids

Large preferential incorporation of 12*t*-18:1-^{3}H occurred in both major phospholipid components (PE and PC). This generally agrees with studies in which PHSOFA-fed rats had considerably more 12*t*-18:1 in liver and plasma phospholipids relative to the quantity in the diet (6). *trans* Fatty acids resemble saturated, rather than *cis*, acids in their incorporation into lipids as evidenced by the selective incorporation of 9*t*- and 11*t*-18:1 at PE-1 and PC-1 in perfused chicken liver (20). Data from hydrolyses of egg yolk phospholipids showed large positive selectivities for the 1-acyl position of both PE (0.86) and PC (0.84), indicating marked exclusion of 9*c*-18:1. The similarity of these 1-acyl selectivity values in yolk PE and PC supports the theory that these fatty acids may be derived from the same pool or compartment (25).

Selectivity values determined for the 2-positions of yolk phospholipids, on the other hand, were negative, although they did not indicate as much discrimination against 12*t*-

18:1-^{3}H as the 1-position did for 9*c*-18:1-^{14}C. Data reported for human plasma PC-1 and PC-2 (0.73 and -0.90, respectively) show selective positioning of 12*t*-18:1 similar to that found here, but much more exclusion of this *trans* isomer from the 2-position (19). Calculated selectivities for 12*c*-18:1 in total yolk phospholipid (0.13) and in the 1-acyl (0.34) and 2-acyl (-0.13) positions were qualitatively the same as in the 12*t*-18:1 feeding (9), but were not as pronounced. Obviously, enzymes responsible for esterifying fatty acids to the phospholipid are sensitive to structural and/or spatial differences in the fatty acids. Acylation rates of *cis* and *trans* octadecenoates with 2-acyl- and 1-acylglycerophospholipids using rat liver microsomal acyl-CoA:phospholipid acyltransferase have led to the suggestion that transfer to the 1-position is quite sensitive to configurational differences in fatty acids, whereas transfer to the 2-position is not (26,27). This may explain why the PE-2 and PC-2 data do not display as large a selectivity as the 1-position in yolk phospholipids, but it does not agree with human plasma PC-2 data that shows almost total exclusion of 12*t*-18:1. Differences must exist between human and hen enzyme systems that allow 12*t*-18:1 to be placed in the 2-position of yolk phospholipid.

Cholesteryl Esters

Selectivity for *cis* fatty acids has been demonstrated in cholesteryl esters (CE) from rat liver microsomes (28), human blood lipids (19,29) and egg yolk lipids (30). When Sgoutas et al. (7) incubated 9*t*-18:1-^{3}H and 9*c*-18:1-^{14}C with rat liver microsomes, the ^{3}H/^{14}C ratio dropped from 3.92 in the incubated mixture to 1.22 in the recovered CE. Previously, acyl CoA: cholesterol-*O*-acyltransferase from rat liver microsomes was shown to esterify 9*c*-18:1 to cholesterol almost 3 times faster than 9*t*-18:1 (28). Human plasma CE had selectivities of -0.74 and -0.68, respectively, for 9*t*- and 12*t*-18:1 when compared to 9*c*-18:1 (19,29).

It was unexpected, but interesting, that more 12*t*-18:1-^{3}H than 9*c*-18:1-^{14}C was incorporated into yolk CE (selectivity, 0.27). Perhaps lecithin:cholesterol acyltransferase selectively removes the 12*t*-18:1 from PC-2 to esterify free cholesterol. This also would account for the more negative selectivity in PC-2 than in PE-2.

Although hen plasma contains significant amounts of CE in low density and high density lipoprotein (31,32), mechanisms exist that allow deposition of large amounts of free cholesterol, but only small amounts of CE, in the yolk. If the esterified fatty acid is hydrolyzed during deposition, sterol-ester hydrolase selectivity would play a role in determining the fatty acid composition of the CE in the yolk. This enzyme is reported to hydrolyze *cis*-unsaturated cholesteryl esters, especially cholesteryl oleate, to a greater extent than *trans* fatty acid cholesteryl esters (33,34), which would result in a positive selectivity value for yolk CE.

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Lipids of Dermatophytes

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ABSTRACT

This investigation deals with phosphatides and fatty acid content of *Epidermophyton floccosum*, *Microsporum cookie* and *Trichophyton mentagrophytes* during different phases of growth. Total phosphatide content of these dermatophytes decreased with age, which was reflected in constituent major phosphatides. The zwitterionic and anionic phospholipids tended to maintain a constant ratio. Short chain fatty acids increased significantly with age in *E. floccosum* whereas these fatty acids represented a minor fraction of the total fatty acids in *M. cookie* and *T. mentagrophytes*. The ratio of saturated to unsaturated fatty acids increased 4-fold during growth in *E. floccosum*, whereas this increase was marginal in *M. cookie*. This ratio decreased in *T. mentagrophytes*.

INTRODUCTION

The principal phosphatides of dermatophytes have been reported to be phosphatidylcholine (PC), phosphatidylethanolamine, (PE) and phosphatidylserine (PS), whereas triacylglycerol (TG) and free fatty acids (FFA) form the major fraction of neutral lipids (1-6). The major fatty acids of these pathogenic fungi have been reported to be palmitate (16:0), stearate (18:0), and linoleate (18:2) (3,4). Lipids of pathogenic fungi have been suggested to have a role in the process of dermatomycosis (4) and their fatty acids are reported to be allergens (7,8). Therefore, 3 dermatophytes, *Epidermophyton floccosum*, *Microsporum cookie* and *Trichophyton mentagrophytes*, representative of each dermatophyte genera, were selected for a comparative study of their phospholipid and fatty acid pattern during different phases of growth.

MATERIALS AND METHODS

The source of *E. floccosum* was as described earlier (3), whereas *M. cookie* and *T. mentagrophytes* were obtained from the Public Health Laboratory Service, Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, London. These cultures were maintained, grown and harvested as described earlier (9). Growth curves of these fungi were monitored by plotting their dry weights against the number of days. After harvesting, the cells were blotted dry, lyophilized and weighed. Lyophilized mycelium was stirred twice for 6 hr in 20 vol of chloroform/methanol mixture (2:1, v/v). The homogenates were stirred and then filtered. The 2 filtrates were combined and washed as described by Folch et al. (10).

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Phosphatides were analyzed and quantitated as we have described (2). Phospholipids were separated from total lipids either by acetone precipitation or by silicic acid column chromatography and their methyl esters were prepared by Prabhudesais' method (11), briefly outlined next. The phospholipid (100 μg of lipid-P) of each sample was dissolved in 5 ml of chloroform/methanol mixture (3:7, v/v). The tubes were placed in ice and 0.2-0.3 ml of thionyl chloride was added with constant shaking. After complete mixing, the tubes were placed at room temperature for 1 hr to achieve complete conversion. To separate 2 layers, 1 ml of water was added. The upper phase was discarded and the lower chloroform layer was washed 3-4 times with water, to remove any traces of thionyl chloride. The chloroform extract was then dried under vacuum or a stream of nitrogen at a low temperature. Fatty acid methyl esters were resolved in a Pye Unichem 104 Gas Liquid Chromatogram with a 10% diethylene glycol succinate (DEGS) (on 100-200 mesh Diatomite C-AW) column at 180 C. Nitrogen was used as the carrier gas with a flow rate of 40 ml/min. Fatty acids were identified by comparing their retention times with those of standards. Amounts of fatty acids were calculated by triangulation.

All organic solvents were of the highest purity available or distilled before use. Standard fatty acid methyl esters were obtained from Applied Science Laboratories Inc., State College, PA.

RESULTS AND DISCUSSION

Variation of phospholipid composition during different growth phases of *E. floccosum*, *M. cookie* and *T. mentagrophytes* was investigated. The dermatophytes were harvested in early log, midlog, early stationary and late stationary phases of growth for phospholipid

TABLE I

Individual Phospholipid Contents during Growth Phases of Dermatophytes[a]

Phospholipid fraction (mg/g dry wt)	*E. floccosum*				*M. cookie*				*T. mentagrophytes*			
	Growth phases											
	EL (10)[c]	ML (20)	ES (30)	S (40)	EL (5)	ML (15)	ES (25)	S (50)	EL[b] (15)	ML (30)	ES (45)	S (60)
TPL	13.90[d]	8.20	6.60	2.80	15.90	14.30	9.80	7.80	28.60	35.90	16.20	14.40
LPC	0.90	0.50	0.66	0.24	1.10	1.03	0.50	1.80	2.87	3.80	2.10	2.30
PC	5.80	3.70	2.85	1.12	8.03	6.23	5.20	3.20	10.01	13.90	5.60	6.90
PS	2.20	1.20	0.93	0.32	1.20	1.20	0.90	0.50	2.70	3.50	2.10	1.50
PI	0.40	0.35	0.24	0.07	0.40	0.89	–	0.20	1.70	0.43	0.20	0.30
PE	2.98	1.25	0.96	0.34	1.30	2.07	1.40	1.20	5.50	5.20	3.20	2.50
CL	0.45	0.40	0.34	0.21	0.81	0.91	0.40	0.35	1.60	0.95	0.24	0.28
UK[e]	1.37	0.80	0.73	0.47	3.20	1.93	1.50	1.00	14.00	8.20	1.70	1.50

[a]Abbreviations: TPL, total phospholipids; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CL, cardiolipin; UK, unknown components; EL, early logarithamic phase; ML, midlogarithamic phase, ES, early stationary phase; S, stationary phase.

[b]Average of one batch, analyzed in duplicate.

[c]Figures in parenthese represent the number of days of growth.

[d]All values are means of 3 independent batches, analyzed in duplicate.

[e]Constituted by 2-3 different components.

analysis. Total phospholipids decreased in *E. floccosum* and *M. cookie* throughout the growth cycle, similar to *M. gypseum* (9) and *T. rubrum* (1), whereas in *T. mentagrophytes*, this trend was noted only after the midlog phase (Table I).

Upon examining individual phosphatides (Table I) of these fungi during different growth phases, a pattern similar to total phospholipids was observed. The major phosphatides, PC, PS and PE, decreased during growth of *E. floccosum* and *M. cookie*, whereas in *T. mentagrophytes*, although PE content decreased with age, PC and PS levels decreased after midlog phase. However, PS and PE content remained constant in *M. gypseum* (9), whereas in *T. rubrum* (1), PE exhibited a decrease and PC and PS maintained constant levels with aging. Apparently, *E. floccosum*, *M. cookie* and *T. mentagrophytes* tended to maintain a constant ratio between the fully neutral (choline-containing phosphatides) and the slightly anionic (PE) plus strongly anionic (PS, PI and CL) phosphatides. The ratio either increases, as in *E. floccosum*, or decreases, as in *M. cookie* and *T. mentagrophytes*, during the midlog and stationary phases. Nevertheless, in the stationary phase, it comes to the initial value as of the early log phase. Maintenance of such a ratio between zwitterionic and anionic phospholipids was also reported in *Neurospora crassa* (12). An internal compensatory mechanism presumably is operative in all these fungi to maintain constant polarity on the membranes in order to keep their functional properties intact.

Variations in fatty acids of dermatophytes with age have been investigated very little (13). Table II presents the variation in fatty acid composition of phosphatides in these dermatophytes with increasing age. A notable feature of the fatty acid composition is the abundant presence (20% of the total) of short chain fatty acids (10:0, 12:0 and 14:0) in *E. floccosum*, whereas in *M. cookie* and *T. mentagrophytes*, they are present in minor and negligible amounts, respectively. During early log phase, the predominant long chain fatty acids are 16:0 and 18:2 in *E. floccosum* and *M. cookie*, but in *T. mentagrophytes*, in addition to these, 18:0 also was identified. The amount of 18:0 in *T. mentagrophytes* was 3- to 4-fold more than in *E. floccosum* or *M. cookie*. Striking alterations in long chain fatty acids were evident between early log and stationary phases only in *E. floccosum*. Palmitate and linoleate levels of *E. floccosum* decreased with concomitant increase of short chain fatty acids; however, the decrease was

TABLE II

Fatty Acid Composition of Phosphatides during Growth Phases of Dermatophytes[a]

Chain length	*E. floccosum*				*M. cookie*				*T. mentagrophytes*			
	Growth phases											
	EL (10)	ML (20)	ES (30)	S (40)	EL (5)	ML (15)	ES (25)	S (50)	EL (15)	ML (30)	ES (45)	S (60)
	(Percent of total)											
10:0	11.95[b]	8.13	18.23	39.28	t	t	3.05	4.44	t	t	t	t
12:0	4.76	3.12	6.18	22.38	t	t	0.31	–	t	t	t	t
14:0	3.16	0.42	0.86	3.37	t	1.03	0.59	0.63	t	t	t	t
16:0	26.47	22.85	24.86	13.55	27.29	28.85	25.53	32.58	34.73	32.79	31.00	27.53
16:1	2.03	1.56	0.82	1.56	t	1.38	1.64	0.87	1.89	6.78	3.15	1.44
16:2[c]	1.10	1.05	0.39	4.79	2.16	1.49	2.07	1.99	1.72	–	1.01	0.92
18:0	4.99	5.74	3.90	1.43	6.52	7.11	6.27	4.19	21.67	8.40	11.76	2.99
18:1	2.08	5.18	4.63	3.19	7.89	11.87	6.16	3.95	3.13	3.89	3.75	2.99
18:2	47.02	46.86	40.01	11.23	55.89	50.02	55.51	53.80	36.89	51.77	51.04	53.73
S/U	1.03	0.67	1.18	4.00	0.51	0.58	0.56	0.72	1.23	0.96	0.75	0.69

[a]Abbreviations: t, trace amounts; S, saturated fatty acids; U, unsaturated fatty acids. All other abbreviations are as given in Table I.

[b]All values are mean of 3 independent batches, analyzed in duplicate.

[c]Tentative identification.

almost 4-fold for linoleate, whereas the level of palmitate decreased by one-half of the initial value. A 4-fold increase in the ratio of saturated to unsaturated fatty acids was observed in *E. floccosum* when early log phase was compared to stationary phase. The increase was only 0.5-fold for *M. cookie*, but it decreased to one-half of its initial value in *T. mentagrophytes*. Except for *T. mentagrophytes* fungus, these observations are similar to those reported for bacteria (14). All the changes observed in phospholipid fatty acids are possible reflections of the organism's response to maintain membrane functions during different growth phases. The response is, however, highest in *E. floccosum*, slight in *M. cookie* and negligible in *T. mentagrophytes*.

Currently, work is being done in this laboratory both at whole cell and enzymatic levels to delineate the mechanism of glycerophosphatide metabolism in these pathogenic fungi.

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Effect of High Fat/High Erucic Acid Diet on Phosphatidate Synthesis and Phosphatidate Phosphatase in the Subcellular Fractions of Rat Heart and Liver

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ABSTRACT

Rats of weaning age were fed for a period of 1, 3 or 6 weeks either a control diet (laboratory stock diet) or a semisynthetic diet containing 20% by weight of either mustard seed oil (1/3 of the total fatty acids were comprised of erucic acid) or corn oil (2/3 of the total fatty acids consisted of linoleic acid). Mitochondrial and microsomal fractions were isolated from the hearts and livers of these rats, and the rate of acylation of *sn*-[U-^{14}C]glycerol 3-phosphate (P) was examined using palmitoyl-CoA or erucoyl-CoA as the acyl donor. In addition, activities of phosphatidate phosphatase of the mitochondrial, microsomal and soluble fractions were assayed. Studies on the acylation of glycerol 3-P with palmitoyl-CoA demonstrated that feeding of the high fat/high erucic acid diet for 1, 3 or 6 weeks significantly increased the rate of formation of monoacylglycerol 3-P by the cardiac subcellular fractions as compared to the control. The rate of formation of diacylglycerol 3-P also increased but to a lesser degree. Feeding the high fat/high linoleic acid diet tended to increase acylation of glycerol 3-P by cardiac subcellular fractions. However, neither high fat diet influenced acyltransferase activities of the hepatic subcellular fractions or phosphatase activities of the cardiac and hepatic fractions. Studies on the acylation of glycerol 3-P with erucoyl-CoA demonstrated that the rate of acylation was ca. 1/10 that measured using palmitoyl-CoA in all experiments; in particular, the formation of diacylglycerol 3-P was extremely slow, suggesting that erucoyl-CoA is an unsuitable substrate for the position-2 of the monoacylglycerol 3-P. The rate of acylation by the cardiac and hepatic subcellular fractions was not influenced by the feeding of the high-fat diets. The rate of glycerol 3-P acylation by both cardiac and hepatic mitochondrial fraction was ca. 2/3 of the rate of acylation by the respective microsomal fraction. In addition, the ratio of monoacyl- to diacylglycerol 3-P synthesized by the mitochondrial fraction was smaller than that by the microsomal fraction. These results suggest that acylation of glycerol 3-P by the mitochondria cannot be attributed to the action of the contaminating microsomal enzymes.

INTRODUCTION

Feeding young rats a diet rich in an oil containing a large amount of erucic acid produces a temporal but large accumulation of heart triglyceride (TG), as well as hyperlipemia and other biochemical changes (1-3). Our analyses indicated that, when the rats were fed for 1 wk on a diet containing mustard seed oil (20%), of which 37% of the total fatty acids was erucic acid, 1/2 of the total TG fatty acids that were accumulated in the heart was comprised of erucic acid. By contrast, feeding of corn oil, of which 62% of the total fatty acids consisted of linoleic acid, did not cause excessive accumulation of linoleic acid in the heart lipids (4). When the hearts of these rats were perfused in vitro, a greater amount of exogenous labeled fatty acid, either palmitic acid or erucic acid, was incorporated into tissue lipids of the rats which had received a diet rich in erucic acid than those which had received a diet rich in linoleic acid (4). Moreover, our stereospecific analyses of the molecular structure of the heart TG demonstrated that, despite the enormous increase in the erucic acid content of heart TG under these experimental conditions, this fatty acid was not distributed randomly in the molecule, i.e., 90% of the total fatty acids that were found being attached at the *sn*-2 position of the TG molecule in the heart consisted of fatty acids other than erucic acid (5).

These results have raised the following, unanswered questions: (a) whether activities of some TG-synthesizing enzymes in the heart or liver may be altered by the high-fat diet, thus causing the change in the heart TG level and plasma TG content, and, (b) whether positional specificity of the acylating reaction is altered, so that erucic acid can preferentially be transacylated to the *sn*-1 or *sn*-3 position of the TG molecule. This paper describes the results of our study, which was carried out in an attempt to explore mechanisms responsible for some of the metabolic changes we have stated.

In addition, the rate of mitochondrial phosphatidate synthesis was compared to that of microsomal synthesis, both in the heart and liver, since (a) mitochondrial and microsomal acylation of glycerol 3-phosphate (P) may respond differently to various experimental interventions (6,7), and (b) in some species of

animals, the rate of mitochondrial synthesis has been shown to be so low that the action by the contaminating microsomal enzyme can account for the entire activity of mitochondrial phosphatidate synthesis (8,9).

MATERIALS AND METHODS

Materials

Palmitic and erucic acids (99% pure), coenzyme A lithium salt (more than 85% pure) and fatty-acid-free (< 0.005%) bovine serum albumin, fraction V, were purchased from Sigma Chemical Co., St. Louis, MO; *sn*-[U-^{14}C]-glycerol 3-P, disodium salt (120 Ci/mol; 98% pure) and Econofluor were obtained from New England Nuclear Corp., Boston, MA. N-Hydroxysuccinimide and dicyclohexylcarboimide were supplied by Eastman Organic Chemicals, Rochester, NY, and lithium aluminum hydride by BDH (British Drug Houses) Laboratories, Poole, England. Ethyl acetate, tetrahydrofuran and other chemicals were purchased from Fisher Scientific, Fairlawn, NJ, or Baker Chemicals, Phillipsburg, NJ, and were of the highest purity available. Coenzyme A esters of fatty acids were synthesized according to the Al-Arif and Blecher method (10); their purity was determined by ultraviolet (UV) spectrophotometry. Silica-Gel-G-precoated thin layer chromatography plates (0.25 mm thick) were purchased from Brinkman Instruments, Rexdale, Ontario.

Animals and Diets

Male albino rats (40-60 g) of a Sprague-Dawley strain at weaning (3-wk-old) were purchased from Biobreeding Laboratory (Ottawa) and immediately fed the test diets (3). One group of animals was fed a Purina laboratory stock diet, the fat content of which was 4.5% by weight. Two other groups were fed a semisynthetic diet containing 20% casein, 20% sucrose, 30% corn starch, 2% vitamin mixture, 4% salt mixture, 4% Brewer's yeast and 20% test oils. The test oils used were mustard seed oil and corn oil obtained from supermarkets. The fatty acid composition of the test oils was determined by gas chromatography and reported previously (3,4); mustard seed oil contained palmitic (3.4%), stearic (20.9%), oleic (17.7%), linoleic (10.2%), gadoleic (10.4%) and erucic (36.6%), and corn oil contained palmitic (9.7%), oleic (25.9%), linoleic (62.1%) and linolenic (0.9%). The variation of these determinations was ±4% of the mean values (4). All diets were fed ad libitum and the animals had free access to water. Rats were fed the different diets for periods of 1, 3 or 6 wk. Body weight, food intake, heart rate and basal metabolic rate were measured in some rats and the results have been reported elsewhere (3,4). These dietary regimens are similar to those employed by some other investigators (11-13). Since a semisynthetic diet with a low fat content was not used as a control diet in this study, the possibility exists that the effect of the high-fat diets may result from factors other than the fat content of the diets. At the end of each experimental period, the animals were decapitated and the hearts and the livers were excised.

Subcellular Fractionation

The excised hearts and livers were finely chopped with scissors, suspended in an ice-cold solution containing 0.25 M sucrose and 0.02 M Tris-HCl, pH 7.4, and homogenized with a motor-driven Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 800 G for 10 min. The supernatant was further centrifuged at 10,000 G for 10 min to sediment the mitochondrial fraction, and this fraction was washed once and spun at 8,000 G for 10 min. The postmitochondrial supernatant was centrifuged at 24,000 G for 15 min to sediment light mitochondria and lysosomes, then centrifuged again at 159,000 G for 45 min to obtain the microsomal pellet and a cell sap fraction (6,14). Both mitochondrial and microsomal fractions were suspended in a sucrose-Tris medium and their protein content was determined (15). Activities of marker enzymes were measured in these fractions. Ratios of the activities found in the mitochondrial, lysosomal and microsomal fractions were: 21:1:1 (cytochrome c oxidase) (16); 3:5:1 (acid phosphatase) (17) and 0.3:0.6:1 (rotenone-insensitive NADPH cytochrome c reductase) (18).

Acyltransferase Assay

The assay of acyl-CoA:*sn*-glycerol 3-P-O-acyltransferase (EC 2:3:1:15) and acyl-CoA: monoacylglycerol 3-P acyltransferase (EC 2.3.1.52) was performed as described previously (19). The reaction mixture contained, in a final vol of 2.0 ml: 100 μmol of Tris-HCl buffer, pH 7.4; 10 mg of bovine serum albumin; 6.0 μmol of *sn*-glycerol 3-P; 1.0 μCi of *sn*-[U-^{14}C]glycerol 3-P and 0.3 μmol of palmitoyl-CoA or erucoyl-CoA. The reaction was started, after a 5-min equilibrium period at 37 C, by the addition of the mitochondrial or microsomal fraction (0.5 mg of protein). The incubation was terminated after 5 min (microsomal fraction) or 10 min (mitochondrial fraction) by the addition of 3.0 ml of *n*-butanol, saturated with 0.1 M boric acid. These assay condi-

tions were previously found to be optimal for measurements of the acyltransferase (19); the rate of acylation was constant for an incubation period of 10 min and up to 1.3 mg of protein (6,19).

The butanol extract, after an addition of 4.0 ml of 0.1 M boric acid, saturated with butanol, was centrifuged at 10,000 G for 10 min to break the emulsion (20). The butanol phase was washed once with 6.0 ml of 0.1 M boric acid saturated with butanol. The washed butanol phase was dried under nitrogen gas and the residue dissolved in chloroform/methanol (2:1, v/v). The lipid extract was applied to thin layer chromatographic plates. The chromatograms were developed with a solvent system composed of chloroform/methanol/3.5 M NH_4OH (65:35:8, v/v/v) (21). The radioactive products were localized by exposure to X-ray films for at least 3 days. The silicic acid containing the lipids was scraped from the plates into counting vials and Econofluor was added to each sample; their radioactivities were determined by a Beckman scintillation spectrometer LS-150. The degree of quenching was similar among samples under these conditions. However, the measured radioactivities were corrected for quenching by feeding the data, together with their external standard ratios, into a Wang computer. The counting efficiency for ^{14}C was more than 90%. The products of acylation were verified by comparing their R_F values with those of the authentic standards. Upon mild alkaline hydrolysis, the 2 products of the reaction produced one spot on paper chromatography with phenol/water (100:40, w/w). The R_F value of this spot corresponded to that of glycerol 3-P (19). Our previous study established that the recovery of phosphatidate, which was subjected to the butanol extraction and thin layer chromatographic procedure, was over 90% (19).

Phosphatase Assay

The activity of phosphatidate phosphatase (EC 3.1.3.4) was determined by measuring the release of Pi from diacylglycerol 3-P (phosphatidic acid), as described previously (14,22, 23). The incubation mixture was prepared immediately before each experiment and contained 3.0 μmol of sodium phosphatidate; 360 μmol of Tris-acetate buffer, pH 7.4; 1 μmol dithiothreitol; 4.0 μmol of $MgCl_2$; and a subcellular fraction (1 mg of protein) in a final vol of 2.0 ml. Phosphatidate was sonicated at 20 KHz and 150 W at 4 C (Biosonik B-10-II, Bronwill Scientific, Rochester, NY) to produce a homogeneous suspension.

After incubation for 30 min at 37 C, the reaction was stopped by the addition of 2.0 ml of 12% (w/v) trichloroacetic acid. After removal of the precipitated protein by centrifugation, the Pi in the reaction mixture was determined by the Berenblum and Chain method (24). These conditions were found to be optimal for the assays of particulate and soluble phosphatidate phosphatase; the rate of reaction was linear with respect to time (180 min) and amounts of enzyme (1 mg) (23).

RESULTS

The rates of acylation of glycerol 3-P by both cardiac mitochondrial and microsomal fractions were significantly increased during feeding of a mustard seed oil diet compared to those of the rats fed a chow diet. Feeding a corn oil diet tended to increase the rates of acylation of glycerol 3-P by the isolated mitochondrial and microsomal fractions, although this effect was less uniform during a 6-wk period than was the effect of feeding the mustard seed oil diet (Table I). The effect of the diets is most likely a result of the difference in their fat contents, although the possibility remains that some other factors in the diets may contribute to the change.

When the assays were carried out using palmitoyl-CoA as the acyl donor of the reaction, the rate of acylation of glycerol 3-P by the cardiac mitochondrial fraction was 2/3 that by the cardiac microsomal fraction. The ratio of monoacyl- to diacylglycerol 3-P synthesis by the mitochondrial fraction was ca. 3, whereas the same ratio by the microsomal fraction was between 4 and 5 (Table I). This product ratio is one of the properties by which acylation reactions of subcellular fractions can be characterized (6,19,20,25), although the precise factors controlling the ratio are unknown.

The hepatic subcellular fractions prepared from the rats which had received the 2 types of high-fat diets synthesized diacylglycerol 3-P and monoacylglycerol 3-P from glycerol 3-P and palmitoyl-CoA at rates similar to the control rates (Table II). The hepatic mitochondrial and microsomal fractions synthesized a significantly greater amount of diacylglycerol 3-P than did the cardiac fractions. Thus, the ratio of monoacyl- to diacylglycerol 3-P synthesis was significantly different from that of the heart and ranged from ca. 0.5 (mitochondrial synthesis) to nearly 1 (microsomal synthesis) (Table II). The rate of microsomal acylation was 1.6-fold greater than the rate of mitochondrial reaction.

The rate of acylation of glycerol 3-P by the subcellular enzymes were dependent upon the kind of acyl donor. Thus, when erucoyl-CoA

TABLE I

Effects of Different Diets on the Formation of Monoacyl- and Diacylglycerol 3-Phosphate by Mitochondrial and Microsomal Fractions of Rat Heart

	1 wk (n=6)			3 wk (n=4)			6 wk (n=6)		
	Chow	Corn	Mustard	Chow	Corn	Mustard	Chow	Corn	Mustard
Mitochondria									
MAG3P[a]	2.37 ± .16	3.14 ± .29*	3.27 ± .13*	2.05 ± .17	2.73 ± .40	2.84 ± .21*	2.47 ± .14	3.27 ± .24*	3.33 ± .25*
DAG3P	.78 ± .06	1.04 ± .09*	.98 ± .04*	.79 ± .05	1.07 ± .17	1.14 ± .11*	.77 ± .08	1.16 ± .06*	1.06 ± .05*
Total	3.15 ± .22	4.18 ± .38*	4.24 ± .16*	2.84 ± .21	3.80 ± .57	3.98 ± .32*	3.24 ± .17	4.42 ± .26*	4.39 ± .30*
MAG3P/DAG3P	3.04	3.02†	3.34†	2.59†	2.55	2.49†	3.21	2.82	3.14
Microsomes									
MAG3P	4.52 ± .35	4.88 ± .30	5.81 ± .49*	3.52 ± .24	4.80 ± .09*	5.75 ± .33*#	4.41 ± .23	5.17 ± .30	5.48 ± .27*
DAG3P	1.05 ± .15	.87 ± .08	1.12 ± .20	.68 ± .14	1.28 ± .17*	1.39 ± .21*	.98 ± .09	1.35 ± .26	1.48 ± .20*
Total	5.57 ± .38	5.75 ± .25	6.93 ± .65*	4.19 ± .28	6.08 ± .24*	7.14 ± .53*	5.39 ± .20	6.52 ± .52	6.96 ± .40*
MAG3P/DAG3P	4.30	5.61†	5.18†	5.18†	3.75	4.14†	4.5	3.83	3.7

[a]Abbreviations are: MAG3P (monoacylglycerol 3-phosphate), DAG3P (diacylglycerol 3-phosphate), Total (MAG3P + DAG3P), chow (laboratory stock diet), corn and mustard (semisynthetic diet containing 20% by weight of either corn oil or mustard seed oil). Significant differences ($p < 0.05$) are indicated by * (different from the "chow") and by # (different from the "corn"). †Indicates a significant difference between the mitochondrial and microsomal ratios. Palmitoyl-CoA was used as the acyl donor for the assays, the detail of which is described in Materials and Methods. The values are expressed as means ± SEM/min/mg of protein.

TABLE II

Effects of Different Diets on the Formation of Monoacyl- and Diacylglycerol 3-Phosphate by Mitochondrial and Microsomal Fractions of Rat Liver

	1 wk (n=4)			3 wk (n=2)			6 wk (n=4)		
	Chow	Corn	Mustard	Chow	Corn	Mustard	Chow	Corn	Mustard
Mitochondria									
MAG3P[a]	1.35 ± .22	1.70 ± .18	1.87 ± .10	2.07 ± .16	2.16 ± .09	1.92 ± .08	1.71 ± .20	1.97 ± .05	2.41 ± .19*
DAG3P	2.79 ± .19	3.91 ± .82	4.18 ± .90	3.74 ± .23	4.01 ± .87	3.99 ± .23	2.86 ± .41	3.16 ± .13	3.73 ± .12*#
Total	4.14 ± .20	5.61 ± .97	6.05 ± .94	5.81 ± .40	6.17 ± 1.06	5.91 ± .31	4.57 ± .61	5.13 ± .13	6.14 ± .10*#
MAG3P/DAG3P	.48†	.43†	.45†	.55	.54	.48	.60	.62	.65
Microsomes									
MAG3P	4.59 ± .45	3.77 ± .33	3.70 ± .33	4.21 ± .54	3.38 ± .08	3.20 ± 1.30	4.02 ± .43	4.76 ± .97	4.71 ± .20
DAG3P	4.63 ± .93	3.98 ± .50	4.57 ± .93	5.29 ± 1.81	4.50 ± 1.22	4.51 ± .32	5.43 ± 1.50	5.13 ± 1.38	5.06 ± 1.40
Total	9.22 ± .69	7.75 ± .20*	8.21 ± 1.02	9.50 ± 2.54	7.88 ± 1.30	7.71 ± .46	9.44 ± 1.85	9.89 ± 2.33	9.77 ± 1.27
MAG3P/DAG3P	.99†	.95†	.81†	.80	.75	.71	.74	.93	.93

[a]Abbreviations are: MAG3P (monoacylglycerol 3-phosphate), DAG3P (diacylglycerol 3-phosphate), Total (MAG3P + DAG3P), chow (laboratory stock diet), corn and mustard (semisynthetic diet containing 20% by weight of either corn oil or mustard seed oil). Significant differences ($p < 0.05$) are indicated by * (different from the "chow") and by # (different from the "corn"). †Indicates a significant difference between the mitochondrial and microsomal ratios. Palmitoyl-CoA was used as the acyl donor for the assays, the detail of which is described in Materials and Methods. The values are expressed as means ± SEM/min/mg of protein.

was used as the acyl donor, the rate of reaction was 1/10 or less than the rate of reaction observed when palmitoyl-CoA was used (Table III). Furthermore, both cardiac and hepatic subcellular fractions synthesized a minute amount of diacylglycerol 3-P, indicating that erucoyl-CoA is an unsuitable substrate for acylating monoacylglycerol 3-P, which presumably contains an erucoyl moiety as a result of the first step of the acylating reaction.

Table III also lists the results of assays of phosphatidate phosphatase activities of the subcellular fractions; both data shown in Table III are those obtained from the assays using the rats which received different diets for 3 wk. Since there was no significant change in these enzyme activities, the data obtained from the rats fed for 1 or 6 wk, as well as SEM value, are omitted from the table.

DISCUSSION

The rates of reaction of phosphatidate and diglyceride formation determined in our assay systems were much higher than the rate of change in the heart TG level observed during feeding a mustard seed oil diet (4). In the experiment, in which the heart TG level was determined, the level increased from 2.8 mg/g of the control value to 14.1 mg/g within 1 wk of feeding (4). If we assume that the change (11.3 mg/g) had occurred in 5 days, then the rate of change in heart TG would be 14 μmol/120 hr, or 1.94 nmol/min/g of heart. Assuming that 1 g of the heart muscle contains 53 mg of mitochondria (26), this value corresponds to 36.6 pmol of TG formation/min/mg of mitochondrial protein. This is ca. 1/10 of the rate of glycerol 3-P acylation with erucoyl-CoA as the acyl donor (Table III), or 1/100 of glycerol 3-P acylation with palmitoyl-CoA (Table I).

Our previous analyses carried out by using the isolated, perfused and nonworking heart indicated that the rates of incorporation of [^{14}C] palmitate or [^{14}C] erucate into tissue TG, diglyceride and phosphoglycerides were ca. 450, 50 and 90 (in nmol/g/30 min), respectively (27). When we compare these data with those obtained from the present study, by assuming again that there are 53 mg of mitochondria/g of heart tissue, 3 nmol of glycerol 3-P acylated/min/mg mitochondrial protein would correspond to the rate of acylation of 4770 nmol/g wet weight of heart/30 min of perfusion. A similar calculation, assuming that there are 4 mg microsomes/g of heart (23,28,

TABLE III

Effects of Different Diets on (a) The Rate of Acylation of Glycerol 3-Phosphate with Erucoyl-CoA and (b) Phosphatidate Phosphatase Activity of Cardiac and Hepatic Subcellular Fractions of Rat[a]

	Chow diet	Corn oil diet	Mustard seed oil diet
(a)			
Heart mitochondria			
Monoacylglycerol 3-P formation	0.21	0.28	0.25
Diacylglycerol 3-P formation	0.08	0.09	0.15
Heart microsomes			
Monoacylglycerol 3-P formation	0.21	0.28	0.27
Diacylglycerol 3-P formation	0.13	0.10	0.09
Liver mitochondria			
Monoacylglycerol 3-P formation	0.20	0.33	0.26
Diacylglycerol 3-P formation	0.04	0.11	0.25
Liver microsomes			
Monoacylglycerol 3-P formation	0.26	0.32	0.08
Diacylglycerol 3-P formation	0.14	0.21	0.09
(b)			
Phosphatidate phosphatase			
Heart mitochondria	6.61	7.31	6.22
Heart microsomes	7.87	8.88	8.76
Heart cell sap	5.30	5.78	4.95
Liver mitochondria	7.96	6.83	6.52
Liver microsomes	6.21	4.84	5.75
Liver cell sap	4.69	5.13	4.69

[a]Rats were fed 3 different diets for 3 weeks; subcellular fractions were prepared and the assays were carried out as described in the text. The results are means of 2 independent determinations each carried out in duplicate and expressed as nmol of substrate transformed/min/mg of protein. For simplicity, the results obtained from the rats fed for 1 or 6 weeks are not tabulated.

29), reveals that the rate of acylation of 5 nmol/mg microsomal protein/min equals that of 600 nmol/g of heart/30 min. In other words, the values reported in this paper are more than 10 times greater than the rates of acylation previously measured in the perfused heart.

These calculations reveal that it is unlikely that activities of glycerol 3-P acyltransferase and phosphatidate phosphatase serve as a rate-controlling function for lipid synthesis. Consequently, although we observed an increase in activities of glycerol 3-P acyltransferase of the heart of the rat which had consumed the high-fat diet, the increase may not be necessarily causally related to the increased TG content of the heart. In support of this hypothesis, other investigators observed that the Km value of glycerol 3-P acyltransferase for acyl-CoA esters is lower than the Km of palmitoylcarnitine acyltransferase (30), and therefore, it is probably the substrate availability that controls the flux of fatty acids to phosphatidate. Further support is provided by our finding that there was no change in activity of cardiac phosphatidate phosphatase, a second step of the de novo synthetic pathway of tissue glycerolipids. Recent evidence suggests that the alteration in TG concentration in heart, which occurs during feeding of a high-fat/high-erucic-acid diet, may be related to a change in the rate of TG breakdown (31), or in the rate or chain-shortening reaction of erucic acid (32,33).

Feeding a diet rich in erucic acid induced hypertriglyceridemia and a high-erucic-acid content of the plasma TG (3,34). However, we found no change in the activities of glycerol 3-P acyltransferase and phosphatidate phosphatase of hepatic subcellular fractions (Tables II and III). Other investigators also found that the activity of hepatic glycerol 3-P acyltransferase was not influenced by dietary modification (35). On the other hand, the activity of soluble phosphatidate phosphatase of the liver was found to increase in diabetes (36), after ingestion of diets rich in fructose (37) or lard (35), and also, following ethanol ingestion (38). Glenny et al., in addition, reported that the capacity of the liver to synthesize TG was increased when rats were fed on diets enriched with TG containing predominantly saturated and monounsaturated fatty acids (35). However, as in the case of the heart already discussed, the level of theoretical activities calculated from the results of our assays of phosphatidate synthesis and hydrolysis is ca. 10-fold the rate of lipid synthesis by the normal in situ or perfused liver. The latter value, although it varies depending on the conditions of experiment, is on the order of 3 μmol/g of liver/hr (39-42). In contrast, the rate of mitochondrial acylation would equal 16.2 μmol/g/hr, assuming that 60 mg of mitochondria exist/g of liver (43,44) and that the rate of acylation is 4.5 nmol/mg mitochondrial protein/min (Table II), whereas the rate of microsomal acylation would be 21.6 μmol/g/hr, assuming that 40 mg of microsomes exist/g of liver (44-46) and the rate of acylation is 9 nmol/mg of microsomal protein/min. The rate of phosphatidate phosphatase/g of liver is of a similar magnitude. Therefore, these calculations make it difficult to postulate that these enzymes play a major role in controlling the rate of hepatic TG synthesis.

The distinct substrate specificity of the glycerol 3-P and monoacylglycerol 3-P acyltransferase reactions catalyzed by rat liver mitochondria and microsomes was reported previously by several investigators (20,47,48). Our study shows that both cardiac and hepatic enzymes are strongly discriminatory against erucoyl-CoA. Since the first acylation of glycerol 3-P takes place at position-1 of the glycerol moiety (19,20,47,48), the acyl acceptor of the second reaction of phosphatidate synthesis, under our assay conditions, must have been *sn*-1-erucoyl-glycerol 3-P. The second reaction proceeded at an even slower rate than the first reaction (Table III), indicating that the erucoyl-CoA is particularly a poor substrate for the *sn*-2-position of the glycerolipid molecule. Such a view agrees with the analysis of positional distribution of the TG molecules of the hearts of rats fed the mustard seed oil diet (5). In that work, 90% of the erucoyl moiety was found to attach to positions-1 and -3 of the triacylglycerol molecule.

The sites of synthesis of phospholipid components of the mitochondrial membranes are well characterized in the mammal (49). Mammalian mitochondria synthesize only mono- and diphosphatidylglycerol and phosphatidic acid; other phospholipids are synthesized by and transferred from the endoplasmic reticulum to the mitochondria (49,50). However, the rate of mitochondrial phosphatidate synthesis in some species of animals is so low that the existence of an independent mitochondrial enzyme was rightly questioned (8,9). In the rat heart, phosphatidate synthesis by the mitochondria not only is more active than can be accounted for by the cross-contaminating microsomal enzyme (Table I), but also exhibits distinct substrate specificity (47,51) as well as different responses to the lack of insulin (7), to thyroid hormones, aging of the animal (6) and to an addition of albumin (6,52), compared to microsomal synthesis. The results presented in this paper also support the view that there

are separate phosphatidate synthesizing enzymes in the mitochondria of the rat heart and liver.

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Antilipidemic Activity of 4-Oxo-Functionalized Ethyl 6-Chlorochroman-2-carboxylate Analogs and a Related Tricyclic Lactone in Three Rat Models

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ABSTRACT

The synthesis of ethyl *cis*-6-chloro-4-hydroxychroman-2-carboxylate (IV) and 6-chloro-4-hydroxychroman-2-carboxylic acid lactone (V) are reported. The antilipidemic properties of these compounds in 3 rat models were compared to the activity obtained for the previously synthesized related analogs ethyl 6-chlorochroman-2-carboxylate (II), ethyl 6-chlorochromanone-2-carboxylate (III) and clofibrate (I). The biologically most interesting analog, ethyl 6-chlorochroman-2-carboxylate (II) like clofibrate (I), was an effective antitriglyceridemic and anticholesterolemic agent in Triton WR-1339 hyperlipidemic rats, sucrose-fed hyperlipidemic rats and chow-fed normolipemic rats. Ethyl 6-chlorochromanone-2-carboxylate (III) was found to be active only after 7 days of administration to sucrose-fed rats. In sucrose-fed, male Sprague-Dawley rats, the comparative effects of these analogs on various hepatic drug parameters also were carried out. Consistent with previous findings, results obtained with these compounds provide evidence showing that changes in hepatic HMG-CoA reductase activity bear no relationship to serum cholesterol lowering in the sucrose-fed model.

INTRODUCTION

We have previously reported that clofibrate (I) is anticholesterolemic and antitriglyceridemic in Triton WR-1339-induced hyperlipidemic, sucrose-fed hyperlipidemic and normolipemic rat models (1,2). In Triton-induced hyperlipidemic rats, ethyl 6-chlorochroman-2-carboxylate (II) was equally as active as I, whereas ethyl 6-chlorochromanone-2-carboxylate (III) was inactive in this model (3). To further explore the effect of structural modification at the 4-position on antilipidemic properties, ethyl-*cis*-6-chloro-4-hydroxy-chroman-2-carboxylate (IV) and its tricyclic lactone, 6-chloro-4-hydroxychroman-2-carboxylic acid lactone (V), were synthesized and their pharmacological profiles examined in 2 hyperlipidemic rat models and in normal animals. Lactone V was of interest since other clofibrate-related lactones (1,4,5) have been found to possess some of the antilipidemic and metabolic properties associated with the parent compound, clofibrate. In this paper, the comparative biological profiles of I-V (Fig. 1) are reported and an improved synthesis for the biologically most interesting antilipidemic analog II is described.

MATERIALS AND METHODS

Analytical Methods

Infrared (IR) spectra were recorded on a Beckman 4230 Spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian A-60A spectrometer or with a Bruker Model HX-90E spectrometer equipped with Fourier transform capability with tetramethylsilane as an internal standard. Melting points were taken in open capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was done with Silica Gel FG (Analtech, Inc.), and the chromato-

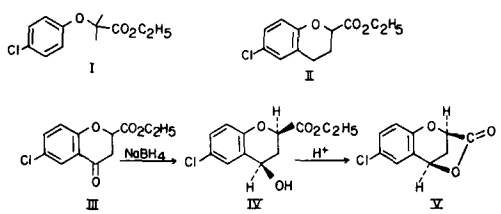

FIG. 1. Structures of clofibrate and related chroman analogs. Key: I, clofibrate; II, ethyl 6-chlorochroman-2-carboxylate; III, ethyl 6-chlorochromanone-2-carboxylate; IV, ethyl *cis*-6-chloro-4-hydroxychroman-2-carboxylate; and V, 6-chloro-4-hydroxychroman-2-carboxylic acid lactone.

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grams were sprayed with 10% H_2SO_4 containing 5% $Ce(SO_4)_2$, and then air-dried. Compounds were detected by ultraviolet (UV) light. Microanalyses was performed by Galbraith Laboratories, Inc., Knoxville, TN.

Synthetic Methods

Ethyl 6-chlorochroman-4-one-2-carboxylate (III). To a solution of VI (6) (20 g; 0.079 mol) in 100 ml of glacial acetic acid and 150 ml of ethyl acetate was first added 1 ml of an aqueous solution of ferrous chloride ($X \cdot H_2O$; Mallinckrodt; 20 mg/ml) followed by 200 mg of platinum oxide catalyst. The mixture was shaken immediately in a Parr Hydrogenator under an initial hydrogen pressure of 60 psi until the rapid uptake of hydrogen stopped. The catalyst was filtered and the filtrate was concentrated under reduced pressure. The resulting oily residue crystallized and the solid was recrystallized from 500 ml of boiling hexane, yielding 18.3 g (91%) of crystals mp 81-83 C, lit. (6) mp 78-79.5 C.

Ethyl cis-6-chloro-4-hydroxychroman-2-carboxylate (IV). To a stirred solution of chromanone III (2.45 g; 0.01 mol) in 50 ml of ethanol (abs) and 25 ml of distilled 1,2-dimethoxyethane maintained at 0-4 C in an ice bath was added, by portions, solid $NaBH_4$ (0.09 mg; 0.0025 mol) over a 10-min period. Additional $NaBH_4$ (0.019 mg; 0.0005 mol) was added to the reaction mixture if starting material was present after 15 min (monitored by TLC [Silica Gel GF; toluene:CH_2Cl_2:EtOAc, 5:5:1, v/v/v]). After a total reaction time of 30 min, 5% HCl solution was added by drops until a pH of 6 was obtained. The solvent was concentrated under reduced pressure, and the solid was dissolved in CH_2Cl_2. The organic layer was washed with 5% HCl followed by saturated $NaHCO_3$ solution, dried ($MgSO_4$) and concentrated under reduced pressure affording an oil which crystallized upon standing. The solid was recrystallized from petroleum ether (60-100 C)-ethyl acetate yielding 2.3 g (89.8%) of crystals mp 82-83 C. IR (cm^{-1}) 3500 (OH), 1760 (CO_2Et), NMR: $CDCl_3$, δ 1.25 (t, 3, J = 7.5 Hz $CH_2\underline{CH_3}$), 2.17-2.50 (m, 2, methylene at C_3), 2.6-3.2 (broad singlet, 1, OH), 4.18 (q, 2, J = 7.5 Hz, $\underline{CH_2}CH_3$), 4.55-4.95 (m, 2, C_2 and C_4 methine), 6.70-7.35 (m, 3, aromatic).

Elemental analysis $C_{12}H_{13}O_4Cl$, calcd. C, 56.15; H, 5.10; Cl, 13.81; found C, 56.36; H, 5.24; Cl, 13.83.

6-Chloro-4-hydroxychroman-2-carboxylic acid lactone (V). A solution of IV (500 mg; 2.2 mmol) in 50 ml of xylene containing *p*-toluene sulfonic acid (10 mg) was heated to reflux for 30 min. The solvent was cooled and washed with 10 ml of saturated $NaHCO_3$ solution, dried ($MgSO_4$) and concentrated under reduced pressure affording a crystalline residue. Recrystallization from *n*-heptane yielded 380 mg (83%) of crystals mp 122-124 C. IR (cm^{-1}) 1800 (lactone carbonyl), NMR:$CDCl_3$, δ 2.40-2.65 (m, 2, C_3 methylene), 4.75-5.00 (m, 1, C_2 methine), 5.12-5.45 (m, 1, C_4 methine), 6.65-7.40 (m, 3, aromatic).

Elemental analysis $C_{10}H_7O_3Cl$, calcd. C, 57.02; H, 3.35; Cl, 16.83; found C, 56.90; H, 3.15; Cl, 17.00.

Ethyl 6-chlorochroman-2-carboxylate (II) (6). Crude 6-chlorochroman-2-carboxylic acid (6) was esterified and distilled according to the procedure described by Witiak et al. (6). To a solution of the crude distillate (20 g) in ethanol was first added 1 ml of an aqueous solution of ferrous chloride ($X \cdot H_2O$; Mallinckrodt; 20 mg/ml) followed by 200 mg of platinum oxide. The mixture was shaken immediately in a Parr hydrogenator and continued until no further pressure drop was observed. The catalyst was filtered and the filtrate concentrated under reduced pressure. The resulting oil was distilled (bp 110-116 C, 0.04 mm) as reported (6), affording pure II. The AMX pattern attributable to contamination of II by VII, which previously was observed in the 90 MHz proton spectrum, was now absent. For the methine and 2 vinyl proton resonance signals of VII, J_{AM} = 10 Hz, J_{AX} = 2 Hz, H_{MX} = 4.5 Hz with δA = 6.4, δM = 5.9 and δX = 5.4.

Biological Methods

Determination of antilipidemic activity. Triton WR-1339-induced hyperlipidemic rat model (7). Methods were identical to those previously reported (4). Hyperlipidemia was induced by IP injection of Triton WR-1339 (oxyethylated *tert*-octylphenolformaldehyde polymer, Ruger Chemical Co., Philadelphia, PA). Male albino Sprague-Dawley rats were housed in groups of 5 and were fed Purina Laboratory Chow and water ad libitum for a 2-wk stabilizing period. After this period, the rats were redistributed by weight into 4 experimental groups of 10 rats each (housed in groups of 5). At random, 2 experimental groups (B and D) were fasted for 24 hr and then injected IP with 225 mg of Triton/kg dissolved in 0.15 M NaCl to give a concentration of 62.5 mg/ml. The 2 control groups (A and C) of comparable weight also were fasted and received only the vehicle (0.25% aqueous methyl cellulose), whereas the remaining groups (B and D) received test compounds in vehicle. Compounds were dispersed in the vehicle to obtain a con-

centration of 8.33×10^{-3} mmol/ml, providing a total screening dose of 0.124 mmol/kg for compounds IV and V. Each rat received 2 2-ml doses by gastric intubation, the first immediately after the Triton injection and the second 20 hr later. Fasting was continued during the post-Triton period.

At 43 hr after Triton administration, the rats were anesthetized with ethyl ether; blood was drawn from the abdominal aorta, and serum was obtained after centrifugation at 500 G for 10 min.

Serum triglycerides were determined by Eggstein's method (8), and serum cholesterol was analyzed by the Holub and Galli method (9).

Sucrose and normolipemic rat models (10, 11). Methods were identical to those previously reported (2).

Sucrose-fed rat model. Male albino Sprague-Dawley rats weighing 240-260 g were housed in a facility with alternating 12-hr light and dark cycles. The animals were fed a diet consisting of 63% sucrose, 28% vitamin-free casein, 4% vitamin fortification mixture, 5% salt (USP XIV), and 5% cellulose and allowed free access to tap water. After a prefeeding period of 4 days, groups of animals were given oral injections of each compound (I-V) (0.4 mmol/kg) twice daily for 7 consecutive days. Control animals were injected with corresponding volumes of vehicle (0.25% methyl cellulose). Animals were killed 12 hr after the last dose; blood was drawn from the abdominal aorta and allowed to clot at room temperature and serum was collected by centrifugation at 500 G for 10 min. Blood samples (0 and 4 days) were collected via orbital plexus from ether anesthetized rats. Serum triglycerides were determined by Eggstein's method (8), and serum cholesterol was determined by the Allain et al. method (12).

Normolipemic rat model. Male, albino Sprague-Dawley rats (Harlan Laboratory, Cumberland, IN) weighing 150-180 g were fed Purina Laboratory Chow and received free access to water. All animals were maintained in a facility with alternating 12-hr light and dark cycles. Prior treatment of all compounds (0.4 mmol/kg) or a requisite vol of 0.25% methyl cellulose were administered orally, twice daily for 7 consecutive days. Injection vol were 1.0 ml/100 g of body weight. Animals were killed 16-18 hr after the last dose. Blood samples were collected and analyzed as indicated in the sucrose-fed rat model.

Preparation of microsomes. The homogenization of livers and preparation of microsomes were done as described previously (4,11).

HMG-CoA reductase (EC 1.1.1.34) assay. The assay from mevalonic acid formation from D,L-3-hydroxy-3-methylglutaryl-3-[^{14}C]-CoA was carried out by procedures identical to those described previously (4).

Ethylmorphine N-demethylase assay. The assay of formaldehyde liberated from ethylmorphine was done using procedures described previously (13). Incubation mixtures contained 5 mg of microsomal protein, 10 μmol of ethylmorphine, an NADPH-generated system (13) and 60 μmol of Tris (pH 7.4) in a final vol of 0.3 ml. Reactions were terminated after 10 min of incubation at 37 C.

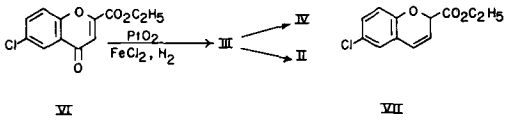
NADPH cytochrome c reductase (EC 1.6.2.a) assay. The assay of cytochrome c reduction in liver microsomes was done using the Phillips and Langdon method (14).

Methods of analysis in liver. Hepatic microsomal cytochrome P-450 cytochrome b_5 were estimated by the Kinoshita and Horie procedure (15). Microsomal protein was assayed by the Lowry et al. method (16). Cholesterol was isolated by saponification (17) and measured by the Parekh and Jung method (18). Triglyceride content was determined by Saloni's method (19).

RESULTS

Synthetic Aspects

An improved synthesis (5) for ethyl 6-chlorochroman-4-one-2-carboxylate (III) was accomplished in high yield by ferrous-chloride-promoted hydrogenation of ethyl 6-chlorochromone-2-carboxylate VI (6) on platinum oxide catalyst (20,21) (Fig. 2). Reduction of chromanone III with sodium borohydride in ethanol and dimethoxyethane yielded IV in 87% yield. Acid-catalyzed lactonization of IV in boiling xylene followed by washing with dilute bicarbonate solution afforded tricyclic lactone V in 83% yield. During the reported (6) Clemmensen reduction of III to II, a minor product ($\leq$ 5%), likely VII, which is not easily separated by physical methods, can be detected (TLC and NMR) and removed by conversion to



VI VII

FIG. 2. Scheme for the chemical synthesis of clofibrate-related chroman analogs. Key: II, ethyl 6-chlorochroman-2-carboxylate; III, ethyl 6-chlorochromanone-2-carboxylate; IV, ethyl *cis*-6-chloro-4-hydroxychroman-2-carboxylate; VI, ethyl 6-chlorochromone-2-carboxylate; and VII, ethyl 6-chlorochrom-3-ene-2-carboxylate.

II via catalytic hydrogenation ($FeCl_2 \cdot PtO_2$) of the esterified reaction mixture.

Biological Aspects

The parent compounds, clofibrate (I) and chroman (II) were shown to be equally effective at the same dose as anticholesterolemic and antitriglyceridemic agents in the Triton WR-1339-induced hyperlipidemic rat model (3). Conversely, chromanone III was ineffective in this model (3). However, reduction of the 4-keto function of III affording the *cis*-hydroxy ester IV and subsequently its corresponding tricyclic lactone V resulted in the selective restoration of antitriglyceridemic activity lost by insertion of a keto function into II (Table I).

A comparison of the antilipidemic properties of these analogs (I-V) in sucrose-fed rats is presented in Table II. Again, clofibrate (I) and chroman II were effective as triglyceride and cholesterol-lowering agents at both 4- and 7-day time periods. Additionally, chromanone III was an effective antilipidemic agent only after 7 days of treatment. In this model, compounds IV and V failed to exhibit antilipidemic effects after 7 days.

Since clofibrate (I) and analogs II and III showed anticholesterolemic activities in Triton or sucrose-fed hyperlipidemic animal models, they were further evaluated in normolipemic rats (Table III). While clofibrate was effective as an anticholesterolemic and antitriglyceridemic agent after 4 and 7 days of treatment, chroman II was only effective after 4 days of drug administration. Chromanone III was inactive after 4 or 7 days of drug treatment.

The metabolic effects of analogs I-V in livers obtained from sucrose-fed rats after 7 days of drug treatment are given in Table IV. Only clofibrate was observed to significantly reduce liver triglyceride levels. None of these agents modified liver cholesterol levels, even though compounds I-IV modified the activity of hepatic microsomal HMG-CoA reductase. While compounds I-III lowered the activity of this enzyme system, analog IV significantly elevated

TABLE I

Comparative Effects of 4-Hydroxy-ester IV and Tricyclic Lactone V on Serum Cholesterol and Triglyceride Levels (mg/dl) in Triton-Induced Hyperlipidemic Male Sprague-Dawley Rats[a]

Lipid analyzed	Compd.	Control group (A)[b]	Drug-treated control (B)[b]	Triton hyperlipidemic (C)[b]	Drug-treated Triton hyperlipidemic (D)[b]
Cholesterol	IV	70.7 ± 11.6	76.1 ± 16.5	105 ± 13.6	103 ± 9.6
	V	86.7 ± 9.18	81.9 ± 10.7	116 ± 9.1	112 ± 11.5
Triglyceride	IV	24.1 ± 7.8	19.6 ± 8.6	41.6 ± 11.7	22.6 ± 5.2[c,d]
	V	19.5 ± 9.26	18.4 ± 6.73	44.1 ± 19.1	19.9 ± 5.46[c,d]

[a]All animals were given a total screening dose of 0.124 mmol/kg of compound.
[b]Mean ± SD; n=10
[c]Statistically significant; $p < 0.05$; comparison of groups C and D.
[d]Statistically significant; $p < 0.05$; comparison of groups A and D.

TABLE II

Comparative Effects of Clofibrate (I) and Analogs II-V on Serum Cholesterol and Triglyceride Levels (mg/dl) in Sucrose-Fed Male Sprague-Dawley Rats

Expt. no.	Compd.	Serum cholesterol[a] Zero-day	4-day	7-day	Serum triglyceride[a] Zero-day	4-day	7-day
1	Control	88 ± 11	77 ± 13	82 ± 6	110 ± 29.8	139 ± 70	98.6 ± 45.4
	I	85 ± 10	37 ± 7(8)[b]	36 ± 8[b]	116 ± 38.6	31.7 ± 13.6(8)[b]	4.2 ± 10[b]
	II	79 ± 4	45 ± 7[b,c]	39 ± 9[b,c]	124 ± 19.9	44.3 ± 15.3[b,c]	28.9 ± 24.8[b,c]
	III	85 ± 8	76 ± 14(9)	64 ± 13[b]	130 ± 51.2	105 ± 48.9(9)	78.3 ± 18.2[b]
2	Control	77 ± 17	–	76 ± 16	77.3 ± 46.2	–	78.9 ± 31.5
	I	77 ± 19	–	34 ± 9[b]	95.8 ± 53.2	–	23.1 ± 15.8[b]
	IV	90 ± 6	–	85 ± 17	83.7 ± 45.3	–	71.1 ± 27.9
	V	87 ± 13	–	71 ± 12	76.0 ± 24.0	–	86.8 ± 36.6

[a]Values reported as mean ± SD; n = 10, unless indicated.
[b]Statistically significant, $p < 0.05$ (response of drugs vs zero, 4 or 7 day control).
[c]Statistically significant, $p < 0.05$ (comparison of clofibrate group vs drug group).

TABLE III

Comparative Effects of Clofibrate (I) and Analogs II and III on Serum Cholesterol and Triglyceride Levels (mg/dl) in Laboratory Chow-Fed Male Sprague-Dawley Rats

Compound	Serum cholesterol[a]			Serum triglyceride[a]		
	Zero-day	4-day	7-day	Zero-day	4-day	7-day
Control	71 ± 8	66 ± 5	66 ± 5	126 ± 29	94 ± 30	118 ± 40
I	73 ± 9	52 ± 8[b]	47 ± 5[b]	151 ± 14	60 ± 28	69 ± 20[b]
II	69 ± 6	57 ± 7[b]	61 ± 8	91 ± 28	58 ± 30[b]	117 ± 28
III	70 ± 8	66 ± 11	69 ± 11	149 ± 44	71 ± 42	104 ± 64

[a]Values reported as mean ± SD; n = 10.

[b]Statistically significant, $p < 0.05$ (response of drug vs zero, 4 or 7 day control).

HMG-CoA reductase activity. In agreement with previous observations, clofibrate (I) administration produced elevations in liver-to-body-weight ratio, liver weight, microsomal protein, cytochrome P-450 and the activity of NADPH cytochrome c reductase. Chroman II only caused an increase in the activities of ethylmorphine N-demethylase and NADPH cytochrome c reductase, whereas analogs IV and V only lowered cytochrome P-450 content. All other hepatic parameters remained unchanged following treatment with analogs III-V.

DISCUSSION

While the 5-chloro- and 5-phenyldihydrobenzofurans selectively lowered elevated cholesterol levels in Triton-induced hyperlipidemic rats (5), 6-substituted-chromans (3) and a clofibrate-related tricyclic enol-lactone (22) like clofibrate, showed both anticholesterolemic and antitriglyceridemic activities at the same dose (0.124 mmol/kg) in this model. However, at ½ this dose, both clofibrate and the enol-lactone only exhibited antitriglyceridemic activity (22). These findings are not unlike most other analogs of clofibrate synthesized in our laboratories, including IV and V, which at the 0.124 mmol/kg dose exhibited selective antitriglyceridemic activity in Triton-induced hyperlipidemic rats. In the sucrose-fed rat model, a clofibrate-related 5-chlorobenzofuran photodimer exhibited weak, but selective, antitriglyceridemic activity (2) whereas a clofibrate-related spirolactone (1) and the 6-phenyl- and 6-cyclohexyl chromans (1) were inactive at the same dose (0.4 mmol/kg). Conversely, in our study, the 6-chlorochroman II, like clofibrate, exhibited both serum cholesterol and triglyceride lowering properties, whereas IV and V were inactive. These differential effects between the 2 animal models most likely reflect differences in mechanisms resulting in hyperlipidemia and substantiate our proposal suggesting that clofibrate-related analogs may be used as mechanistic probes (23). In this regard, we should like to point out that no observable correlation exists between analog activity in the Triton, sucrose-fed, or normal rat models.

So far, our studies have revealed that only clofibrate and the 6-chlorochroman analog II are effective in all 3 animal models. However, it should be noted that II was inactive in normal rats at 7 days, indicating that this analog only exhibited a transient effect. Others have provided evidence that clofibrate administration may have a transient effect in the liver with regard to liver ribosomal protein synthesis and an age-related diminution in the clofibrate effect on liver microsomal protein concentrations, and inductive effects on liver microsomal drug metabolism (13,24). In our laboratories, we have reported that clofibrate (I) and related chloro analogs produce hepatic microsomal enzyme induction after chronic treatment of normolipemic (13,25) and sucrose-fed animals (1,2). Apparently, effects on hepatic drug metabolism are unrelated to the antilipidemic action of clofibrate and related analogs. In this study, only the 6-chlorochroman II exhibited properties associated with hepatic enzyme induction albeit these effects were less pronounced than with clofibrate in the sucrose-fed (Table IV) and normal (unpublished observations) rat models.

Consistent with previous findings using a photodimer (2) of ethyl 5-chlorobenzofuran-2-carboxylate and ethyl 6-phenylchroman-2-carboxylate (3), results obtained with analogs II-IV provide evidence that changes in hepatic HMG-CoA reductase activity bear no relationship to serum-cholesterol lowering in the sucrose-fed model. In fact, IV produced a 2-fold elevation in HMG-CoA reductase activity with no change in serum cholesterol levels.

TABLE IV

Comparative Effects of Clofibrate (I) and Analogs II-V on Various Hepatic Parameters after Chronic Administration to Sucrose-Fed Male Sprague-Dawley Rats

Parameters[a]	Control[b]	Clofibrate[c] (I)	II[c]	III[c]	IV[c]	V[c]
Liver cholesterol (mg/g)	2.61 ± 0.33	2.69 ± 0.30	3.28 ± 0.72	2.68 ± 0.16	–	–
	2.95 ± 1.04	2.43 ± 0.69	–	–	2.42 ± 1.00	2.52 ± 0.35
Liver triglycerides (mg/g)	7.74 ± 1.70	2.52 ± 0.98[d]	8.44 ± 1.77	8.5 ± 1.60	–	–
	9.87 ± 4.82	4.02 ± 1.03[d]	–	–	11.3 ± 9.03	12.4 ± 11.3
HMG-CoA reductase	6.60 ± 2.11	3.15 ± 0.53[d]	3.42 ± 1.18[d]	3.67 ± 0.28[d]	–	–
(nmol/mg/h)	10.7 ± 4.75	7.36 ± 4.29	–	–	16.6 ± 5.99[d]	5.48 ± 2.70
Liver/body wt (%)	4.63 ± 0.28	5.52 ± 0.45[d]	4.64 ± 0.12	4.43 ± 0.33	–	–
	3.69 ± 0.21	5.13 ± 0.32[d]	–	–	3.76 ± 0.26	3.70 ± 0.16
Liver wt (g)	12.73 ± 1.55	14.18 ± 2.23	11.85 ± 0.97	12.24 ± 0.98	–	–
	10.44 ± 0.88	13.31 ± 1.25[d]	–	–	10.67 ± 0.54	10.29 ± 0.54
Microsomal protein (mg/g)	33.8 ± 3.00	40.8 ± 2.7[d]	34.0 ± 4.0	32.6 ± 3.9	–	–
	28.2 ± 2.30	32.4 ± 3.60[d]	–	–	28.1 ± 2.67	30.2 ± 3.82
Ethylmorphine N-demethylase	10.8 ± 0.84	11.9 ± 3.23	17.6 ± 1.77[d]	12.8 ± 2.32	–	–
(nmol HCHO formed/mg/min)	7.26 ± 1.28	7.03 ± 1.34	–	–	7.53 ± 0.99	6.61 ± 0.62
NADPH-Cytochrome c reductase	78.4 ± 15.0	74.3 ± 20.1	116.0 ± 27.2[d]	73.9 ± 5.1	–	–
(nmol/mg/min)	89.9 ± 7.40	152.0 ± 19.5[d]	–	–	86.7 ± 10.5	83.9 ± 13.9
Cytochrome b_5 (nmol/mg prot.)	0.133 ± 0.028	0.089 ± 0.039	0.125 ± 0.028	0.136 ± 0.018	–	–
	0.208 ± 0.024	0.223 ± 0.022	–	–	0.162 ± 0.039	0.196 ± 0.033
Cytochrome P-450	0.395 ± 0.079	0.627 ± 0.086[d]	0.478 ± 0.062	0.416 ± 0.068	–	–
(nmol/mg prot.)	0.697 ± 0.063	0.931 ± 0.151[d]	–	–	0.590 ± 0.049[d]	0.562 ± 0.033[d]

[a]Values of the parameters are reported as the mean ± SD; n = 5. These animals were randomly selected from the treatment groups indicated in Table III. Values were determined after completion of a 7-day drug treatment.

[b]Control animals received a requisite volume of vehicle.

[c]The drug was given orally twice daily (0.4 mmol/kg).

[d]Significant differences from the control ($p < 0.05$) for drug treated vs control animals.

These findings are consistent with evidence provided by others suggesting that serum cholesterol lowering is associated with a modification of lipoprotein turnover, rather than a specific inhibition of the hepatic cholesterol biosynthetic pathway (26).

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Thromboxane B_2 Biosynthesis and Phospholipids Hydrolysis in Platelets from Hypercholesterolemic Rabbits

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ABSTRACT

Thromboxane B_2 biosynthesis from arachidonic acid was increased in platelets from hypercholesterolemic rabbits. The enzymic activity of phospholipase A_2 which releases arachidonic acid, the precursor for the biosynthesis of thromboxane B_2, showed hardly any change in hypercholesterolemic platelets. Phospholipase C and diglyceride lipase activities also were not changed in platelets from hypercholesterolemic rabbits. Furthermore, phospholipid concentration in platelets were not increased in this state. Thus, we conclude that the supply of precursor for thromboxane B_2 biosynthesis was not increased in platelets from hypercholesterolemic rabbits as compared to controls. These results suggest that the enzyme activity of thromboxane B_2 biosynthesis may be enhanced in platelets from hypercholesterolemic rabbits.

INTRODUCTION

The metabolism of arachidonic acid in platelets is of particular importance, since prostaglandins formed from this polyenoic fatty acid are believed to play a central role in platelet function (1). Thromboxanes are extremely potent in inducing platelet aggregation (2), and they also stimulate smooth muscle contraction in blood vessels (3). Thromboxane A_2, the major component involved in platelet aggregation, is extremely unstable. Therefore, thromboxane B_2, which is produced by nonenzymatic oxidation of thromboxane A_2, was found as a stable product of arachidonic acid metabolism in platelets (1). Although the accelerated generation of thromboxane A_2 in platelet-rich plasma from atherosclerotic rabbits was observed by the bioassay system, this mechanism has not been demonstrated (4).

It is known that nonesterified arachidonic acid acts as precursor for prostaglandin synthesis (5,6). However, such a free form is extremely low in the intracellular levels (7,8). Nonesterified arachidonic acid involved in prostaglandin synthesis seems to arise mainly from the hydrolysis of phospholipids. In order to examine the mechanism of promotion of aggregation in platelets of atherosclerotic animals, we investigated thromboxane B_2 biosynthesis as well as phospholipid hydrolysis in platelets from hypercholesterolemic rabbits.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Arachidonic acid (60.2 mCi/mmol) was purchased from Radiochemical Center, Amersham, England. [4-^{14}C]Cholesterol (50 mCi/mmol), [4-^{14}C]cholesteryloleate (50 mCi/mmol), and myo[^{14}C(U)]inositol (200 mCi/mmol) were purchased from New England Nuclear, Boston, MA. Arachidonic acid, cholesteryl oleate, oleic acid, 1-palmitoyl-lysophosphatidylcholine, dipalmitoyl-phosphatidylcholine, bovine serum albumin (essentially fatty-acid-free), phospholipase A_2 (naja-naja venom), *Clostridium welchii* phospholipase C, ATP and CoA were obtained from Sigma Chemical Co., St. Louis, MO. Silica Gel 60 HR and Kiesel Gel 60 F-254 thin layer plates were from Merck, Darmstadt, G.F.R. Prostaglandins E_2, $F_{2\alpha}$, A_2 and thromboxane B_2 were gifts from Ono Pharmaceutical Co. Ltd., Osaka, Japan. All other chemicals were of analytical grade.

Hypercholesterolemic Rabbits

Hypercholesterolemia was induced in 12 rabbits weighing 3-4 kg by feeding them pellet food containing 1% cholesterol daily for 3 months (9). They were divided into 4 groups.

Preparation of Platelet Microsomes

Blood was collected into a 3.8% solution of sodium citrate (9 vol blood/1 vol citrate). Platelet-rich plasma was prepared by centrifugation of citrated blood at 200 G for 10 min. The resultant supernatant was collected by centrifugation at 2,000 G for 20 min. The precipitate obtained was washed twice with Tyrode's solution, suspended in 10 mM Tris-HCl buffer (pH 7.4), and disrupted by sonication (2 30-sec treatments with 2-min intervals). The microsomal preparation was prepared by differential centrifugation (2,000 G, 15 min; 12,000 G, 15 min; 105,000 G, 1 hr). The pellets after the final centrifugation were suspended in the same buffer and stored in a small aliquot at -70 C.

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Preparation of Labeled Phospholipid

Phosphatidylcholine (PC) containing [1-^{14}C] arachidonic acid in the 2-position of the glycerol backbone was synthesized by the following procedure (10-12). The reaction mixture contained 2 μCi of [1-^{14}C] arachidonic acid as ammonium salts, 100 μmol of potassium phosphate buffer (pH 7.4), 20 μmol of ATP, 2 μmol of CoA, 10 μmol of $MgCl_2$, 12.5 μmol of reduced glutathione, 0.9 μmol of 1-palmitoyllysophosphatidylcholine and ca. 1 mg of rat liver microsomes which was prepared by the Land and Merkel procedure (10) in a final vol of 1 ml. After incubation at 37 C for 3 hr, lipids were extracted by the Bligh and Dyer method (13) and the radiolabeled PC was isolated by thin layer chromatography (TLC) using Silica Gel 60 HR plates developed with chloroform/methanol/glacial acetic acid/water (25:15:4:2, v/v) at 4 C as described by Dodge and Phillips (14). After exposure to iodine vapor, the area corresponding to authentic PC was scraped and extracted with chloroform.

When this labeled PC was digested with najanaja venom, radioactivity greater than 95% was released as free arachidonic acid. This indicated that the most of the labeled [1-^{14}C] arachidonic acid was in position C-2.

Preparation of Labeled Platelets

Platelet-rich plasma (10^{10} cells) was incubated with 0.2 μCi of [1-^{14}C] arachidonic acid bound to 10% bovine serum albumin without fatty acid at 37 C for 1 hr (15). After the incubation mixture was cooled in an ice-cold bath and centrifuged at 2,000 G for 20 min, the precipitate was washed twice with Tyrode's solution (16). The pellet was suspended in 0.05 M glycine-NaOH buffer (pH 9.5) and used as labeled platelets. The efficiency of incorporation was about 20%.

Assay Procedure of Thromboxane B_2 Synthesis

The conversion of arachidonic acid to thromboxane B_2 was measured by quantitative radio-TLC. The reaction medium contained the following components: 0.1 M Tris-HCl, pH 8.0, 2×10^9 count of washed platelet, 0.1 μCi of [^{14}C] arachidonic acid and 5 μg unlabeled arachidonic acid in 1.0 ml. The reaction mixture was incubated at 37 C for 10 min and the reaction was stopped by the addition of 10% formic acid to bring it to pH 3.5-3.0. The mixture was extracted 3 times with ethyl acetate. The extract was pooled and evaporated at 40 C in a stream of nitrogen. The residue was redissolved in acetone and applied to Silica Gel G plate and developed in ethyl acetate containing 1% acetic acid (17). The spots on the plate were revealed by iodine vapor. The zones of each component on the plate were scraped and counted on a liquid scintillation counter.

Assay of Phospholipase A_2 Activity

Two substrates, labeled PC or platelets, were used for the determination of phospholipase A_2 activity. When labeled PC was used as the substrate, nonradioactive PC dissolved in chloroform/methanol (2:1, v/v) was added as the carrier (10,000 cpm/200 nmol). After the solvent was evaporated under nitrogen, the buffer was added to the residue and sonicated for 3 min. The reaction was then initiated by addition of platelets. The reaction mixture contained 0.05 M glycine-NaOH buffer (pH 9.5), 10 mM $CaCl_2$ (18,19), 200 nmol (10,000 cpm) of substrate, and 1 mg protein of platelets (sonicated twice for 20 sec each) in a final vol of 1.0 ml. The incubation was carried out at 37 C for 10 and 20 min. Lipids were extracted by the Bligh and Dyer method (13) and applied to Silica Gel HR thin layer plates developed with chloroform/methanol/glacial acetic acid/water (25:15:4:2). The spots were made visible in UV light after spraying a solution of 2,7-dichrolofluorescein (33.3 mg/100 ml of 2 mM NaOH). The zone corresponding to PC and neutral lipid was scraped. The neutral lipid fraction was rechromatographed by thin layer plate developing with petroleum ether/diethyl ether/acetic acid (80:30:1, v/v). More than 97% of radioactivity in neutral lipid was detected in free fatty acid, so the neutral lipid zone was counted on the liquid scintillation counter as released arachidonic acid.

Phospholipase A_2 activity also was measured in labeled platelets as the substrate. The reaction system was the same as already described, except that the labeled platelets in place of PC and 10 μg of epinephrine were added. Incubation was carried out at 37 C for 15 min and subsequent assay methods were the same as already mentioned.

Assay of Phospholipase C and Diglyceride Lipase Activities

Phospholipase C and diglyceride lipase activities were measured by the methods of Rittenhouse-Simmons (20) and Bell et al. (21), respectively. The hydrolysis of [^{14}C] myoinositol phosphatidylinositol (PI) was determined. [^{14}C] myoinositol PI 20 μM was incubated with platelet sonicate supernate (500 μg protein), 2 mg deoxycholate, 5 mM Ca^{2+}, and 50 mM Tris-HCl (pH 7.0) for 60 min at 37 C in a final vol of 1.0 ml. Labeled diglyceride was obtained

by treating 2-[1-^{14}C] arachidonyl-phosphatidylcholine with *Cl. welchii* phospholipase C. As determination of diglyceride lipase activity, platelet microsomes (200 μg protein) were incubated in 50 mM Tris-HCl, pH 7.0, containing 100 mM NaCl, 5 mM $CaCl_2$, 12 mM reduced glutathione, and 20 μM 2-[1-^{14}C] arachidonyl diglyceride for 30 min at 37 C.

Assay of Cholesteryl Esterase Activity

Cholesteryl esterase activity in platelets was measured by Stoke's method (22). For the hydrolysis of cholesteryl esters, platelet mitochondria (1 mg protein) was incubated with 3 μM [4-^{14}C] cholesteryl oleate in 0.1 M citrate-0.2 M potassium phosphate buffer (pH 4.0) for 2 hr at 37 C in a final vol of 1.0 ml. Esterification of cholesterol: incubation mixture contained platelet mitochondria (1 mg protein), 2 μM [4-^{14}C] cholesterol, 4 μM oleic acid, and 0.1 M citrate-0.2 M potassium phosphate (pH 7.0). Incubation continued for 2 hr at 37 C in a final vol of 1.0 ml.

Analysis

The cholesterol content was determined enzymatically according to the Richmond procedure (23). Phospholipids were determined by analyzing the phosphorus content (24,25). Fatty acid methyl esters were prepared by a borontrifluoride-methanol reagent (26). Methyl esters and dimethylacetals were separated by TLC at 4 C on a Silica Gel 60 HR plate developed with toluene containing butylated hydroxytoluene. The separated derivatives were extracted with chloroform containing 500 μg/ml of BHT (15) and analyzed by gas liquid chromatography using a Shimadzu Gas Chromatograph (model GC-4APTF) equipped with a column (0.3 × 200 cm) of 3% EGSS-X on Chromosorb W (80-100 mesh). The column temperature was 160 C and the nitrogen flow rate was 50 ml/min. The peaks were identified from the retention times of known methyl esters and the percentage distribution was determined from the proportions of the peak area. Trace or unidentified components were not calculated.

Protein was determined by the Lowry et al. method (27) with bovine serum albumin as standard.

Triplicate samples from each pooled platelet were analyzed in all experiments. Values given in the Table are means of 4 hypercholesterolemic groups and 4 control groups. Student's t-test was used to analyze the results.

RESULTS

Hypercholesterolemia and Thromboxane B_2 Synthesis

As shown in Table I, when rabbits were fed a cholesterol diet for 3 months, the cholesterol quantity was increased in both plasma and platelets. On the other hand, although phospholipids in plasma were increased in hypercholesterolemic state, those in platelets were almost unchanged.

The biosynthesis of thromboxane B_2 by washed platelets was increased about 3-fold in hypercholesterolemic rabbits as compared to control. The apparent Km values for arachidonic acid were 19 μM (hypercholesterolemia), 50 μM (control), respectively.

Phospholipase A_2 Activity

The activity of phospholipase A_2 in platelets of control and hypercholesterolemic rabbits was assayed using 1-palmitoyl-2-[1-^{14}C] arachidonyl PC or labeled platelets as the substrate. When 1-palmitoyl-2-[1-^{14}C] arachidonyl PC was used as a substrate, the release of radioactive arachidonic acid increased during incubation for 10 and 20 min, but there were hardly any differences between control and platelets from

TABLE I

Lipid Quantity and Thromboxane B_2 Synthesis in Hypercholesterolemic Rabbits

		Cholesterol[a]	Phospholipid[a]	Thromboxane B_2 Synthesis[b]
Control	Plasma	78	70	
	Platelet	55	178	0.70
HCR[c]	Plasma	745[d]	410[d]	
	Platelet	150[e]	172	2.22[e]

[a]Units are mg/dl of plasma and μg protein of platelet.

[b]Unit is nmol/10 min/2 × 10^9 cells.

[c]Hypercholesterolemic rabbit fed on a cholesterolemic diet.

[d]$P<0.001$ compared with control.

[e]$P<0.01$ compared with control.

hypercholesterolemic rabbits (Table IIA).

We also measured phospholipase A_2 activity using platelets labeled with [1-^{14}C] arachidonic acid as the substrate. As shown in Table IIB, about half of the radioactivity labeled with [1-^{14}C] arachidonic acid was incorporated into PC in both control and hypercholesterolemic platelets. Analysis by TLC before and after incubation indicated that the arachidonic acid was released mainly from PC phosphatidylethanolamine and phosphatidylserine. The total amount of radioactive arachidonic acid released showed almost no change between control and hypercholesterolemic platelets.

Phospholipase C and Diglyceride Lipase Activities

The hydrolysis rate of PI was not increased in platelets from hypercholesterolemic rabbits (Table III). As shown in Table III, the activity of diglyceride lipase also was unchanged in platelets from hypercholesterolemic rabbits as compared to controls.

Cholesteryl Esterase Activity

The optimal pH was determined from preliminary experiments. Assays of the hydrolysis of cholesteryl esters were done at pH 4.0 and esterification of cholesterol was determined at pH 7.0. The hydrolysis of cholesteryl esters was decreased but esterification of cholesterol was increased in platelets from hypercholesterolemic rabbits (Table IV). We measured cholesteryl ester concentration in platelets. Cholesteryl ester concentration in platelets from hypercholesterolemic rabbits (70 μg/mg protein) was much higher than control (8 μg/mg protein). A previous report suggested that the decreased arachidonic acid in platelets from canines fed a cholesterol diet was the result of use of arachidonic acid for cholesteryl ester synthesis (28). In our experiments, cholesterol esterification was promoted and cholesteryl esters were increased in platelets. But, as shown in Table VB, there was no arachidonic acid in fatty acid composition of platelet cholesterol esters in both groups.

Fatty Acid Composition of Platelet Phospholipids

Arachidonic acid derived from phospholipids of platelets was most abundant in the phosphatidylethanolamine fraction in both control and hypercholesterolemic platelets (Table VA). Cholesterol supplementation of the diet caused consistent alteration in the fatty acid composition of the platelet phospholipids including increases in the percentages of 16:1 (palmitoleic acid) and 18:1 (oleic acid) and decreases in the percentages of 18:0 (stearic

TABLE II

Phospholipase A_2 Activity in Platelets from Hypercholesterolemic Rabbits

A[a]

Incubation time (min)	Control	HCR
	Radioactivity released (%)	
10	22.6	22.9
20	36.0	35.0

B[a]

		LPC[b]	SM	PC	PI	PS	PE	AA
		Radioactivity (%)						
Control	Pre[c]	0.9	1.1	47.2	17.5	13.3	20.0	
	Post	1.0	0.7	17.9	16.2	8.2	3.8	52.3
HCR[d]	Pre	1.0	1.1	61.7	7.5	9.2	19.7	
	Post	1.1	0.6	26.1	5.2	4.9	4.9	57.1

[a]Enzymic activity were determined as described in Materials and Methods, except that 1-palmitoyl-2-[1-^{14}C] arachidonyl phosphatidylcholine in A and platelets labeled with [1-^{14}C] arachidonic acid in B were used as substrate, respectively.

[b]LPC: lysophosphatidylcholine; SM: sphingomyeline; PC: phosphatidylcholine; PI: phosphatidylinositol; PS: phosphatidylserine; PE: phosphatidylethanolamine; AA: arachidonic acid.

[c]Pre: preincubation; post: postincubation.

[d]HCR: platelets from hypercholesterolemic rabbits.

TABLE III

Phospholipase C and Diglyceride Lipase Activities

	Phospholipase C[a]	Diglyceride lipase[b]
Control	6.72	6.40
HCR[c]	6.86	6.74

[a]Fatty acid released nmol/mg protein/60 min.

[b]Fatty acid released nmol/mg protein/30 min.

[c]Platelets from hypercholesterolemic rabbits.

TABLE IV

Cholesterol Esterase Activity

	Hydrolysis of cholesteryl esters (%)	Esterification of cholesterol (%)
Control	68.9	0.26
HCR[a]	27.5[b]	0.52[b]

[a]Platelets from hypercholesterolemic rabbits.

[b]$P<0.01$ compared to control.

TABLE V

Fatty Acid Composition of Platelet Phospholipids (A) and of Cholesteryl Esters (B)

A

		16:0	16:1	18:0	18:1	18:2	18:3	20:4	22:1
PC	C[a]	38.0	2.0	19.0	9.8	25.6	1.6	2.6	1.6
	H[b]	35.5	2.4	16.5	10.7	31.0[c]	1.2	2.5	trace
PI	C	23.6	4.2	36.1	10.4	4.9	trace	13.2	7.6
	H	28.2	7.0[d]	24.9[d]	14.1[c]	7.0	trace	10.3[c]	8.6
PS	C	10.9	0.8	42.0	7.0	21.7	trace	13.4	4.4
	H	10.9	3.7[d]	36.3[c]	14.0[d]	20.0	trace	10.0[c]	4.5
PE	C	9.2	1.4	23.3	6.5	14.5	trace	43.3	2.0
	H	11.9	3.2[d]	22.2	7.1	14.3	trace	37.0[c]	4.3[d]

B

	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:0	20:1	22:1
C[a]	18.3	3.4	5.7	13.0	4.2	5.8	4.1	10.5	10.5	26.1
H[b]	17.1	3.1	6.8	12.6	7.8[c]	6.2	4.8	9.4	5.9[c]	26.0

[a]Control.

[b]Hypercholesterolemia.

[c]$P<0.05$ compared to control.

[d]$P<0.01$ compared to control.

acid) and 20:4 (arachidonic acid).

DISCUSSION

The arachidonic acid released from phospholipids by phospholipase A_2 was rapidly converted into prostaglandins or prostaglandin-related substances. The formation of prostaglandins could be increased by many stimulatory factors, one of which may result from the increasing amount of polyunsaturated fatty acids through the deacylation of phospholipids by the action of phospholipase A_2. The phospholipase A_2 activities in healthy human (18) and normal rabbit platelets (29) were reported previously. In this study, we examined the phospholipase A_2 activity in platelets from hypercholesterolemic rabbits. Arachidonic acid was released mainly from PC, phosphatidylserine and phosphatidylethanolamine in platelets from both control and hypercholesterolemic rabbits. The rate of arachidonic acid released from these phospholipids in platelets from hypercholesterolemic rabbits was not significantly changed as compared to that of control rabbits (Table II). We also found no differences in the phospholipid content of platelets from either control or hypercholesterolemic rabbits (Table V). The total amount of released arachidonic acid, therefore, was not increased in platelets from hypercholesterolemic rabbits. Most workers have assumed that arachidonic acid metabolism is triggered by activation of phospholipase A_2. But, recently, a specific phospholipase C has been described in human platelets which cleaves inositol phosphate from PI when the cells are stimulated by thrombin (20). In that case, accumulation of diglyceride was observed. Arachidonic acid was released from this diglyceride by the action of diglyceride lipase (21). In our experiment, diglyceride lipase and phospholipase C activities also were unchanged in platelets from hypercholesterolemic rabbits. Increased aggregation of platelets from atheroschlerotic rabbits was reported previously (4). With regard to the increased aggregation of platelets from hypercholesterolemic rabbits, it was considered that the enzymic activities of thromboxane biosynthesis were enhanced or that the supply of precursor for thromboxane biosynthesis was promoted as the genesis. But, the enhanced activities of phospholipase A_2, phospholipase C and diglyceride lipase were not observed in the platelets from hypercholesterolemic rabbits. These results suggest that the release of arachidonic acid from phospholipids was unchanged and that the enzymic activities of thromboxane B_2 biosynthesis may be accelerated in platelets from hypercholesterolemic rabbits. Further experiments on prostaglandin endoperoxidase and thromboxane synthetase are needed to elucidate the detailed mechanisms of enhanced conversion of arachidonic acid to thromboxane

B_2 by platelets from hypercholesterolemic rabbits.

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The Prevention of Alcoholic Fatty Liver Using Dietary Supplements: Dihydroxyacetone, Pyruvate and Riboflavin Compared to Arachidonic Acid in Pair-Fed Rats

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ABSTRACT

Male Sprague-Dawley rats were fed for 30 days a high-fat liquid ethanol diet with dihydroxyacetone, pyruvate and riboflavin added as supplements (AMA-). Plasma triglyceride (TG) levels were 6-fold greater in these rats than in those fed and alcohol with without the supplements (AA-). The liver TG content in rats fed the AMA- diet was similar to that of rats fed a control diet (CA-) in which alcohol was replaced with isocaloric amounts of dextrose. Livers of rats fed the AA- diet had 3 times more TG than controls. Alcohol ingestion also enhanced the hepatic content of cholesteryl esters (CE) and phospholipids (PL). These lipids were reduced to levels found in livers of rats fed the control diet (CA-) when dihydroxyacetone, pyruvate and riboflavin were included in the alcohol diet. The fatty acid compositions of TG, CE and PL from livers of rats fed the AMA- diet were similar to those of corresponding lipids from rats fed the control diet (CA-) but differed from compositions when fed the alcohol diet (AA-). Regardless of the diet fed, TG had the same fatty acid composition in plasma and liver. The same was true of PL fatty acid composition. However, the fatty acid composition of CE differed between liver and plasma. The major fatty acid in liver CE was 18:1 whereas in plasma it was arachidonic acid (20:4). Reduced fatty liver was observed in an earlier study when rats were fed ad libitum an ethanol diet containing 20:4. In the present study, we pair-fed the same diet and fatty liver was not reduced. Dihydroxyacetone, pyruvate and riboflavin did not prevent alcohol-induced fatty liver when 20:4 was included in the AMA- diet. Our results confirm that dietary dihydroxyacetone, pyruvate and riboflavin prevent alcohol-induced fatty liver, and show that this effect may result from increased mobilization of fat from liver.

INTRODUCTION

The level of hepatic triglycerides (TG) becomes elevated in alcoholic fatty liver. This condition develops in rats even when they are fed nutritionally adequate diets containing alcohol (1,2). Until recently, attempts to reduce alcohol-induced fatty liver by dietary means were unsuccessful (3). However, we observed a significant reduction in fatty liver when rats were fed an alcohol diet with arachidonic acid (20:4) (4). In our earlier study, the alcohol diet containing 20:4 was fed ad libitum (4); in the present study, the same diet was pair-fed to determine if this method of feeding also reduces liver TG content.

Stanko et al. (5) reported that fatty liver was prevented by including dihydroxyacetone, pyruvate and riboflavin in an alcohol diet. When we examined the results of Stanko et al. and compared them to those of other studies, it was uncertain whether the inclusion of dihydroxyacetone, pyruvate and riboflavin prevented or only reduced fatty liver (4). In our study, we show that the inclusion of these supplements in an alcohol diet fed to rats not only prevents fatty liver, but also maintains the fatty acid compositions of the hepatic lipids at control levels.

Since fatty liver is either reduced or prevented by feeding ethanol diets containing 20:4, or dihydroxyacetone, pyruvate and riboflavin, respectively, we examined the effect of feeding these supplements on the hepatic TG content. Furthermore, we analyzed the content and fatty acid composition of plasma lipids to determine whether mobilization of fat from liver was increased under conditions in which fatty liver was prevented.

MATERIALS AND METHODS

Animals and Diets

Fifteen male Sprague-Dawley rats weighing ca. 300 g were obtained from Hilltop Lab Animals, Inc. (Chatsworth, CA). Rats were housed individually in stainless steel cages and fed Wayne Lab Blox diet (Allied Mills, Chicago, IL) ad libitum for 9 days. Animals were divided into 5 groups of 3 each. Each group consumed one of the following 5 Lieber/DeCarli liquid rat diets (Bio Mix-711, Bio-Serv, Inc., Frenchtown, NJ): (a) control (CA-), (b) ethanol (AA-), (c) ethanol with 7 wt % of the fat as arachidonic acid (AA+), (d) ethanol diet with 22 g/l dihydroxyacetone, 22 g/l pyruvate and 2.2 g/l riboflavin (AMA-), and (e) the contents of the AMA- diet with 7 wt % of the fat as 20:4 (AMA+). All diets were individually pair-fed to

animals in the AA- group for 30 days. Rats in the AA- group were fed ad libitum throughout the experiment. The amount of diet consumed by each rat in the AA- group was fed to a corresponding rat in each of the remaining diet groups. Dihydroxyacetone and pyruvate were obtained from Sigma (St. Louis, MO). Riboflavin was purchased from ICN Pharmaceuticals, Inc. (Cleveland, OH). Arachidonic acid (70% pure) was purchased from Nu-Chek Prep., Inc. (Elysian, MN). The fatty acid composition of the arachidonic acid was the same as reported previously (4). Butylated hydroxytoluene (BHT, 0.02%) was added to the fat used for the preparation of the diets. Diets were mixed according to supplier's instructions with the exception of the additions of 20:4, dihydroxyacetone, pyruvate and riboflavin. Diets were kept at 5 C under nitrogen and used within 72 hr. Fatty acid analysis of the diets indicated no loss of unsaturated fatty acids during either storage or feeding. Rats were fed fresh diets every morning. All rats consumed an average of 72.1 ml of diet/day. Rats in the AA-, CA-, AA+, AMA- and AMA+ groups gained a total of 41, 62, 23, 43 and 47 g in body wt, respectively, for the 30-day period.

Quantitation of Lipids from Liver and Plasma

After 30 days of feeding, rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/ml/300 g rat), exsanguinated and plasma isolated (6). Livers were removed, rinsed in ice-cold saline, blotted, trimmed free of mesentary tissue and weighed. Small portions of liver (ca. 0.5 g) were cut from the central portion of the left lobe and weighed. These portions were homogenized in ice-cold saline. Lipids were extracted from liver homogenates and plasma as described by Folch et al. (7). Lipid extracts were protected from the oxidation of unsaturated fatty acids by adding BHT (0.02%) and leaving in a nitrogen atmosphere. Phospholipids (PL), TG and cholesteryl esters (CE) were separated by thin layer chromatography on Silica Gel G plates using benzene as the developing solvent. Fatty acid methyl esters were prepared and quantitated as described previously (4).

RESULTS AND DISCUSSION

Liver Lipid Content

The lipid content of livers from rats fed each of the 5 diets is shown in Table I. The amounts of TG, CE and PL were greater in livers from rats fed the ethanol diet (AA-) than from rats fed the control diet (CA-). The most significant differences were in the levels of TG ($p<0.01$) and CE ($p<0.05$). Ethanol feeding increases the levels of hepatic TG (4,5,8-14), PL (4,12) and CE (8). However, the contents of liver total lipids, TG, CE and PL were reduced when rats were fed dihydroxyacetone, pyruvate and riboflavin with ethanol (AMA-) (Table I). Stanko et al. observed that addition of dihydroxyacetone, pyruvate and riboflavin to the diet prevented ethanol-induced accumulation of TG in the liver of rats pair-fed a twice-concentrated Liber/DeCarli liquid diet (5). In their study, the values for the hepatic TG content of rats fed the control diet were considerably greater than those observed in other studies (4,8). Hence, it was uncertain if the dietary additives prevented or only reduced

TABLE I

Content of Liver Lipids from Rats Fed Various Diets[a]

Diet	TL	TG	CE	PL
CA-	30.3 ± 6.3[c,i]	14.6 ± 5.9[d,k]	0.7 ± 0.2[f]	14.4 ± 0.8[g]
AA-	76.8 ± 6.4[h,i]	55.2 ± 3.1[k,l]	3.3 ± 0.7[e,f]	18.6 ± 1.3
AA+	74.8 ± 11.0[b,c]	63.9 ± 9.6[d,j]	1.9 ± 0.5	17.3 ± 4.0
AMA-	37.1 ± 0.9[b,h]	18.3 ± 2.3[j,l]	0.8 ± 0.2[e]	15.5 ± 1.1
AMA+	60.7 ± 15.9	42.0 ± 16.2	2.0 ± 0.7	17.4 ± 0.5[g]

[a]Rats were fed control (CA-), alcohol (AA-), alcohol with arachidonic acid (AA+), alcohol with dihydroxyacetone, pyruvate and riboflavin (AMA-), and AMA- with arachidonic acid (AMA+) diets. The content of the various lipid classes (TL=total lipids, TG=triglycerides, CE=cholesteryl esters and PL=phospholipids) are given as mg fatty acid methyl esters/g wet wt of livers. The wt of the methyl esters approximate the wt of TG. However, the true wt of PL is ca. 25-30% greater than the wt of methyl esters. The wt of CE is ca. twice the wt of CE methyl esters given. The TG, CE and PL fractions were separated from TL of livers by thin layer chromatography. These were quantitated by the analysis of their fatty acid methyl esters by gas liquid chromatography using methyl pentadecanoate as an internal standard (4). Each value represents the mean ± SEM of average values from duplicate analyses of livers from 3 animals. Values with a common superscript are significantly different using the two-tailed t-test with: [b-g]$p<0.05$, [h-k]$p<0.01$, [l]$p<0.001$.

TABLE II

Content of Plasma Lipids from Rats Fed Various Diets[a]

Diet	TL	TG	CE	PL
CA-	2.3 ± 0.5	1.1 ± 0.3	0.39 ± 0.04	1.04 ± 0.05
AA-	2.3 ± 0.2[b]	0.6 ± 0.2[d]	0.64 ± 0.09[f]	1.24 ± 0.19
AA+	2.2 ± 0.1[c]	0.59 ± 0.02[e]	0.46 ± 0.03[g]	0.76 ± 0.20
AMA-	4.9 ± 0.9[b,c]	3.7 ± 1.1[d,e]	0.32 ± 0.02[f,g]	1.04 ± 0.05
AMA+	2.6 ± 0.3	1.0 ± 0.3	0.43 ± 0.04	1.02 ± 0.01

[a]Descriptions of the diets and abbreviations are given in Table I. Total lipids were separated from plasma and various classes were quantitated as described in Table I. Each value represents the mean ± SEM of average values of mg fatty acid methyl esters/ml plasma from duplicate analyses of plasma from 3 animals. Values with a common superscript are significantly different using the two-tailed t-test with $p<0.05$.

hepatic TG accumulation in alcohol-fed animals (4). The results of this study with rats pair-fed normal concentrations (prepared according to the suppliers' instructions) of the Lieber/DeCarli diets confirm the conclusions of Stanko et al. that the inclusion of dihydroxyacetone, pyruvate and riboflavin (AMA-) reduces the liver TG content to control levels in alcohol-fed rats (Table I). Our values for the liver TG content were lower in rats fed either the CA- or AMA- diet (Table I) than those reported by Stanko et al. (5). This difference may result from the sensitivity of the methods used for quantitating TG. Stanko et al. used a spectrophotometric method, whereas in our study, a more sensitive gas liquid chromatographic (GLC) procedure was used. Analysis of hepatic lipids showed that feeding dihydroxyacetone, pyruvate and riboflavin also caused a reduction in the levels of PL and CE in ethanol-fed rats (Table I).

Only hepatic total lipids (TL), and TG esters were quantitated in the earlier study by Stanko et al. on the prevention of alcohol-induced fatty liver (5). From their results, we concluded that TG levels increased, whereas PL and CE levels decreased in livers of rats fed the alcohol diet without supplements (4). This contradicts results of other studies which show that not only TG (4,5,8-14), but also PL (4,12), cholesterol and CE (8) increase in the liver with alcohol consumption. Our results show increased levels of these liver lipids in rats fed an alcohol diet (AA-) (Table I).

Dietary 20:4 reduces hepatic TG and PL when fed ad libitum in an alcohol diet (4). However, such a reduction in these liver lipids was not seen when rats were pair-fed an alcohol diet containing 20:4 (AA+) (Table I). This difference in liver lipid contents between rats fed ad libitum and pair-fed is probably related to the amount of diet consumed. Ad libitum feeding provides more nutrients and a continuous food supply to the liver, whereas pair-feeding produces periods of food deprivation and fasting. Such cyclic deprivation-feeding programs can significantly influence various metabolic reactions in the liver (15).

Although dietary dihydroxyacetone, pyruvate and riboflavin prevented fatty liver, when 20:4 was also included (AMA+), fatty liver was not prevented (Table I). Dihydroxyacetone and 20:4 could serve as precursors of lipids. However, the interrelationships between these compounds in lipid synthesis and in alcohol-induced fatty liver are unknown.

Plasma Lipid Contents

Plasma TG levels were lower in rats fed an alcohol diet with (AA+) or without (AA-) 20:4 than in those fed the control diet (CA-) (Table II). This was true even when the hepatic TG levels were higher in the alcohol-fed rats (AA-, AA+) than in controls (CA-) (Table I). Baraona and Lieber (16) have observed high levels of lipoproteins of density <1.019 in alcohol-fed rats. Since the major component of lipoproteins of density <1.019 is TG, increased levels of TG should be present in the plasma in their study. The difference in plasma TG levels observed in these 2 studies can be attributed to the method of feeding. In the investigation by Baraona and Lieber (16), plasma lipoproteins were analyzed postprandially and gastric intubation used to force-feed rats before sacrifice. In our study, rats were not force-fed. Our results show that in animals fed an alcohol diet ad libitum, plasma TG are reduced below control values.

When dihydroxyacetone, pyruvate and riboflavin were fed with alcohol (AMA-), plasma TG levels were elevated 6-fold from those of alcohol-fed animals (Table II). Although the control rats (CA-) and those fed the AMA- diet consumed the same amount of food and had similar levels of hepatic TG (Table I), their plasma TG levels were significantly different.

The plasma TG content of control (CA-) rats was only 1/3 as high as in the AMA- group (Table II). This difference could be a result of the time interval between diet consumption and sacrifice. The rats in this study were provided with diet every morning including the day of sacrifice. Rats that were pair-fed the control diet always consumed their food within the first few hr after feeding, then fasted during most of the day. Rats fed the AMA- diet consumed diet all day as if they were fed ad libitum. This cyclic feeding/fasting regimen is likely to influence plasma lipid levels. If rats were fed the CA- diet ad libitum, their plasma lipid levels may have been elevated as high as in rats fed the AMA- diet.

The content of plasma total lipids was similar in all diet groups except for the AMA- group (Table II). The increased level of total lipids in the AMA- group was caused by elevated plasma TG. The plasma of rats fed alcohol (AA-, AA+) contained higher levels of CE than those of the control (CA-) or AMA- diet groups. The plasma levels of PL were not significantly altered in rats fed the various diets (Table II). The differences observed in the relative levels of TG, CE and PL in plasma may reflect variations in the levels of lipoproteins.

Rats in the AA- and AMA- groups had food available to them throughout a 24-hr period every day during the experiment. Animals in those 2 groups consumed similar amounts of diet/day. However, plasma TG levels of these groups differed by 6-fold (Table II). Triglycerides of the plasma originate from diet fat (chylomicrons) or from liver (lipoproteins). The low levels of plasma TG in rats given AA- and AA+ diets indicate that alcohol may reduce intestinal absorption of fat or inhibit TG mobilization from liver. We have observed that intestinal absorption of fat was essentially complete (90-95%) by analysis of the fecal fat content of rats fed the Lieber/DeCarli ethanol diet. Others also have reported that fat absorption is unaffected by alcohol (16). A decrease in intestinal absorption of fat would reduce, rather than accentuate, the development of fatty liver, because the TG that accumulates in liver of alcohol-fed animals is mostly of diet origin (4,9). Hence, it is likely that the lower plasma TG content of rats in the AA- diet group reflects a reduced mobilization of TG from the liver. On the other hand, the elevated plasma TG content of rats fed the AMA- diet could result from increased release of TG from liver. The results of the analysis of the fatty acid compositions of TG from liver and plasma, discussed in the following sections, further support that plasma TG of rats fed the AMA- diet are not of direct origin from dietary fat.

Liver Lipid Fatty Acid Composition

The fatty acid composition of liver TL (Table III), TG (Table IV), PL (Table V) and CE (Table VI) were altered by the type of diet fed. Each lipid class had a characteristic fatty acid composition: 18:0 was a major fatty acid in PL (Table V), whereas relatively low levels of this acid were present in TG (Table IV) and CE (Table VI). Oleic acid was a major component

TABLE III

Fatty Acid Composition of Liver Total Lipids[a] from Rats Fed Various Diets

Acid	Group				
	CA-	AA-	AA+	AMA-	AMA+
16:0	19.3 ± 0.6	16.3 ± 1.3	15.0 ± 0.2	20.3 ± 1.1	19.4 ± 1.7
16:1	1.9 ± 0.4	1.8 ± 0.2	1.8 ± 0.1	3.3 ± 0.7	2.3 ± 0.4
18:0	12.5 ± 2.6	8.2 ± 0.4	5.9 ± 1.4	11.5 ± 0.4	9.0 ± 2.5
18:1	31.8 ± 5.9	43.6 ± 0.6	36.3 ± 1.8	33.0 ± 1.1	30.9 ± 4.5
18:2	11.4 ± 0.6	17.1 ± 1.0	16.8 ± 1.3	13.0 ± 1.2	13.9 ± 1.5
20:4	16.6 ± 3.1	9.3 ± 0.03	15.2 ± 1.4	13.9 ± 0.4	18.0 ± 3.4
24:0	T	0.7 ± 0.2	2.8 ± 0.4	T	1.8 ± 0.4
22:6	2.6 ± 0.6	1.3 ± 0.2	1.3 ± 0.1	1.7 ± 0.2	1.6 ± 0.2
$\frac{18:2}{20:4}$	0.69 ± 0.15	1.85 ± 0.10	1.14 ± 0.17	0.94 ± 0.08	0.85 ± 0.20
$\frac{16:0}{16:1}$	10.6 ± 1.5	9.3 ± 0.3	8.3 ± 0.6	6.7 ± 1.3	8.6 ± 1.2

[a]Values are given as the percentage of total fatty acids. The total lipids were extracted from liver and their fatty acid methyl esters were prepared and analyzed by gas liquid chromatography (4). See Table I for diet abbreviations. Percentages of total fatty acids are given as the mean ± SEM of average values from duplicate analyses of liver from 3 rats in each diet group. Values of 0.5% or less are given as T. The values for 24:0 also include 22:4 and 24:1.

TABLE IV

Fatty Acid Composition of Liver Triglycerides from Rats Fed Various Diets[a]

Acid	Group				
	CA-	AA-	AA+	AMA-	AMA+
16:0	23.8 ± 0.7	16.8 ± 1.1	15.7 ± 0.8	24.9 ± 1.8	22.8 ± 3.4
16:1	4.7 ± 1.8	2.1 ± 0.3	2.1 ± 0.2	5.0 ± 1.1	3.3 ± 0.7
18:0	1.4 ± 0.1	1.9 ± 0.4	1.1 ± 0.2	1.3 ± 0.1	1.1 ± 0.1
18:1	54.3 ± 0.5	51.0 ± 0.4	42.1 ± 1.3	53.0 ± 0.5	42.0 ± 0.2
18:2	14.5 ± 1.3	19.3 ± 0.6	19.7 ± 1.0	13.7 ± 1.8	17.7 ± 0.9
20:4	0.9 ± 0.4	3.5 ± 0.8	10.0 ± 0.7	0.6 ± 0.3	7.6 ± 1.5
22:4	T	1.1 ± 0.2	3.2 ± 0.5	T	T
22:5	T	T	0.9 ± 0.4	T	T
22:6	T	0.6 ± 0.2	0.6 ± 0.3	T	0.5 ± 0.3
18:2/20:4	18.7 ± 5.7	8.0 ± 1.0	2.0 ± 0.2	17.9 ± 0.4	2.5 ± 0.5
16:0/16:1	6.7 ± 1.3	8.2 ± 0.5	7.2 ± 0.7	5.4 ± 1.0	7.2 ± 0.6

[a]Values are given as the percentage of total fatty acids. Triglycerides were isolated from the total lipids of liver by thin layer chromatography and their fatty acid methyl esters were prepared and analyzed by gas liquid chromatography (4). See Table I for diet abbreviations. Percentages of fatty acids are given as the mean ± SEM of average values from duplicate analyses of liver from 3 rats in each diet group. Values of 0.5% or less are given as T.

TABLE V

Fatty Acid Composition of Liver Phospholipids from Rats Fed Various Diets[a]

Acid	Group				
	CA-	AA-	AA+	AMA-	AMA+
16:0	17.5 ± 0.5	13.3 ± 1.1	13.8 ± 0.4	16.3 ± 0.2	17.0 ± 2.2
16:1	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	1.2 ± 0.1	0.9 ± 0.2
18:0	23.8 ± 0.6	28.2 ± 1.8	24.6 ± 0.5	23.8 ± 1.4	23.0 ± 0.4
18:1	9.7 ± 0.5	10.2 ± 0.3	11.9 ± 0.4	11.3 ± 0.4	9.8 ± 0.8
18:2	8.4 ± 0.1	9.8 ± 0.8	7.3 ± 0.2	11.5 ± 1.1	7.7 ± 0.8
20:4	32.0 ± 1.2	30.1 ± 0.3	35.5 ± 1.0	29.5 ± 1.1	34.5 ± 1.8
24:0	0.9 ± 0.7	T	T	T	1.0 ± 0.4
22:6	4.0 ± 0.8	4.0 ± 0.3	3.3 ± 0.7	3.1 ± 0.3	2.8 ± 1.4
18:2/20:4	0.26 ± 0.009	0.69 ± 0.34	0.21 ± 0.01	0.40 ± 0.05	0.23 ± 0.03
16:0/16:1	32.8 ± 8.3	34.8 ± 3.9	25.3 ± 2.8	14.2 ± 1.2	20.8 ± 2.6

[a]Values are given as the percentage of total fatty acids. Phospholipids were isolated from the total lipids of liver by thin layer chromatography and their fatty acid methyl esters were prepared and analyzed by gas liquid chromatography (4). See Table I for diet abbreviations. Percentages of fatty acids are given as the mean ± SEM of average values from duplicate analyses of liver from 3 rats in each diet group. Values of 0.5% or less are given as T. The values for 24:0 also include 22:4 and 24:1.

(50%) of CE and TG, whereas this acid was present in only small levels (10%) in PL (Table V). As observed previously (4), the fatty acid composition of TG from rats fed the AA- diet and AA+ diets was found to be different from that of control (CA-) rats (Table IV), and was similar to the fatty acid composition of dietary fat (4).

The percentage composition of various fatty acids in the total lipid, TG, PL and CE from livers of rats fed the AMA- diet was similar to the corresponding lipids from livers of control rats (Tables IV-VI). Hence, the inclusion of dihydroxyacetone, pyruvate and riboflavin not only prevented fatty liver (Table I), but also prevented changes in the composition of the fatty acids of TG (Table IV), CE (Table V), and PL (Table VI) caused by alcohol feeding.

The relative levels of 16:1 in liver TG and CE in rats fed the AA- diet were lower than in controls (CA-) (Tables IV and VI). Reduced levels of 16:1 reflect decreased hepatic desaturase activity in alcohol-fed rats (4,17,18). The levels of 16:1 in liver TG and CE of rats fed the

TABLE VI

Fatty Acid Composition of Liver Cholesteryl Esters from Rats Fed Various Diets[a]

Acid	Group				
	CA-	AA-	AA+	AMA-	AMA+
16:0	21.2 ± 3.0	8.5 ± 0.1	7.2 ± 1.6	18.2 ± 3.5	13.2 ± 3.6
16:1	4.8 ± 0.9	2.1 ± 0.3	2.1 ± 0.3	5.2 ± 0.7	4.2 ± 1.0
18:0	2.7 ± 0.5	2.4 ± 0.5	1.6 ± 0.7	5.4 ± 3.0	2.8 ± 0.6
18:1	50.8 ± 3.2	68.6 ± 1.5	64.6 ± 2.4	47.4 ± 8.2	55.2 ± 5.4
18:2	9.4 ± 0.8	10.8 ± 0.6	11.2 ± 0.6	8.8 ± 1.3	9.2 ± 0.8
20:4	8.4 ± 0.5	5.1 ± 1.2	11.2 ± 0.5	10.2 ± 2.3	11.9 ± 0.4
$\frac{18:2}{20:4}$	1.11 ± 0.06	2.38 ± 0.47	1.02 ± 0.10	0.96 ± 0.09	0.78 ± 0.09
$\frac{16:0}{16:1}$	4.6 ± 0.6	4.1 ± 0.5	3.3 ± 1.1	3.6 ± 0.8	3.2 ± 0.3

[a]Values are given as the percentage of total fatty acids. Cholesteryl esters were isolated from the total lipids of liver by thin layer chromatography and their fatty acid methyl esters were prepared and analyzed by gas liquid chromatography (4). See Table I for diet abbreviations. Percentages of fatty acids are given as the mean ± SEM of average values from duplicate analyses of liver from 3 rats in each diet group. Values of 0.5% or less are given as T.

AMA- or CA- diets were similar ($p>0.05$) (Tables IV and VI). Increased levels of 16:1 suggest that the alcohol-induced inhibition of hepatic desaturase activity may be reversed by dietary dihydroxyacetone, pyruvate and riboflavin. The levels of both 16:0 and 16:1 increased in liver TG and CE in rats fed the AMA- diet. Hence, the ratio of 16:0 to 16:1 was not significantly altered ($p>0.05$) from the ratio observed with rats fed the AA- diet (Tables IV and VI). It is likely that the synthesis of both 16:0 and 16:1 is stimulated in the livers of rats fed the AMA- and control diets as compared to those fed the AA- diet.

The ratio of the levels of 18:2 to 20:4 was several-fold greater in liver total lipids of rats fed the AA- diet than in controls when rats were fed ad libitum (4). Such an increase in the ratio also was observed in our study in which rats were pair-fed the same diets (Table III). Furthermore, as observed earlier (4), the 18:2/20:4 ratio from liver TG was smaller in alcohol-fed rats (AA-) than in controls (Table IV). The 18:2/20:4 ratio returned to control values in liver TL (Table III), TG (Table IV), PL (Table V) and CE (Table VI) when dihydroxyacetone, pyruvate and riboflavin were fed with ethanol (AMA-).

When 20:4 was fed with alcohol (AA+), the 18:2/20:4 ratios in liver TL, TG and PL were similar to those from corresponding lipids of rats fed the AMA+ diet (Table III-V). Although rats were pair-fed in this study, the values for the 18:2/20:4 ratios in their liver lipids were similar to those from rats fed the AA+ diet ad libitum (4). Fatty liver was reduced when rats were fed the AA+ diet ad libitum (4), but was not when they were pair-fed the same diet (Table I). These findings suggest that the reduction of fatty liver observed upon feeding the AA+ diet (4) could be related to increased diet consumption and not to the changes in the relative levels of 18:2 and 20:4 in hepatic lipids.

Plasma Lipid Fatty Acid Composition

Plasma fatty acids of rats fed the CA-, AA-, AA+ or AMA+ diets contained relatively more 20:4 (Table VII) than when compared to the level in their liver lipids (Table III). Plasma lipid contained low levels of 20:4 when rats were fed the AMA- diet (Table VII). The levels were only 50 or 25% of those observed in plasma of rats fed the CA- or AA+ diet groups, respectively. The ratio of 18:2 to 20:4 was significantly increased ($p<0.05$) in plasma of rats fed the AMA- diet because of the reduced levels of 20:4 (Table VII). The enhanced 18:2/20:4 ratio must be a result of the presence of a relatively large amount of TG (Table II) containing trace levels of 20:4 (Table VIII).

In each diet group, the composition of various fatty acids from plasma TG (Table VIII), and PL (Table IX) were similar to the fatty acid composition of corresponding lipids from liver (Tables IV and V). In rats fed the AMA- diet, the fatty acid composition of plasma TG (Table VIII) was similar to that of liver TG (Table IV) but different from the composition of dietary fat (4). This finding suggests that the plasma TG of rats fed the AMA- diet could have been derived from liver TG, rather than directly from dietary fat as chylomicrons. This conclusion is further

TABLE VII

Fatty Acid Composition of Plasma Total Lipids from Rats Fed Various Diets[a]

Acid	Diet group				
	CA-	AA-	AA+	AMA-	AMA+
16:0	17.2 ± 1.4	13.6 ± 1.2	14.3 ± 0.5	19.0 ± 1.7	15.8 ± 1.1
16:1	1.7 ± 0.4	1.0 ± 0.2	1.2 ± 0.1	2.9 ± 0.6	1.5 ± 0.3
18:0	9.7 ± 0.5	12.8 ± 2.1	11.8 ± 0.7	6.6 ± 0.5	10.4 ± 1.1
18:1	30.4 ± 2.3	25.9 ± 5.6	21.1 ± 1.5	39.3 ± 2.6	23.4 ± 2.9
18:2	16.8 ± 0.3	15.8 ± 1.2	11.3 ± 0.6	19.7 ± 1.6	11.5 ± 0.6
20:4	20.8 ± 1.2	26.6 ± 5.7	36.9 ± 1.5	9.1 ± 0.9	31.8 ± 3.9
24:0	T	0.5 ± 0.1	0.5 ± 0.3	T	1.3 ± 0.4
22:6	0.5 ± 0.3	1.2 ± 0.1	T	T	0.82 ± 0.04
18:2/20:4	0.81 ± 0.05	0.69 ± 0.22	0.31 ± 0.03	2.28 ± 0.37	0.37 ± 0.06
16:0/16:1	10.9 ± 1.7	13.5 ± 1.3	12.6 ± 1.0	6.2 ± 1.4	11.0 ± 1.4

[a]Values are given as the percentage of total fatty acids. The total lipids were extracted from plasma and their fatty acid methyl esters were prepared and analyzed by gas liquid chromatography (4). See Table I for diet abbreviations. Percentages of total fatty acids are given as the mean ± SEM of average values from duplicate analyses of plasma from 3 rats in each diet group. Values of 0.5% or less are given as T. The values for 24:0 also include 22:4 and 24:1.

TABLE VIII

Fatty Acid Composition of Plasma Triglycerides from Rats Fed Various Diets[a]

Acid	Diet group				
	CA-	AA-	AA+	AMA-	AMA+
16:0	17.1 ± 1.0	15.3 ± 0.9	11.7 ± 1.5	18.1 ± 0.7	18.0 ± 1.5
16:1	2.3 ± 0.5	1.3 ± 0.4	1.4 ± 0.1	3.1 ± 0.5	3.3 ± 0.4
18:0	2.2 ± 0.5	2.7 ± 0.4	2.0 ± 0.2	1.7 ± 0.1	1.7 ± 0.2
18:1	56.8 ± 1.1	56.6 ± 1.3	50.1 ± 0.7	55.7 ± 0.5	46.0 ± 1.2
18:2	17.9 ± 0.5	18.7 ± 0.7	20.4 ± 0.3	18.1 ± 0.5	19.6 ± 0.7
20:4	1.4 ± 0.1	2.7 ± 0.8	10.5 ± 1.2	0.9 ± 0.2	9.4 ± 0.8
18:2/20:4	13.5 ± 1.6	8.1 ± 2.0	2.3 ± 0.4	22.8 ± 3.7	2.1 ± 0.1
16:0/16:1	8.2 ± 1.5	16.0 ± 6.1	8.3 ± 1.0	6.1 ± 0.9	5.6 ± 0.9

[a]Values are given as the percentage of total fatty acids. Triglycerides were isolated from the total lipids of plasma by thin layer chromatography and their fatty acid methyl esters were prepared and analyzed by gas liquid chromatography (4). See Table 1 for diet abbreviations. Percentages of fatty acids are given as the mean ± SEM of average values from duplicate analyses of plasma from 3 rats in each diet group. Values of 0.5% or less are given as T.

supported by the levels of 16:1 in plasma TG. Dietary fat contained only trace levels of 16:1 (4). The plasma of rats fed the AMA- diet not only contained higher levels of 16:1 (Table VII) but also contained large amounts of TG (Table II). Since 16:1 is mainly synthesized in the liver, our results suggest that, when rats were fed the AMA- diet, triglycerides produced in the liver were released to the plasma. Livers of rats fed the AMA- diet may have an increased capacity to mobilize fat, thereby reducing fatty liver. This conclusion is consistent with the hypothesis that alcohol promotes fatty liver by blocking the release of lipoproteins (10,19).

It is possible that plasma lipids are of hepatic origin since, in rats fed the various diets, the fatty acid compositions of plasma TG or PL were similar to those of corresponding liver lipids. However, this conclusion cannot be extended to CE. The fatty acid composition of plasma CE (Table X) was significantly different from that of liver CE in each dietary condition used. In liver CE, the levels of 18:1 were high (50-60%), whereas in plasma, these levels were low (10-20%) (Tables VI and X). Furthermore, 20:4 comprised ca. 50-70% of the total acids of plasma CE whereas only low levels (10%) of 20:4 were found in liver CE. These differences

TABLE IX

Fatty Acid Composition of Plasma Phospholipids from Rats Fed Various Diets[a]

Acid	Diet group				
	CA-	AA-	AA+	AMA-	AMA+
16:0	19.8 ± 0.9	16.9 ± 1.0	19.7 ± 1.1	20.0 ± 1.6	20.8 ± 0.2
16:1	0.50 ± 0.03	0.42 ± 0.04	0.57 ± 0.05	0.82 ± 0.41	0.68 ± 0.04
18:0	21.7 ± 0.3	26.4 ± 2.0	22.9 ± 0.7	21.7 ± 1.1	22.9 ± 0.9
18:1	11.9 ± 0.8	12.2 ± 1.1	12.0 ± 0.2	14.6 ± 0.2	11.8 ± 1.3
18:2	13.9 ± 0.9	13.9 ± 0.8	8.3 ± 0.4	17.8 ± 1.3	7.2 ± 0.7
20:4	25.5 ± 0.8	24.9 ± 0.7	32.6 ± 0.7	21.9 ± 0.4	32.7 ± 1.9
24:0	1.1 ± 0.1	1.0 ± 0.2	0.9 ± 0.3	T	T
22:6	1.7 ± 0.2	1.6 ± 0.2	0.9 ± 0.4	1.0 ± 0.1	T
18:2/20:4	0.56 ± 0.05	0.56 ± 0.05	0.25 ± 0.02	0.82 ± 0.07	0.22 ± 0.03
16:0/16:1	40.6 ± 4.2	42.4 ± 3.1	37.6 ± 4.4	26.5 ± 5.9	30.7 ± 1.7

[a]Values are given as the percentage of total fatty acids. Phospholipids were isolated from the total lipids of plasma by thin layer chromatography and their fatty acid methyl esters were prepared and analyzed by gas liquid chromatography (4). See Table I for diet abbreviations. Percentages of total fatty acids are given as the mean ± SEM of values from duplicate analyses of plasma from 3 rats in each diet group. Values of 0.5% or less are given as T. The values for 24:0 also include 22:4 and 24:1.

in fatty acid compositions of CE suggest that, if hepatic CE are released to plasma, acyl exchange reactions involving 20:4 may have occurred during circulation, altering specifically the fatty acid composition of plasma CE. Alternately, liver may synthesize different species of CE. Those containing large amounts of 18:1 may be retained whereas those with high levels of 20:4 may be released from the liver. These processes must occur even during the development of fatty liver because the levels of 18:1 in liver CE and of 20:4 in plasma CE were also high in rats fed AA- and AA+ diets.

In summary, results of our study confirm the earlier reports of Stanko et al. (5) that the addition of dihydroxyacetone, pyruvate and riboflavin to an ethanol diet fed to rats prevents fatty liver. We have shown that the fatty acid composition of liver lipids also was maintained at control levels when this diet was fed. Because TG do not accumulate in the liver in spite of feeding alcohol, dietary supplementation with these compounds must have altered the metabolism so as to stimulate the mobilization of TG from liver. Our results on the analysis of the content and fatty acid composition of lipids from plasma and liver suggest that increased

TABLE X

Fatty Acid Composition of Plasma Cholesteryl Esters from Rats Fed Various Diets[a]

Acid	Diet group				
	CA-	AA-	AA+	AMA-	AMA+
16:0	10.1 ± 3.1	6.0 ± 0.2	6.6 ± 0.4	8.7 ± 1.3	7.2 ± 0.7
16:1	1.6 ± 0.03	1.0 ± 0.1	1.2 ± 0.2	4.1 ± 0.8	1.7 ± 0.4
18:0	0.6 ± 1.1	0.5 ± 0.1	T	T	1.2 ± 0.5
18:1	12.6 ± 1.1	13.0 ± 1.5	8.8 ± 0.6	20.3 ± 3.7	11.2 ± 1.2
18:2	14.3 ± 0.9	14.7 ± 0.3	7.1 ± 0.1	15.1 ± 1.6	5.6 ± 0.2
20:4	59.2 ± 4.6	63.7 ± 1.1	75.1 ± 0.6	47.7 ± 3.7	72.2 ± 2.2
18:2/20:4	0.25 ± 0.03	0.22 ± 0.02	0.09 ± 0.003	0.33 ± 0.06	0.08 ± 0.006
16:0/16:1	6.4 ± 2.1	6.2 ± 0.4	5.9 ± 0.9	2.3 ± 0.4	4.9 ± 1.3

[a]Values are given as the percentage of total fatty acids. Cholesteryl esters were isolated from the total lipids of plasma by thin layer chromatography and fatty acid methyl esters were prepared and analyzed by gas liquid chromatography (4). See Table I for diet abbreviations. Percentages of total fatty acids are given as the mean ± SEM of values from duplicate analyses of plasma from 3 rats in each diet group. Values of 0.5% or less are given as T.

mobilization of hepatic lipids to plasma could have occurred when rats were fed the AMA-diet. The capacity of dietary 20:4 to reduce alcohol-induced fatty liver apparently depends on feeding the diet ad libitum (4), because when rats were pair-fed AA+ diet, fatty liver was not reduced. Moreover, although fatty liver was abolished in rats when pair-fed an ethanol diet containing dihydroxyacetone, pyruvate and riboflavin, addition of 20:4 to such a diet did not prevent fatty liver. Hence, our study demonstrates the importance of the roles played by the mode of feeding and the type of the dietary supplements in reducing alcohol-induced fatty liver.

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The Role of Lipid Peroxidation during Chronic and Acute Exposure to Ethanol As Determined by Pentane Expiration in the Rat

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ABSTRACT

Weanling rats were fed one of 3 diets containing 0, 11 or 200 international units (IU) dl-α-tocopherol acetate/kg diet for 4 weeks. Following this period, the drinking water was replaced with an 18% solution of ethanol (v/v). An isocaloric D-glucose solution was substituted for the drinking water of a control group of rats fed the vitamin-E-deficient diet for 4 weeks. The 4 treatment groups were maintained on the diet and drinking regimen for 20 weeks. Basal levels of expired pentane were determined at weeks 0, 1, 3, 5, 7 and 9. Chronic ethanol consumption did not influence basal pentane production during the 9-week treatment. Basal levels of expired pentane were affected by dietary vitamin E. Rats supplemented with vitamin E had basal pentane levels less than one-half of the level of rats fed a vitamin-E-deficient diet ($p < 0.001$). After 14 weeks of treatment, the 2 groups of rats fed a vitamin-E-deficient diet were administered p.o. an acute dose of 6 g of ethanol/kg body wt. Pentane expired above basal levels during the following 4-hr period correlated with the amount of hepatic triglycerides determined at the conclusion of the experiment. The etiology of ethanol toxicity is a complex and multifactorial system made up of many biological variables that influence lipid peroxidation. The appropriate choices of experimental designs and methods are important in examining the role of lipid peroxidation.

INTRODUCTION

The etiology of ethanol toxicity is thought by some investigators to include lipid peroxidation. Di Luzio (1) reported that antioxidant pretreatment inhibited acute ethanol-induced fatty livers in rats and concluded that the hepatotoxicity of ethanol strongly involved lipid peroxidation (2). Further studies in vitro by Di Luzio and colleagues provided evidence to support the occurrence of lipid peroxidation in ethanol toxicity. Acute ethanol administration triggered accumulation of liver triglycerides and increased peroxide values (3), elevated liver malonaldehyde production in liver homogenates (4), and caused the formation of conjugated dienes in isolated mitochondria and microsomes (5), whereas antioxidant treatment diminished these changes.

A controversy over the role of lipid peroxidation was begun when Hashimoto and Recknagel (6) reported no increased conjugated dienes in rat liver mitochondrial or microsomal lipids following administration of a single dose of ethanol. Other investigators using in vitro assays have questioned the ethanol-induced occurrence of lipid peroxidation. In contrast to these cited reports, administration of an acute dose of ethanol neither caused an increase in liver peroxide values (7) nor elicited liver malonaldehyde production (8,9). Studies of chronic ethanol consumption by rats have continued the controversy by providing evidence in favor of (10-12) and against (13) the involvement of lipid peroxidation in the toxicity of ethanol.

The observation by Riely et al. (14) of the evolution of ethane by mice after being injected with carbon tetrachloride has led to the use of monitoring expired hydrocarbons, such as pentane and ethane, as an index of in vivo lipid peroxidation. In vitro evidence suggesting that an acute dose of ethanol induces lipid peroxidation has stimulated several investigators (15-17) to monitor expired pentane and ethane during ethanol intoxication of lab animals. All 3 studies reported elevated pentane or ethane levels in rat breath following administration. Litov et al. (16) showed that rats given vitamin E in the diet had a smaller increase in expired pentane than rats fed a vitamin-E-deficient diet. Our study examined the effects of chronic consumption of ethanol and administration of an acute dose of ethanol during the chronic exposure on pentane production by the rat.

MATERIALS AND METHODS

Animals and Treatments

Male Sprague-Dawley rats (Simonsen Laboratories, Inc., Gilroy, CA) obtained at 3 wk of age were housed as pairs in hanging wire cages and were kept on a 14-hr light and 10-hr dark cycle at 22-24 C.

The basal diet was a powdered, vitamin-E-deficient diet (18) that contained additional amounts of nutrients as follows (g/kg diet): manganese sulfate, 0.167; cupric sulfate, 0.016; calcium phosphate (dibasic), 7.46; magnesium sulfate, 1.64; pyridoxine HCl, 0.004; choline

chloride, 0.250; and calcium pantothenate, 0.003. Two other diets were prepared by supplementing the basal diet with either 11 international units (IU) or 200 IU dl-α-tocopherol acetate/kg of diet. Rats were fed one of the 3 diets ad libitum for 4 wk. After 4 wk, an 18% ethanol solution (v/v) was substituted for the drinking water in 3 dietary groups of 6 rats each. Fluid consumption by the rats was recorded. A D-glucose solution was substituted for the drinking water of a control group of 6 rats fed the vitamin-E-deficient diet for 4 weeks. The caloric content of the D-glucose solution was adjusted on the basis of fluid consumption so that the number of calories consumed was equal to that consumed by the rats given ethanol. The diet and ethanol or D-glucose regimens were continued for 20 wk. After 14 wk of treatment, rats fed the vitamin-E-deficient diet and given ethanol or D-glucose drinking solutions were fasted for 16 hr prior to being administered an acute dose of ethanol. The rats were given a 50% solution of 95% ethanol in normal saline (v/v), 15.6 ml/kg body wt, by stomach tube. This corresponded to a dose of 6 g of ethanol/kg body wt. Ethanol solutions were prepared from bulk 95% ethanol that had been redistilled and passed through a 1 m × 1.5 cm column filled with 6-14 mesh activated charcoal.

Pentane Analysis

Each animal was tested for pentane production after being fasted for 16 hr. Basal pentane was determined at 0, 1, 3, 5, 7 and 9 wk of treatment with an 18% ethanol drinking solution or an isocaloric D-glucose drinking solution. After 14 wk of treatment, pentane levels in the rats fed the vitamin-E-deficient diet and administered an acute oral dose of ethanol were determined. Breath samples were collected at 0 (basal), 0.5, 1, 2, 3 and 4 hr after ethanol administration. Collection of samples of air expired by rats and their subsequent analyses for pentane were done according to the procedure described by Dillard et al. (19). Pentane production is expressed as picomoles (pmol) of pentane produced/100 g body weight/min of breathing. Respiration rates were not measured, but no marked differences in the breathing rate of any of the rats was observed. Statistical analysis of the data was made on a Tektronix 4051 minicomputer system (Tektronix, Beaverton, OR).

Liver and Blood Analysis

Upon completion of the study after 20 wk of treatment with diets and drinking solutions, rats were anesthetized with pentobarbital and blood was removed by heart puncture using heparinized syringes and needles. Four g of fresh liver were used to prepare mitochondrial and microsomal samples for determination of conjugated dienes (6). One-half ml of fresh blood was used to assay for glutathione peroxidase (20). The remaining liver was frozen for later analysis of triglycerides, cholesterol and cholesteryl esters by thin layer chromatography (21) using charring and densitometry.

RESULTS

Basal Pentane

Pentane production at 0 wk of analysis was similar by rats fed the 3 diets containing 0, 11 and 200 IU vitamin E/kg diet for 4 wk (Fig. 1). A one-way analysis of variance using simple and general contrasts between treatment groups was used to determine the significance of the differences between treatments.

The variances in the pentane values of the 4 treatment groups were not homogeneous, as determined by Bartlett's test. Transformation of the pentane values at weeks 1, 3, 5 and 9 of analysis using ln (X +1) was performed prior to doing a one-way analysis of variance. Rats fed a vitamin-E-deficient diet and given ethanol in their drinking water for 9 wk did not have basal pentane levels significantly different from those of rats fed a vitamin-E-deficient diet and given an isocaloric amount of D-glucose in their drinking water for 9 wk. At 9 wk of treatment, rats fed no vitamin E and given the ethanol

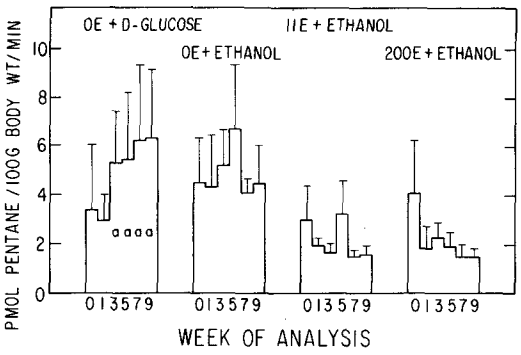

FIG. 1. Mean basal pentane production by rats during the 9-week treatment period of diet (0, 11, or 200 IU dl-α-tocopherol acetate/kg diet) and drinking solution (ethanol as an 18% solution or an isocaloric solution of D-glucose). Prior to the first analysis, each group of rats had been fed its respective diet and water for 4 weeks since weaning. Six rats were in each group except where indicated; a = 5. Error T-bars denote standard deviation.

drinking solution produced a mean of 4.5 ± 1.6 pmol pentane/100 g body wt/min and rats fed no vitamin E and given the D-glucose drinking solution expired a mean of 6.3 ± 2.8 pmol pentane/100 g body wt/min. Simple contrasts between the 2 groups of rats fed vitamin E in the diet showed no significant differences in pentane levels during the 9-wk analysis period, except at week 5. General contrasts between the 2 groups not supplemented with vitamin E and the 2 vitamin-E-supplemented groups demonstrated significant reductions in pentane expiration by vitamin E treatment at week 1 ($p < 0.01$) and at weeks 3, 5 and 9 ($p < 0.001$). A Student's t test was made on the 7-wk pentane values obtained for the groups of rats given the ethanol drinking solution and fed either 0, 11 or 200 IU vitamin E/kg diet. Vitamin E in the diet significantly decreased ($p < 0.001$) basal pentane levels.

The different diet and drinking regimens did not affect the growth rates of rats within the groups. The mean body wt ± standard error for rats fed the vitamin-E-deficient diet and given either glucose or ethanol drinking solutions for 9 wk were 359 ± 14 g and 360 ± 13 g, respectively. Rats given the ethanol drinking solution for 9 wk and fed diets supplemented with 11 or 200 IU vitamin E/kg diet had a mean body wt of 358 ± 13 g and 364 ± 13 g, respectively.

Acute Ethanol Exposure

An acute dose of 6 g of ethanol/kg body wt p.o. given to rats fed the vitamin-E-deficient diet after 14 wk of treatment with ethanol in the drinking water dramatically increased pentane production above basal levels for a 4-hr period in only 2 of the 6 rats. Pentane levels rose rapidly at 30 min, peaked at 60 min, and slowly declined afterwards. The other 4 rats in this group and the rats fed the vitamin-E-deficient diet and given an isocaloric amount of D-glucose in their drinking water expired insignificant amounts of pentane above basal levels.

Pentane production above basal levels during the 4 hr following ethanol administration was plotted against the amount of liver triglycerides determined after 20 wk of treatment (Fig. 2). There was a linear correlation ($r = 0.96$, $p < 0.001$) between acute ethanol-induced pentane evolution and liver triglyceride levels, both of which were influenced by prior diet and drinking regimen. This correlation results primarily from 2 particular animals that produced large amounts of pentane following an acute dose of ethanol. These 2 rats were later found to have high liver triglyceride levels. There was no correlation between levels of expired pentane and levels of liver cholesterol or cholesteryl esters.

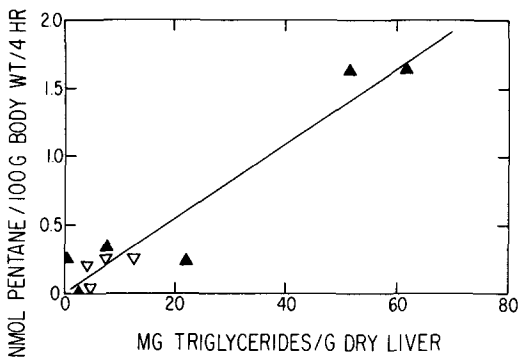

FIG. 2. Linear relationship between expired pentane following administration of an acute dose of ethanol and hepatic triglyceride content determined 6 weeks later ($r = 0.96$, $p < 0.001$). Pentane values are expressed as the amount of pentane expired above basal levels during the 4-hr period following administration of an acute oral dose of 6 g ethanol/kg body wt. Each symbol represents an individual rat pretreated with (▲) a vitamin-E-deficient diet with an 18% ethanol drinking solution and (▽) a vitamin-E-deficient diet with an isocaloric D-glucose drinking solution for 14 weeks prior to administration of the acute oral dose of ethanol.

Liver and Blood Analysis

Liver mitochondria and microsomes prepared from rats in all 4 treatment groups contained no detectable amounts of conjugated dienes.

The amounts of liver triglyceride, cholesterol and cholesteryl esters varied among animals, and these amounts were not related to the diets and drinking treatments (Table I).

Table II displays the glutathione peroxidase activity in whole blood of rats from the 4 treatment groups. Whole blood glutathione peroxidase activity was shown by a one-way analysis of variance to be significantly higher ($p < 0.05$) in rats that were provided a diet containing 200 IU vitamin E/kg diet and an ethanol drinking solution than in rats from any of the other 3 treatment groups.

DISCUSSION

The connection between alcohol consumption and the development of a fatty liver has been well established. However, the basic mechanisms underlying the hepatic injury elicited by ethanol is unclear. Observed effects in hepatocytes following ethanol treatment are the accumulation of triglycerides and structural abnormalities in mitochondria, endoplasmic reticulum and the Golgi apparatus (22). Depending on the experimental conditions used

TABLE I

Liver Lipids of Rats Treated with D-Glucose or Ethanol Drinking Solutions

Treatment[a]	Triglyceride[b]	Cholesterol[b]	Cholesteryl ester[b]
0E + D-Glucose	7.3 ± 3.9[c]	6.2 ± 2.6[c]	1.7 ± 0.8[c]
0E + EtOH	24.2 ± 26.3	3.9 ± 1.7	2.6 ± 4.0
11E + EtOH	16.1 ± 15.3	3.4 ± 1.3	4.1 ± 3.3
200E + EtOH	6.6 ± 4.4[d]	4.5 ± 2.8[d]	3.3 ± 2.2[d]

[a]IU dl-α-tocopherol acetate/kg diet plus an 18% ethanol drinking solution or an isocaloric D-glucose solution for 20 wk.

[b]Mean ± SD expressed as mg/g dry liver for 6 rats.

[c]Value for 4 rats.

[d]Value for 5 rats.

by an investigator, the increase in liver triglycerides can be ascribed to any of 4 major metabolic alterations: (a) impaired hepatic lipoprotein release; (b) increased lipogenesis in the liver; (c) decreased hepatic triglyceride oxidation; and (d) enhanced mobilization of fatty acids from adipose to the liver. Di Luzio (1) suggested that lipid peroxidation was involved in the disturbance of lipid metabolism, since antioxidant treatment was found to ameliorate the damage induced by ethanol.

Numerous studies with rats have been done by various investigators to study the peroxidation of liver lipids following acute, as well as chronic, ethanol intoxication. Initially, the methods used to detect lipid peroxidation were the in vitro assays of determining the peroxide value, malonaldehyde production, and/or conjugated diene formation. Conflicting results have been reported. A controversy has come about that has split investigators into 2 factions: one that supports an important role of lipid peroxidation in ethanol toxicity and one that does not. Comporti (23) has reviewed extensively studies reporting conflicting results.

The in vitro methods used in the past to detect peroxides, malonaldehyde, and conjugated dienes have distinct disadvantages. Special precautions need to be followed to prevent artifactual peroxidation during the preparation of tissue samples for analysis. A major drawback is that the products of lipid peroxidation, peroxides (24), malonaldehyde (25), and conjugated dienes (26), are labile and are rapidly removed in vivo. These products are most easily detected when a highly potent free-radical initiator, such as carbon tetrachloride, is used so that the formation of products (conjugated dienes) exceeds their metabolism (26). In addition, variations in the assay procedures used (5) and the conditions of the experiments contribute to conflicting results from different laboratories.

TABLE II

Blood Glutathione Peroxidase Activity in Rats Treated with D-Glucose or Ethanol Drinking Solutions

Treatment[a]	Whole blood[b]
0E + D-Glucose	11.0 ± 1.5[c]
0E + EtOH	17.8 ± 5.5
11E + EtOH	14.5 ± 5.3
200E + EtOH	23.8 ± 3.7

[a]IU dl-α-tocopherol acetate/kg diet plus an 18% ethanol drinking solution or an isocaloric D-glucose solution for 20 wk.

[b]Mean specific activity ± SD as nmol NADPH oxidized/min/mg protein for 6 rats.

[c]Value for 3 rats.

A powerful technique to study in vivo lipid peroxidation is the measurement of expired pentane and/or ethane. Although pentane is a minor product of autoxidized ω6-unsaturated fatty acids (27), the methodology of gas chromatography allows the detection of pmol amounts of pentane. The high sensitivity is further enhanced by the biologically inert chemical properties of pentane.

An 18% ethanol solution replacing the drinking water of rats did not influence basal pentane levels during the 9-wk diet and drinking regimen. Basal pentane did change with time, but the change was caused by the vitamin E content of the individual diet used. Rats fed the vitamin-E-deficient diets maintained high levels of expired pentane throughout the testing period. As previously reported (28), rats supplemented with dietary vitamin E had basal pentane production diminished to less than one-half the rate of that by rats maintained on a vitamin-E-deficient diet. It has been reported (29) that rats chronically drinking for as long as 260 days a 15% ethanol solution, which provided 30% of their total caloric intakes, did not develop fatty livers. Drinking solutions

containing 18% ethanol or less did not elicit increased pentane production or serious physiological dysfunction, possibly because rats rapidly metabolize ethanol about 2-3 times faster than do humans (30). When 41.5% of the caloric intake of rats was provided by an ethanol drinking solution for 6 wk, a greater release of biliary glutathione disulfide was stimulated (31). Glutathione disulfide recently has been suggested as a sensitive indicator of oxidative stress (32).

Administration of an acute dose of ethanol triggered exhalation of increased amounts of pentane in 2 of the 6 vitamin-E-deficient rats pretreated chronically with ethanol. The chronic ethanol regimen was severe enough to cause 2 of the vitamin-E-deficient rats to be highly susceptible to an acute dose of ethanol, as indicated by elevated levels of expired pentane. The same 2 rats were shown to have been physiologically affected by the chronic ethanol treatment when they were found to have fatty livers 6 wk after treatment. The observation that only some of the rats had altered pentane production suggests that other uncontrolled factors may influence an individual animal's susceptibility to ethanol toxicity.

Ethanol is a weak toxicant as shown by its relatively large oral LD_{50} of 13.7 g/kg of body wt (33). Other in vivo studies (15-17) have shown expired pentane or ethane to increase only moderately following oral administration of > 5 g ethanol/kg body wt as compared to the large amounts expired following administration of a potent toxic prooxidant such as carbon tetrachloride.

Individual animal variation can be of substantial magnitude in ethanol toxicity studies. This study showed that only 2 of 10 rats responded to an acute challenge of ethanol by having significantly increased levels of expired pentane. Burk and Lane (17) reported that following administration of an acute dose of ethanol, the amounts of ethane exhaled by rats had standard deviations greater than 50% within groups.

The etiology of liver dysfunction precipitated by ethanol ingestion constitutes a complex and multifactorial system. There is evidence that genetic factors may predispose an individual to alcoholism (34) and influence the wide variation of response to ethanol. Age and animal species can affect the reaction to ethanol. Other important factors are the dose and time of exposure to ethanol, along with the extent of involvement of each of the 3 major pathways of ethanol metabolism, (a) alcohol dehydrogenase, (b) the catalase system, and (c) the microsomal ethanol oxidizing system. The nutritional status is fundamental to an animal's response since nutrients maintain optimal defenses against toxic challenges. Interwoven among and influenced by these 3 factors is lipid peroxidation. Determining the extent of lipid peroxidation will depend on the experimental conditions used and the ability of the investigator to control these variables. Whether lipid peroxidation is among one of the several causes of ethanol toxicity or whether it is a result of ethanol toxicity is yet to be determined. Further investigation using the sensitive and specific in vivo technique of monitoring expired pentane may lead to a more thorough understanding of the importance of lipid peroxidation in ethanol toxicity.

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METHODS

Purification of Phospholipids by Preparative High Pressure Liquid Chromatography

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ABSTRACT

A method is described for the purification of a number of phospholipids by preparative high performance liquid chromatography (HPLC). Purification of digalactosyl-diglyceride from spinach and egg phosphatidylcholine, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine from its reaction mixture have been resolved. The lipid separation is performed on a polygosil column and the individual compounds are monitored directly by refractive index detection. Chloroform/methanol mixtures are used as eluent systems, providing a wide polarity range to separate the classes of lipids. The developed equipment can be used for columns between 10 and 50 cm long and 4 and 50 mm inner diameter. The flow rate could be varied between 1 and 100 ml/min and applied pressures between 10 and 450 bars.

INTRODUCTION

High performance liquid chromatography (HPLC) has shown great potential for the separation of phospholipids on an analytical scale (1-10). Limited information currently is available concerning the purification of phospholipids on a gram scale (11-12). It would be of great practical use to develop HPLC equipment for the fast separation of lipids in large quantities (20 mg-5 g) using silica gel as a support for the stationary phase. For this purpose, we have constructed an apparatus which can be used for columns between 10 and 50 cm length and 4 to 50 mm inner diameter, with flow rates between 1 and 100 ml/min and pressures of 10 to 450 bars.

The pulse-damping system and the large preparative HPLC column used in the current study were developed because they were commercially unavailable. The choice of eluent for the separation of lipids is restricted to systems such as chloroform/methanol (6,11,12), hexane/isopropanol/water (9,10) and acetonitrile/water (7,8). Lipid was detected in the hexane/isopropanol/water and acetonitrile/water systems by monitoring the absorption between 203 and 214 nm. Because of the ultraviolet (UV) cut-off for chloroform at 345 nm, refractive index detection has to be used for the chloroform/methanol system. As water can give rise to hydrolysis of phospholipids, we have routinely used an eluent with little or no water.

This paper describes the equipment developed and results of the purification of phospholipids from natural sources or reaction mixtures on a gram scale.

MATERIALS AND METHODS

Instrumentation

The eluent was delivered by a Dosa-pro Milton-Roy pump (Paris, France), type Milroyal B, with admissible discharge pressure of 450 bars. The pulse-damping system was a thick-walled, homemade, stainless steel can with a screw-nut cover and 3 tube-inlet connections (Fig. 1). The eluent was filtered through a high pressure, mobile-phase filter with 2-M pores (Chrompack, Middelburg, The Netherlands).

For sample introduction, an injection valve, model 70-10 (Rheodyne, Berkeley, CA) with homemade loops up to 7 ml was used. Analytical chromatographic columns were stainless steel (length 25 cm, id 8 and 16 mm, respectively, packed with Silica Gel Li Chrosorb Si-60, particle diameter 10 μm, Merck, GFR), supplied by Knauer (Berlin [West], GFR). Preparative chromatographic columns, developed and manufactured in cooperation with Chrompack (Middelburg, The Netherlands), were stainless steel, 50 cm length, id 22 and 50 mm (Fig. 2), respectively. The column was dry-packed with silica gel (polygosil 60-1525, Machery-Nagel & Co., Düren, GFR) by adding small amounts of silica during column vibration.

Refractive index measurements were done

with a differential refractometer, Type 51.88 (Knauer, Berlin [West], GFR). The maximal flow for this detector is 10 ml/min. At higher flow rates, a stream splitter had to be used. A multirange pen-recorder (Linseis, type 2377, Knauer, Berlin [West], GFR) was used. The preparative HPLC used in the current study is shown in Figure 3.

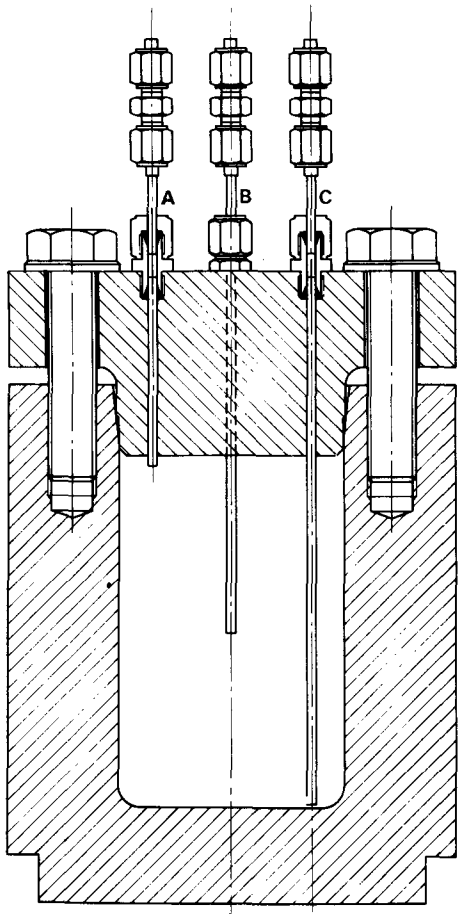

FIG. 1. Schematic representation of the pulse-damping system. The thick-walled, stainless steel can has a screw-nut cover and 3 tube inlets (A, B and C). Tube A is connected to the pump, tube B to a high pressure valve and tube C to the column (by way of the mobile-phase filter and the injector). Starting a chromatographic run, the valve of tube B is open and tube C is disconnected. The running pump fills the pulse-damping system until the eluent leaves this through tube B. The valve of tube B is then closed. Tube C is connected with the column. In principle, the pulse-damping system operates such that the enclosed air column above the lowest point of tube B is compressed when the pump delivers eluent and expands when the pump compartment is refilled.

Solvents

Columns were eluted with varying mixtures of chloroform and methanol. Chloroform, technical grade (Chemproa, 's-Hertogenbosch, The Netherlands) was prepurified by distillation from a 10-ℓ, all glass distillation apparatus with a 1-m Vigreux set-up. Prior to distillation, 0.3 ml of 25% NH_4OH and 50 ml of 100% ethanol were added per 8 ℓ to bind the acidic breakdown products of chloroform and to lend stability. After collection of a 1-ℓ prefraction (waste), 6.5 ℓ of chloroform were collected for final purification. Both the prepurified chloroform and the methanol were freshly distilled before use from separate 10-ℓ distillation systems as already described. The chloroform is stabilized with 0.8% of spectroscopic grade ethanol (v/v). Prior to use, the air from the eluting solvents was removed by evaporation for 3 min at reduced pressure (± 14 mm Hg). This procedure was repeated almost every hour.

Lipid Preparation

The isolation of crude digalactosyl-diglyceride was done according to the methods Carroll (13) and Freeman-Allen (14). Egg phosphatidylethanolamine and egg phosphatidylcholine were isolated and 1,2-dioleoyl-*sn*-glycero-3-

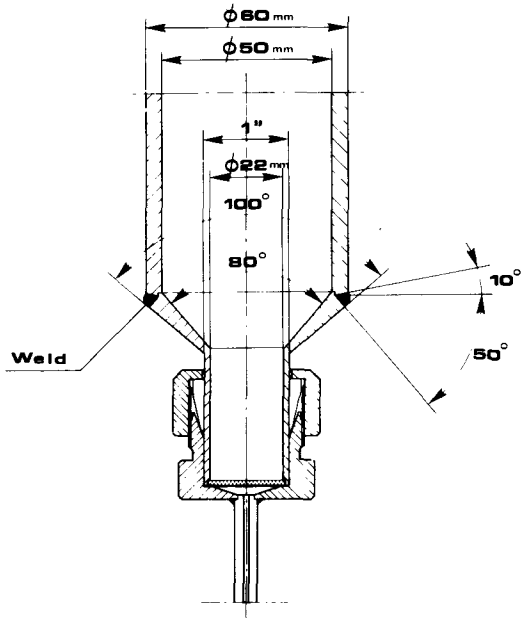

FIG. 2. Schematic representation of the column outlet. The 50-mm inner diameter part of the column is 50 cm long. The column inlet is of exactly the same construction as the column outlet.

phosphocholine was synthesized according to established procedures (15). 1,2-Dioleoyl-*sn*-3-phosphoethanolamine was synthesized as described by Cullis and de Kruijff (16). All lipid samples were prepurified on glass columns filled with silica (Mallinckrodt Silicar CC-4 special) at atmospheric pressure. The collected fractions were evaporated and redissolved in a small vol of chloroform/methanol (1:1, v/v). The fractions were analyzed for purity by HPLC on Silica Gel 60 plates (10 X 10 cm, for nano-TLC, Art. 5633, Merck Darmstadt, GFR). A mixture of chloroform/methanol/water/25% NH_3 in water (65:35:4:1, v/v) was used as the eluting solvent system. Spots were located by spraying the plate with a phosphorus reagent according to Dittmer and Lester (17). The purification results of digalactosyl-diglyceride fractions were compared with those previously reported by Siakotos and Ronser (18).

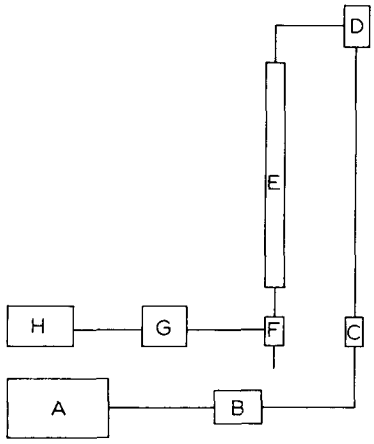

FIG. 3. Schematic drawing of the preparative HPLC equipment. (A) Pump, (B) pulse-damping system, (C) mobile-phase filter, (D) injector, (E) column, (F) column-outlet and stream-splitter, (G) detector, and (H) recorder.

RESULTS AND DISCUSSION

The number of theoretical plates was determined by eluting a 300-mg sample of phosphatidylethanolamine (PE) or phosphatidylcholine (PC) with chloroform/methanol (80:20 for PE; 40:60 for PC, v/v) at a flow rate of 20 ml/min. The 5-cm id column was found to have 6,000 theoretical plates/m. This still is a rather low value and packing methods have to be improved.

The chromatogram, after purification by preparative HPLC of 250 mg crude digalactosyl-diglyceride from spinach (11,12), is shown in Figure 4. In run A (chromatogram given in the insert), fraction 3 was the only one containing digalactosyl-diglyceride. It was shown by HPLC, however, that this fraction was still impure. Therefore, the polarity of the solvent was decreased and the digalactosyl-diglyceride fraction 3 was reinjected in 1.1 ml and rechromatographed as shown in Figure 4B. Fraction 4B3 contained 89 mg of pure digalactosyl-diglyceride.

Complete purification of 5 g of PC from egg yolk, resulting in a white glassy product, was achieved as shown in Figure 5. The different fractions were collected and identified by HPLC as shown in the inserts. Fractions 5a3 and 5a4 (Fig. 5a) were rechromatographed with a chloroform/methanol mixture of lower polarity as shown in Figure 5b. Fractions 5b2 and 5b3 contained 4.32 g pure PC.

The purification of synthetic PC produced by the fatty acid chloride method (15) is demonstrated in Figure 6. Complete purification of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was achieved in 2 steps which are necessary to avoid

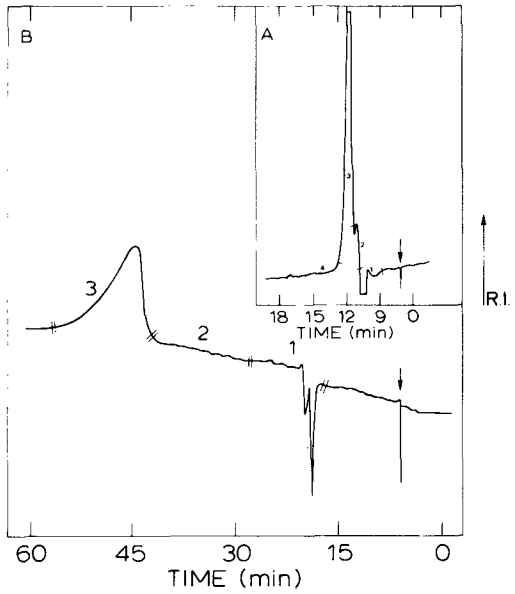

FIG. 4. Purification of digalactosyl-diglyceride from spinach. Column: Polygosil 60-1525, length 50 cm, id 22 mm; sample load: A = 250 mg crude digalactosyl-diglyceride in 1.1 ml solvent; B = 114 mg prepurified digalactosyl-diglyceride in 1.1 ml solvent; eluent system: A = chloroform/methanol, 85:15 v/v; B = chloroform/methanol, 93:7 v/v; flow rate 11.6 ml/min; detection: refractive index at range 8, temperature 20 C. A1, 2 and 4: unidentified contaminants; A3: prepurified digalactosyl-diglyceride; B1 and 2: unidentified contaminants; B3: 89 mg pure digalactosyl-diglyceride.

overloading the column. A purification in one run was demonstrated for 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine when the column was loaded with 1 g of sample (Fig. 7). The PE was formed by phospholipase D treatment of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine in the presence of ethanolamine.

The most time-consuming part in the classic purification procedures of lipid classes and products of lipid synthesis is column chromatography at atmospheric pressure using silica gel as the stationary phase support. This chromatographic procedure can take several days, furthering the possibility of decomposition and oxidation. To avoid such reactions, a very fast and efficient separation is required. A prerequisite for the developed HPLC equipment is the flexibility of the different parts, so that analytical, as well as preparative, columns can be used. An efficient pulse-damping system is required to achieve a high detection sensitivity. The high-pressure pump used with a flow range of 1-100 ml/min is a pulsating pump which caused changes in pressure of 50% in the absence of a damping system. The pressure-damping system described in Figure 1 reduced the pressure changes to less than 5% at a pressure of 200 bars and a flow rate of 20ml/min.

The largest commercially available HPLC columns packed with small (5, 7, or 10 μ) silica gel particles are 25 cm long and 1.6 cm id. The maximal capacity of these columns for lipids is ca. 250 mg. Therefore, columns with ± 5-g capacity were constructed and dry-packed in our laboratory. After some developmental work, the column was constructed as described in Figure 2. The columns were dry-packed with 15-25 μ silica gel particles. This permitted flow rates of 30ml/min at pressures of 250 bars. All purifications were done with an isocratic eluent at room temperature. Before sample injection, the column was equilibrated with the solvent system until a stable baseline was obtained. In the set-up just described, the 5-cm id column was used for more than 60 runs without loss of capacity and reproducibility. The results obtained in this study demonstrate the usefulness of HPLC for the purification of lipids.

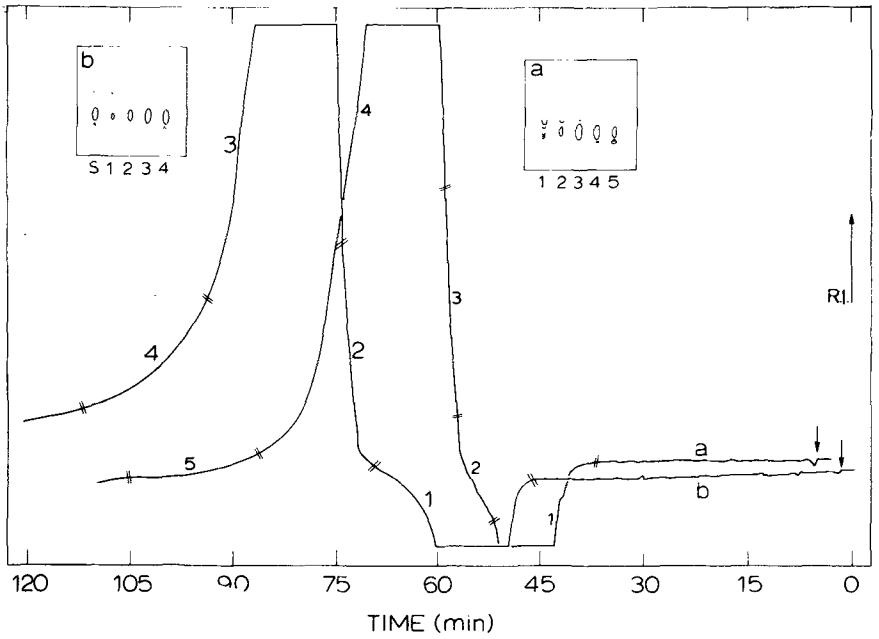

FIG. 5. Purification of phosphatidylcholine from egg yolk. Column: Polygosil 60-1525, length 50 cm, id 50 mm; sample load: a = 5.0 g crude egg phosphatidylcholine in 7 ml solvent; b = 4.5 g prepurified egg phosphatidylcholine in 7 ml solvent; eluent system: a = chloroform/methanol, 60:40 v/v; b = chloroform/methanol, 70:30 v/v; flow rate: a = 17 ml/min, b = 19 ml/min; detection: refractive index at range 16; temperature: 20 C; a1, 2 and 5: phosphatidylcholine and unidentified contaminants; a3 and 4: prepurified phosphatidylcholine; b1 and 4: phosphatidylcholine and unidentified contaminants; b2 and 3: 4.32 g pure phosphatidylcholine.

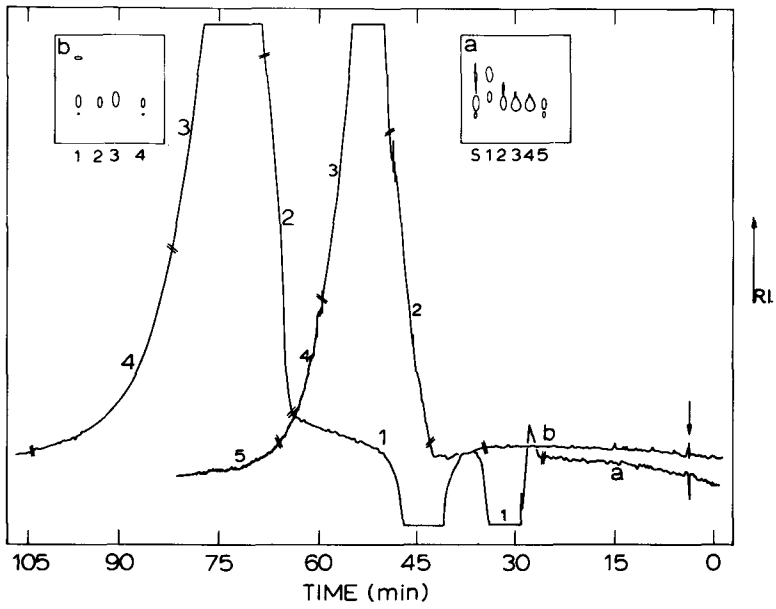

FIG. 6. Isolation of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine from a synthetic mixture. Column: Polygosil 60-1525, length 50 cm, id 50 mm; sample load: a = 3.8 g crude phosphatidylcholine in 7 ml solvent; b = 3.2 g prepurified phosphatidylcholine in 7.0 ml solvent; eluent system: a = chloroform/methanol, 60:40 v/v; b = chloroform/methanol, 65:35 v/v; flow rate: 27 ml/min; detection: refractive index at range 16; temperature 20 C; a1, 2 and 5: phosphatidylcholine and unidentified contaminants; a3 and 4: prepurified phosphatidylcholine; b1, 2 and 4: phosphatidylcholine and unidentified contaminants; b3: 3.12 g pure 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

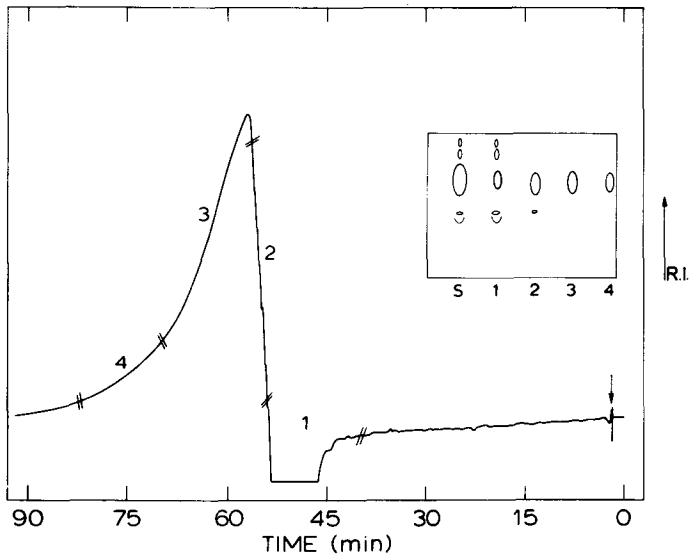

FIG. 7. Isolation of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine from a synthetic mixture. Column: Polygosil 60-1525, length 50 cm, id 50 mm; sample load: 1300 mg crude phosphatidylethanolamine in 5-ml solvent; eluent system: chloroform/methanol, 80:20 v/v; flow rate: 17 ml/min; detection: refractive index at range 16; temperature 20 C; 1 and 2: phosphatidylethanolamine and unidentified contaminants; 3 and 4: 1,100 mg pure 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

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COMMUNICATIONS

Studies on Biochemical Effects of Nitrogen Dioxide: I. Lipid Peroxidation As Measured by Ethane Exhalation of Rats Exposed to Nitrogen Dioxide

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ABSTRACT

This research was in order to follow the periodic fluctuation of lipid peroxidation by a new method in rats exposed to nitrogen dioxide. Wistar male rats were examined for lipid peroxidation as demonstrated by ethane exhalation. In rats continuously exposed to 10 ppm nitrogen dioxide for 2 weeks, the amount of ethane exhaled fluctuated in a complex manner during the exposure. Ethane exhalation decreased slightly after the first day of exposure and then increased rapidly. The maximal values were observed after the fourth day of exposure and then decreased gradually to the initial level. Furthermore, the activity of glutathione peroxidase in lungs of rats exposed to 10 ppm nitrogen dioxide varied symmetrically against the change of ethane formation. Similar changes in ethane exhalation were observed in rats exposed to the lower levels of nitrogen dioxide (0.4, 1.2 and 4.0 ppm) for 4 months. Compared to 10 ppm nitrogen dioxide exposure for 14 days, the characteristics in rats exposed to the low levels (0.4-4.0 ppm) of nitrogen dioxide were: the decline of ethane formation, the delay in alterations, and the tendency toward gradual increase during the longer period exposure.

Nitrogen dioxide is a strong oxidizing agent commonly found in urban air. It is well known that nitrogen dioxide causes pathological lung injury (1,2). Since peroxidation of biological membrane lipids is considered an integral part of cell damage and many toxic processes (3), the toxic action of nitrogen dioxide may result in part from lipid peroxidation. Evidence that exposure to nitrogen dioxide causes in vivo lipid peroxidation was shown by the measurement of conjugated dienes by Thomas et al. (4). Since then, many investigators have tried to detect lipid peroxides in tissues; however, they have been unable to detect them following exposure to nitrogen dioxide (5).

Volatile hydrocarbons are known to appear early during the autoxidation of edible fats (6-8), and ethane is considered to be produced during autoxidation of linolenic acid (9). Riely et al. (10) advanced the use of hydrocarbon gas analysis when they applied the method to a biological system. Since that time, other investigators have monitored ethane production to assess lipid peroxidation in vivo (11-16).

This work was done to confirm the occurrence of lipid peroxidation following exposure of rats to nitrogen dioxide and to follow the patterns of ethane exhalation in rats exposed to nitrogen dioxide.

MATERIALS AND METHODS

JCL:Wistar male rats (8-wk-old) were used for 10 ppm nitrogen dioxide exposure, and 13-wk-old rats were used for 0.4, 1.2 and 4.0 ppm nitrogen dioxide exposure. The 8-wk-old rats were housed in groups of 3 and the 13-week-old rats were housed in groups of 6 in hanging wire-mesh cages in a room kept at a constant temperature of 24-26 C with humidity 50-60%. The 8-wk-old rats were exposed to nitrogen dioxide in a stainless steel and glass chamber (0.4 m^3) as described previously (17), and the 13-wk-old rats were exposed in a larger chamber (2.0 m^3). The rats were subjected to a daily cycle of 14 hr of light and 10 hr dark, and they were fed a commercial stock diet (CE-2) obtained from Japan Clea Co., Ltd. (JCL). This stock diet (CE-2) contains 3.5% (w/w) of crude lipids, and the fatty acid compositions in the crude lipids were 18.3, 21.0, 54.8 and 2.8% of palmitate, oleate, linoleate and linolenate, respectively (K. Kaya, personal communication).

The analytical method for measuring exhaled ethane was described previously (18-20), and the hydrocarbon gas exhaled was expressed in units of pmol ethane exhaled/100 g body weight/min (18-20).

In order to estimate the activities of lung

enzymes, the rat blood was taken from the neck artery under light anesthesia with diethyl ether. The lungs were then removed, washed with saline and stored at -80 C in vials purged with nitrogen. The activities of glutathione peroxidase (21), glutathione reductase (22), glucose-6-phosphate dehydrogenase (23), and 6-phosphogluconate dehydrogenase (24) in lungs were examined using the 100,000 G supernatant obtained from a 15% homogenate prepared in 50 mM Tris-HCl buffer, pH 7.4.

RESULTS

The time course of ethane evolution and the activities of glutathione peroxidase and glutathione reductase following exposure to 10 ppm nitrogen dioxide for 2 wk are shown in Figure 1. The amounts of ethane exhaled fluctuated in a complex manner during the exposure. Ethane was decreased significantly after the first day of the exposure, the level being about 60% of the initial value (100%). This decrease in the early phase also was confirmed in another experiment in which rats were exposed to 4.48 ppm nitrogen dioxide for 1 hr (25). Ethane exhalation increased rapidly after the second day of exposure, and reached the maximal level of 220% of the initial level after the fourth day. Ethane exhalation then decreased gradually and returned to the initial level after the 10th day of exposure. Glutathione peroxidase activity in lungs was depressed significantly after the second day, and then a significant increase occurred 5 to 7 days after the exposure and remained essentially at a plateau until the 14th day. The time course of glutathione reductase activity was also similar to that of glutathione peroxidase. The activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase also fluctuated with a similar time course, and significant increases in activity were observed from the third to the 14th day. The maximal level was ca. 170-200% of the initial value. The changes in ethane exhalation and in the activities of glutathione peroxidase and glutathione reductase during nitrogen dioxide exposure were symmetrical in the course after the third day, as shown in Figure 1.

The time courses of ethane exhalation by rats exposed to nitrogen dioxide at 0 (control group), 0.4, 1.2 and 4.0 ppm for 16 wk are shown in Figure 2. The time courses were similar to those following exposure to 10 ppm nitrogen dioxide. After the first week, ethane evolution was at the maximal levels, and the values obtained by exposure to 0.4, 1.2 and 4.0 ppm nitrogen dioxide corresponded to 108, 135 and 172% of the control group, respectively. Significant differences from the control were observed in the 1.2 ppm ($P < 0.05$) and 4.0 ppm ($P < 0.001$) groups, and then, ethane formation decreased until the fourth week. Significant differences ($P < 0.05$) from control group values were obtained after the second and fourth weeks of exposure to 1.2 and 4.0 ppm nitrogen dioxide. After that time, ethane evolution increased very gradually. Following the 16th wk of exposure, only 4.0 ppm nitrogen dioxide group was significantly ($P < 0.05$) higher than the control group. In addition, the

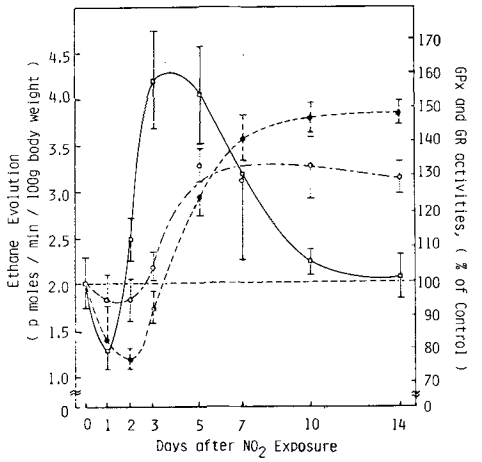

FIG. 1. Periodical variations of ethane evolution in breath and of the activities of glutathione peroxidase and glutathione reductase in lungs of rats exposed to 10 ppm nitrogen dioxide for 2 weeks. □, Ethane evolution; ●, glutathione peroxidase (GPx); ○, glutathione reductase (GR): The values are expressed by mean ± SEM (n = 8-12).

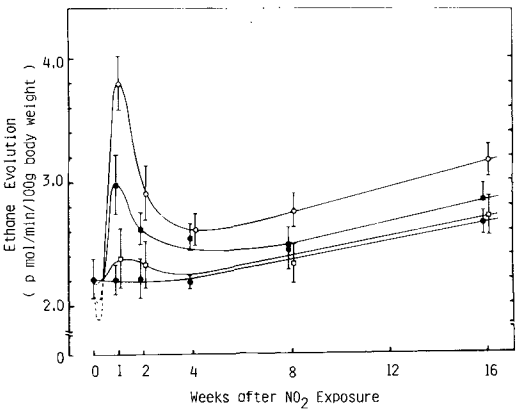

FIG. 2. Periodical variations of ethane evolution in breath of rats exposed to 0.4, 1.2 and 4.0 ppm nitrogen dioxide for 4 months. ●, Control group; □, 0.4 ppm; ■, 1.2 ppm; ○, 4.0 ppm: The values are expressed by mean ± SEM (n = 5-12).

slight decreases within the first week of exposure to nitrogen dioxide were estimated from the result obtained following the 10 ppm nitrogen dioxide exposure; these are shown in Figure 2 as dotted lines. Pentane, derived from peroxides of fatty acids of ω-6 family, was poor as an index of lipid peroxidation by nitrogen dioxide exposure.

DISCUSSION

In the experiment with 10-ppm nitrogen dioxide exposure, the relationship between ethane evolution and the activities of glutathione peroxidase and glutathione reductase indicates that lipid peroxides produced by the exposure to nitrogen dioxide induced glutathione peroxidase and glutathione reductase activities to protect cells from oxidative damage. The induced glutathione peroxidase might decompose lipid hydroperoxides produced mainly in organelle membranes. Although McCay et al. (26) reported that glutathione peroxidase would not reduce hydroperoxides present in membrane, Tappel (27) explored this dilemma and found evidence for a phospholipase that hydrolyzed fatty acid hydroperoxides from phospholipids at rates significantly faster than those of presently known phospholipases. The existence of a phospholipase with a faster hydrolysis rate may explain this discrepancy. Furthermore, McCay et al. (26) presented evidence that glutathione peroxidase may act to prevent the formation of fatty acid peroxides rather than to convert these compounds to nontoxic hydroxy fatty acids. This evidence might explain the symmetrical changes between ethane formation and glutathione peroxidase activity.

The time course of ethane formation was similar to the proliferation of Type II cells in alveolar tissues following 17 ppm nitrogen dioxide exposure (28-30). Our results may correspond to the process in which (a) Type I cells are damaged in an early phase (0-1 day) by nitrogen dioxide exposure, (b) Type II cells proliferation as a repair process begins in 1-3 days, and (c) Type II cell proliferation declines after 3 days. After 3 days, enzymes such as glutathione peroxidase and glutathione reductase that are protective against oxidative stress possibly are induced.

Changes similar to those seen with 10 ppm nitrogen dioxide exposure were observed in rats exposed to lower levels of nitrogen dioxide (0.4, 1.2 and 4.0 ppm, for 16 wk). These results indicate that ethane exhalations by exposure to lower levels of nitrogen dioxide are smaller and the appearances of the peaks were slower than that seen during exposure to 10 ppm level of nitrogen dioxide. The gradual increase of ethane exhalation after 4 wk might result in part from the aging process as reported previously (18). It is interesting that the differences in ethane exhalation among the exposed groups and the control group decreased between the fourth and eighth weeks, and that the differences between the control and exposed groups tended toward an increase again after the eighth wk. From these results, it is possible that a statistically significant difference in ethane formation following exposure to lower levels of nitrogen dioxide for a longer term can be observed. Such an experiment is now underway in our laboratory.

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The Metabolism of 18:0 and 18:2(n-6) by the Ovine Placenta at 120 and 150 Days of Gestation

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ABSTRACT

The effects of the approach of parturition in the sheep on the incorporation of 18:0 and 18:2(n-6) into the placental lipids and on the activities of the Δ9- and Δ6-desaturase enzymes of placental tissue have been studied in vitro. The incorporation of 18:0 into the esterified lipids of placental tissue between the 120th and 150th days of gestation declined markedly; the high level of incorporation of 18:2(n-6) into the esterified lipids of the placenta (some 2-fold higher than 18:0) remained constant over the gestational period. While placental Δ9-desaturase activity was the same at 150 days as at 120 days of gestation, the activity of the Δ6-desaturase enzyme increased significantly. These results are discussed in relation to the fetal demand for fatty acids near term and the differences that exist between the mechanisms of maternal-fetal transfer of 18:0 and 18:2(n-6) in the sheep.

INTRODUCTION

The plasma lipids of the fetal lamb differ extensively in composition from those of the parent ewe (1). In particular, the plasma lipids of the fetal lamb contain very low proportions of 18:2(n-6) and relatively high proportions of 20:3(n-9), a fatty acid characteristically present in the lipids of nonruminant animals under conditions of essential fatty acid deficiency (2). In spite of this, the proportions of 20:4(n-6) in the plasma lipids of the fetal lamb are higher than in those of the ewe. With respect to the relationship between the polyunsaturated fatty acid metabolism of the maternal sheep and its fetus, two important observations recently have been made. Studies in vivo with cannulated, pregnant sheep have shown that the ovine placenta discriminates against the direct transfer of 18:2(n-6) relative to saturated fatty acids into the fetus (1) whereas observations in vitro have shown that the placental tissue possesses an active desaturase system metabolizing 18:2(n-6) to 20:4(n-6) (3). Such studies, to an extent, provide an explanation for the compositional differences between the polyunsaturated fatty acid composition of the plasma lipids of the ewe and her fetus and also indicate a significant role for the placenta in the metabolism and supply of polyunsaturated fatty acids. In the sheep, such a role would be expected to become more marked as the fetus grows and its demands on the very limited maternal supply of essential fatty acids increase. With this question in mind, the possibility that the approach of parturition has an effect on the relative rates of uptake and metabolism of 18:0 and 18:2(n-6) by the placenta, in particular the activities of the Δ9- and Δ6-desaturase systems, respectively, have been studied in vitro. The results of this work are now reported.

EXPERIMENTAL PROCEDURE

Eight pregnant Cheviot ewes were divided into 2 groups each of 4 animals which were killed by a captive bolt pistol at 120 and 150 days of gestation, respectively. Immediately after death, placental cotyledons were excised and placed in ice-cold homogenization buffer (7.5 mM sodium phosphate buffer, pH 7.4, containing 0.32 M sucrose and 1 mM EDTA). After removing the outer capsules and connective tissue, the cotyledons were chopped roughly with scissors and homogenized in a laboratory emulsifier (Silverson Machines Ltd., Chesham, England) in the proportions of 10 g of tissue to 25 ml of homogenization buffer. Any residual connective tissue was then removed by filtration through a 2 mm gauge stainless steel wire gauze. Aliquots of the filtrate were then incubated in a reaction mixture containing 0.5 μCi (ca. 8 nmol) of either [1-^{14}C]-18:0 or [1-^{14}C] 18:2 appropriate for the Δ9- and Δ6-desaturase activities as described (3). After incubation at 37 C for 2 hr, by which time the plateau of incorporation had been reached (R.C. Noble and J.H. Shand, unpublished results), the lipids, including unreacted substrate, were extracted from the reaction mixture by chloroform/methanol according to the Folch et al. method (4) and separated on thin layers of Silica Gel G (E. Merck, Darmstadt, Germany) by development in hexane/ether/formic acid (80:20:1, v/v). The major neutral lipid fractions, i.e., cholesteryl esters, triglycerides, unesterified fatty acids and the partial glycerides, were eluted from the silicic acid by washing with diethyl ether and the phospholipids by washing with acidified methanol. An aliquot of each fraction was dried down and, following dissolution in a toluene-based scintillant, the radioactivity was determined using a liquid scintillation counter (Packard 2425; Packard

Instruments Ltd., Downers Grove, IL). The remainder of the esterified lipid bands were then pooled, transmethylated by refluxing in methanolic HCl (5) and the constituent fatty acid methyl esters separated according to their degree of unsaturation by argentation chromatography (6). Bands corresponding to saturated, monenoic, dienoic, trienoic and tetraenoic fatty acids were eluted with diethyl ether and, following removal of the solvent, their radioactivity was determined as just described. Confirmation of the fatty acid groupings was obtained by the cochromatographic separation of standard methyl esters and subsequent gas chromatographic identification. The remainder of the unesterified fatty acid fraction was transmethylated in the presence of a heptadecanoic acid standard and quantitatively analyzed by gas liquid chromatography (GLC) for the determination of the placental pool sizes of unesterified 18:0 and 18:2(n-6). These data were required for determining the degree of dilution of the fatty acid substrates by their respective placental pools. This enabled the desaturase activities to be calculated in terms of pmol of fatty acid metabolized. The protein contents of the placental homogenates were determined by a modified Lowry et al method (7).

RESULTS

The proportional incorporation in vitro of [1-^{14}C] 18:0 and [1-^{14}C] 18:2(n-6) into the major esterified lipid fractions of the placental tissue from sheep at 120 days of gestation, (about one month prior to parturition) and 150 days of gestation (nominally at birth) are given in Table I. The unesterified fatty acid fraction accounted for the remainder of the radoactivity extracted. At 120 days of gestation, the amount of 18:2(n-6) that was incorporated into the esterified lipid of the placental tissue exceeded the amount of 18:0 that became esterified by nearly 2-fold. Although in both cases the major incorporation was into the phospholipids, there was a significantly higher incorporation of 18:2(n-6) into the phospholipid fraction than of 18:0. At 150 days of gestation, the amount of 18:0 that became incorporated into the total esterified lipids of the placenta was very much lower than at 120 days of gestation (38% compared to 13%). Although this was primarily accounted for by a decrease in the incorporation into the phospholipid fraction, there were significant reductions also in the amounts of 18:0 incorporated into the triglyceride and partial glyceride fractions. In contrast, the amount of 18:2(n-6) incorporated into the total esterified lipids of the placenta at 150 days of gestation remained similar to the values obtained at 120 days (68% compared to 67%). Incorporation of 18:2(n-6) into the phospholipid fraction at 150 days of gestation was similar to that for 120 days. However, there was a significant increase and decrease, respectively, in the proportions of 18:2(n-6) incorpo-

TABLE I

The Incorporation of [1-^{14}C] 18:0 and [1-^{14}C] 18:2(n-6) into the Esterified Lipids of Sheep Placental Tissue in vitro[a]

Fatty acid substrate	Lipid fraction	Gestational age (days) 120	150
[1-^{14}C] 18:0	Cholesteryl esters	1.4 ± 0.2	0.6 ± 0.1
	Triglycerides	9.3 ± 1.7[b]	1.1 ± 0.2[b,c]
	Partial glycerides	1.8 ± 0.2[b]	0.5 ± 0.1[b,c]
	Phospholipids	25.8 ± 1.9[b,c]	10.4 ± 1.5[b,c]
	Mean dpm ^{14}C (± SE) in the total lipids extracted after incubation	602,811 ± 42,844	626,844 ± 44,519
[1-^{14}C] 18:2(n-6)	Cholesteryl esters	1.0 ± 0.3	1.8 ± 0.4
	Triglycerides	15.7 ± 1.8[b]	5.8 ± 0.7[b,c]
	Partial glycerides	2.5 ± 0.5[b]	6.9 ± 0.5[b,c]
	Phospholipids	48.9 ± 2.1[c]	52.0 ± 3.9[c]
	Mean dpm ^{14}C (± SE) in the total lipids extracted after incubation	739,988 ± 56,299	836,064 ± 52,742

[a]Each result is the mean ± SE of 4 observations and is expressed as a percentage of the mean dpm ^{14}C in the total lipids extracted after incubation.

[b]Statistically significant difference ($p < 0.01$) between the incorporation of the same fatty acid at the 2 times of gestation.

[c]Statistically significant difference ($p < 0.01$) between the incorporation of 18:0 and 18:2(n-6) at the same gestation time.

TABLE II

The Δ9- and Δ6-Desaturase Activities of Sheep Placenta in vitro[a]

Activity	Gestational age (days) 120	150
Δ9-Desaturase (pmol of 18:0 metabolized to monenoic fatty acids/min/mg protein)	11.2[c] ± 2.2	8.0 ± 1.5
Δ6-Desaturase (pmol of 18:2(n-6) metabolized to trienoic and tetraenoic fatty acids/min/mg protein)	3.1[b,c] ± 0.1	10.8[b] ± 0.6

[a]Each result is the mean ± SE of 4 observations.
[b,c]Statistical comparisons as defined in Table I.

rated into the partial glyceride and triglyceride fractions at 150 days of gestation. Clearly, the relative differences between the overall incorporation of 18:0 and 18:2(n-6) into the esterified lipids of the placenta had become very much more marked near parturition. There was no significant difference (i.e., p in all cases was greater than 0.01) between the means amounts of ^{14}C in the total lipids extracted from the incubation mixtures.

Measurements in vitro of the activities of the Δ9- and Δ6-desaturase systems in the sheep placenta at 120 days and 150 days of gestation are given in Table II. At 120 days of gestation, the extent of Δ6-desaturase activity was only about 1/3 that of the Δ9-system. No difference was observed in the activity of the Δ9-desaturase system between 120 days and 150 days of gestation. In contrast, the activity of the Δ6-desaturase system at 150 days of gestation was some 3-fold greater than that observed at 120 days.

DISCUSSION

The role of the placenta as a modifying influence, temporary reservoir and supplier of lipid to the developing ovine fetus may be considerable (8,9). Although there is a significant transfer of unesterified fatty acids across the placenta from the maternal to the fetal circulation (1), it has been demonstrated in many species, including the sheep, that such transfer is accompanied by extensive incorporation of the fatty acids into the lipid fractions of the placenta (9). From these findings and those obtained presently, it is now clear that the metabolic influence of the placenta on fatty acid supply to the fetus is very much dependent on the nature of the fatty acids being supplied and may alter considerably with advancing gestation.

Although the fatty acid requirements of the ovine fetus may, in part, be satisfied by direct transfer from the mother, fetal tissues have been shown to possess the ability to synthesize long chain fatty acids de novo from acetate (10); however, this ability would appear to diminish as parturition approaches. The possibility exists, therefore, that a greater fetal demand for preformed, long chain, saturated fatty acids at this time is satisfied by an increased rate of direct transfer from the mother. However, our results with 18:0 demonstrate that any increased fetal requirement for long chain fatty acids is not simply met by an increase in direct maternal-to-fetal transfer. Clearly, there also is a dminished incorporation of maternally derived fatty acids into the esterified lipid fractions of the placenta. The resultant increase in the placental unesterified 18:0 pool and, therefore, the placental-fetal unesterified fatty acid concentration gradient, would allow an increased flow of 18:0 from the placenta into the fetal circulation.

The extent to which maternally derived 18:0 was metabolized by the Δ9-desaturase system in the placental tissue remained unchanged over the last month of gestation. Several reasons exist as to why any such change should be unnecessary. The level of the Δ9-desaturase activity in the placenta, even at one month prior to parturition, has been shown to be relatively high (3). Furthermore, evidence obtained from the liver of the lamb immediately after birth (3,11) would suggest that Δ9-desaturase activity is present in fetal lamb tissue. In addition, 18:1-(n-9) comprises one of the major components of the maternal plasma unesterified fatty acids whose mobilization and subsequent passage across the placenta during the latter part of gestation may be more than sufficient to satisfy fetal requirements.

The situation with regard to 18:2(n-6) contrasts strongly with that for 18:0. The direct transfer of 18:2(n-6) across the ovine placenta into the fetal circulation does not occur to any

significant extent (1). On the other hand, uptake and subsequent conversion to 20:4(n-6) via an active Δ6-desaturase system present in the placenta is extensive (3). Following esterification, in particular into the phospholipids, the 18:2(n-6) and newly synthesized 20:4(n-6) are then exported into the fetal circulation (R.C. Noble and J.H. Shand, unpublished results). Very low activity levels only of the Δ6-desaturase systems have been found in the tissues of the lamb immediately after birth (11). In view of this, any increase in fetal demand for fatty acids of the (n-6) series that occurs during the last month of gestation could most easily be accommodated by an increase in polyunsaturated fatty acid metabolism by the placenta as observed in this investigation. The lack of any overall increase in the incorporation of 18:2(n-6) into the placental lipids over the last month of gestation is a further indication that the increased fetal demand near term is not for 18:2(n-6) per se but for its longer chain metabolites (8). Such a shift in equilibrium toward 20:4(n-6) could be achieved readily through the observed increases in the activity of the placental Δ6-desaturase system.

The increased placental synthesis of 20:4-(n-6) near parturition may not be confined to satisfying an increased fetal requirement at this time. In view of the major role of the prostaglandins in the onset of labor in the sheep (12), the possibility also exists that the increase in the placental Δ6-desaturase activity observed near parturition may be in partial response to an increased requirement for prostaglandin synthesis.

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The Enzymic Synthesis of GM1b: Rat-Brain CMP-N-Acetylneuraminic Acid: Asialo-GM1 Sialyltransferase

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ABSTRACT

An enzyme which catalyzes the transfer of N-acetylneuraminic acid (NeuNAc) to a tetrahexosylceramide (asialo-GM1) in young rat brain is described. The enzymic product is a new monosialoganglioside containing a neuraminidase-labile neuraminic acid, GM1b. The acivity of this sialyltransferase is highest in fetal and young rat brains. The enzyme exhibits a pH optimum of 6.5 in cacodylate buffer. The incorporation of radioactivity into GM1b is stimulated in the presence of asialo-GM1 and CMP-NeuNAc and is dependent on the quantity added. The detergent mixture, Tween 80 and CF54, is required for optimal activity. Recent demonstration of the natural occurrence of GM1b in the free cell types of rat ascites hepatoma cells suggests a functional importance of this CMP-NeuNAc:asialo-GM1 sialyltransferase in the in vivo formation of this novel monosialoganglioside.

ABBREVIATIONS

The symbols of gangliosides are based on the Svennerholm nomenclature system (1). Dihexosylceramide (GL-2a), cer-glc-gal; trihexosylceramide (asialo-GM2), cer-glc-gal-galNAc; tetrahexosylceramide (asialo-GM1), cer-glc-gal-galNAc-gal; GM1, cer-glc-gal (NeuNAc)-galNAc-gal; GM1b, cer-glc-gal-galNAc-gal-NeuNAc.

INTRODUCTION

The biosynthesis of a novel monosialoganglioside, GM1b, containing a neuraminidase-labile neuraminyl group occurred in vitro when asialo-GM1 and CMP-NeuNAc were incubated with an enzyme source from the young rat brain homogenate (2). Using an ultramicroscale permethylation procedure of radioactive GM1b, the position of the neuraminidase-labile NeuNAc group linked to the terminal galactosyl unit was established (3,4).

In this report, we describe the developmental pattern, the properties and the optimal assay conditions for the CMP-NeuNAc:asialo-GM1 sialyltransferase. The significance of this enzymic reaction is discussed in relation to the in vivo operation of the asialoglycolipid pathway for the synthesis of ganglioside.

MATERIALS AND METHODS

Label CMP-[^{14}C]NeuNAc was purchased from New England Nuclear Corp., Boston, MS; Tween 80, Triton CF-54 and other detergents were purchased from Sigma Chemical Co., St. Louis, MO; Sephadex G-25 superfine was obtained from Pharmacia, Piscataway, NJ; the nonradioactive CMP-NeuNAc was prepared by the Brunetti et al. method (5) with the modification of Arce et al. (6); the asialo-GM1 was prepared by acid hydrolysis (0.1 N HCl) of bovine brain gangliosides for 1 hr at 100 C, and was purified by preparative thin layer chromatography (TLC) (7). Nonradioactive GM1b was biosynthesized in large-scale preparation as described previously (4). Sprague-Dawley albino rats were obtained from Charles River Breeding Laboratory, Wilmington, MS. Their body length (rump to crown) is given as a measure of maturity (8).

The standard incubation system for CMP-NeuNAc:asialo-GM1 sialyltransferase contained 0.1 M cacodylate buffer (pH 6.5), 0.2 mg Triton CF-54 and 0.1 mg Tween 80, 0.75 mM asialo-GM1, 0.25 mM CMP-[^{14}C]NeuNAc (sp act 1.64 x 10^6 cpm/μmol), 0.03 ml rat brain homogenate and water to a final vol of 0.1 ml. The reaction was routinely incubated at 37 C for 1 hr. The homogenate used as the enzyme source was prepared by homogenizing brain in 4 vol of a solution containing 0.32 M sucrose and 0.11% (w/w) 2-mercaptoethanol. The enzymic reaction was stopped by the addition of 2 ml of chloroform/methanol (2:1, v/v). After shaking, the reaction mixture was transferred to a column (1 cm diameter) containing 0.8 g of Sephadex G-25 superfine previously equilibrated with chloroform/methanol/water (60:30:4.5, v/v/v). After collecting the filtrate, the column was washed twice with 2.5 ml of the same solvent used for equilibration. The total filtrate was collected and dried under a stream of nitrogen. To the dried residue, 0.2 ml of chloroform/methanol (2:1, v/v)

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containing 5 μg of nonradioactive GM1b was added as carrier, and the total content was chromatographed as a small band on a Silica Gel G plate (250 μm thickness, Analtech Inc., Wilmington, DE) for a 2-hr ascending run at 22 C with chloroform/methanol/water (60:35:8, v/v/v). Detection was with iodine vapor and the area corresponding to GM1b was removed and transferred to a counting vial. Thixotropic powder was added, followed by the addition of 16 ml of scintillation fluid containing 5 g of 2.5-diphenyloxazole and 0.3 g of 1,4-bis(2-(4-methyl-5-phenyloxazolyl)) benzene in 1 ℓ of toluene. The counting efficiency was 83% as determined by the internal standardization technique. Control tubes contained the complete incubation mixture minus asialo-GM1.

RESULTS

Developmental Pattern of Rat Brain CMP-NeuNAc:asialo-GM1 Sialyltransferase

Activity of the CMP-NeuNAc:asialo-GM1 sialyltransferase during development is shown in Figure 1. A rise in enzyme activity is observed from 7 days before birth and reaches a maximal level between birth and 15 days postpartum. Activity then declines sharply to the adult level at 25 days postpartum. The specific enzyme activity, when expressed as nmol ^{14}C-product/mg protein/hr, follows a similar pattern during development. The sp act are 0.85, 1.08, 1.01 and 0.25 at 7 days before birth, at birth, 10 days and 25 days postpartum, respectively.

The wet wt of the rat brains studied increased from ca. 0.1 g in the 15-day fetus to 1.6 g 15 days postpartum. Only small changes in brain wt were observed after 15 days of age.

Requirements and General Characteristics of CMP-NeuNAc:asialo-GM1 Sialyltransferase

The sialyltransferase reaction required the glycolipid substrate, asialo-GM1, Triton CF-54, plus Tween 80, for optimal activity (2). Other detergents were tested for replacing the detergent mixture for optimal activity. At a concentration of 0.3 mg/incubation mixture, Triton CF-54, Cuscum, Triton X-100 and Tween 20 could replace 77, 35, 34 and 21% of the optimal activity, respectively. Sodium deoxycholate, Tween 40 and Tween 80 were relatively inactive.

The sialyltransferase activity in the homogenate of a 5-day-old rat brain was porportional to the amount of protein added up to 0.6 mg. Inhibition of the enzyme activity was observed when the protein concentration was greater than 0.8 mg. Incorporation of radioactivity was stimulated in the presence of asialo-GM1 and was dependent on the quantity added.

The apparent K_m for asialo-GM1 was at 1×10^{-4} M. CMP-NeuNAc stimulated the sialyltransferase activity at the optimal concen-

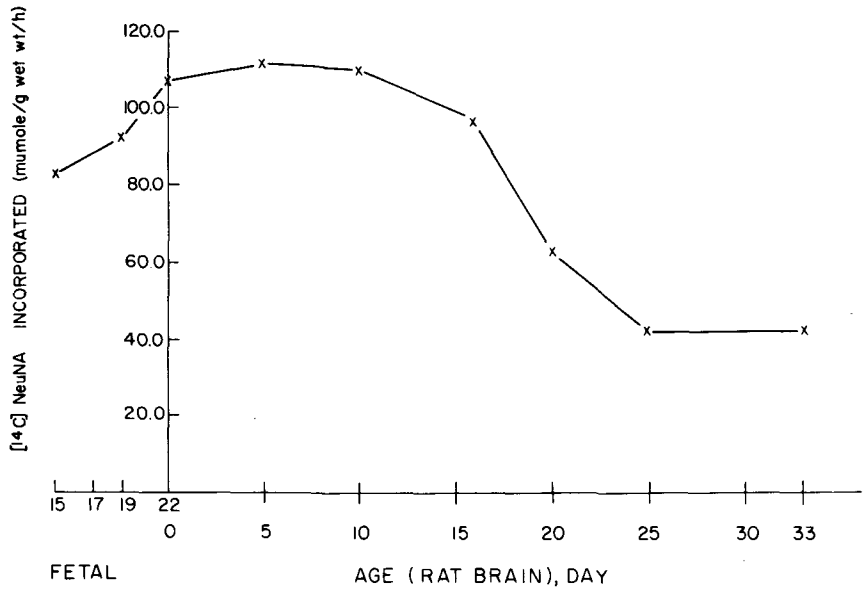

FIG. 1. The activity of CPM-N-acetylneuraminic acid:asialo-GM1 sialyltransferase during development. The enzyme activity was assayed as described in Materials and Methods. Each point is a mean of 3 individual values.

tration of 0.25 mM. Inhibition of enzyme activity was observed at a CMP-NeuNAc concentration greater than 0.25 mM. The sialyltransferase reaction was directly proportional to reaction time for 1 hr and gradually leveled off up to 6 hr. A pH optimum of 6.5 in cacodylate buffer was observed.

The product of the CMP-NeuNAc:asialo-GM1 sialyltransferase has been conclusively identified as GM1b (2,4).

DISCUSSION

With rat brain, incubation in vitro of CMP-NeuNAc and asialo-GM1 resulted in the biosynthesis of a novel monosialoganglioside (GM1b) containing a neuraminidase-labile neuraminyl group (2). Using ultramicroscale permethylation of radioactive GM1b, the structure was subsequently established (3,4).

The sialyltransferase involved in the formation of GM1b was detectable 7 days before birth and reached a maximal level between birth and 15 days postpartum. Adult level of enzyme activity was reached at 25 days postpartum (Fig. 1). The developmental profile of this transferase activity could represent the potential of rat brain to synthesize GM1b in vivo. During rat brain development, the sp act of this sialyltransferase for GM1b formation is slightly higher than the galactosyltransferase for GM1 synthesis (9). Preliminary localization of the enzyme indicates that most of this sialyltransferase activity is in the microsomal fraction. Attempts to solubilize and purify the enzyme have been unsuccessful.

Although the natural occurrence of this novel monosialoganglioside has yet to be proven in brain tissue, the dramatic accumulation of its proposed precursor, asialo-GM1, from patients afflicted with generalized gangliosidoses (10) and in nonadhesive rat ascites hepatoma cells (11) suggests that the synthesis of GM1b in vivo is possible. A most recent demonstration of the natural occurrence of GM1b in nonadhesive rat ascites hepatoma cells (12) has added considerable weight to the in vivo operation of the asialoglycolipid pathway for ganglioside biosynthesis originally proposed by Yip and Dain (7). The asialoglycolipid pathway involves the stepwise synthesis of the glycolipid precursors, dihexosylceramide (GL-2a), asialo-GM2 and asialo-GM1, from glucosylceramide by 3 glycosyltransferases. Asialo-GM1 is then catalyzed to GM1b by a sialyltransferase. The enzymes involved in the formation of asialo-GM2 and asialo-GM1 were present in the nonadehsive rat ascites hepatoma cells (13) and in brain tissue (7,14). Therefore, the brain tissue and the nonadhesive rat ascites hepatoma cells have similar enzymic components for the biosynthesis of GM1b in vivo.

Recently, Stoffyn and Stoffyn (15) have shown that incubation of asialo-GM1 and CMP-NeuNAc in the presence of homogenates from normal and neoplastic tissues resulted in formation of GM1b. In addition, a new disialoganglioside different from GD1a and GD1b was formed.

The accumulation of the asialoglycolipids in neurological disorders and in nonadhesive rat ascites hepatoma, asialo-GM1 in generalized gangliosidoses (10), asialo-GM2 in Tay-Sachs disease (16), and asialo-GM1 and asialo-GM2 in rat ascites hepatoma (11), suggests that GM1b and its related asialoglycolipids may play an important and dynamic role in the metabolism of complex glycosphingolipids.

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Phase Transition of an Unnatural Analog of Phosphatidylcholine: Phosphatidyl-N-isopropylethanolamine

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ABSTRACT

The phase-transition temperature of an aqueous dispersion of dipalmitoyl phosphatidyl-N-isopropylethanolamine was determined using *trans*-parinaric acid as a fluorescent probe. Phosphatidyl-N-isopropylethanolamine has a phase-transition temperature that is 3-4 C higher than the corresponding natural analog of dipalmitoyl phosphatidylcholine.

INTRODUCTION

Manipulation of polar head groups of phospholipids in cell cultures with amino alcohol supplements has been used widely as a technique to assess how this portion of the phospholipid molecule affects the structural and functional properties of biological membranes (1-5). An unnatural amino alcohol, N-isopropylethanolamine (IPE), has been shown to be incorporated de novo (6) into membrane phospholipids as 1,2-diacyl-*sn*-glycero-3-phosphoisopropylethanolamine in rat liver (3) and cultured L-M cells (2). In addition, IPE has been shown to inhibit the incorporation of choline into lipids in isolated hepatocytes (7) and cultured L-M cells (6). Furthermore, growth of L-M cells in the presence of IPE for 24 hr causes a decrease in the microsomal stearoyl-CoA desaturase activity to 40% of the activity found in control cells (8). Similarly, the microsomal 3-hydroxy-3-methylglutaryl-CoA reductase activity was reduced by the supplementation of IPE in the media of cultured glial and neuromal cells (5). A decrease in the activity of 5′-nucleotidase of liver plasma membranes also was observed in rats that were injected intraperitoneally with IPE (9). The exact mechanism(s) involved in the lowering of these specific membrane-bound enzyme activities is unclear at present. However, the fluidity of biological membranes has been implicated in the regulation of a variety of membrane functions (10). In order to test the possibility that IPE could affect the membrane fluidity, we determined whether the replacement of choline by an unnatural polar head group (IPE) on the phospholipid backbone influences the phase transition. Our results show that the phase-transition temperature of dipalmitoyl phosphatidyl-IPE is several degrees higher than its naturally occurring choline analog.

MATERIALS AND METHODS

Dipalmitoyl phosphatidylcholine (DPPC) was purchased from Sigma Chemical Co., St. Louis, MO. The IPE was synthesized as described previously (2). Dipalmitoyl phosphatidyl-IPE (DPP-IPE) was prepared by phospholipase-D-catalyzed transphosphatidylation of DPPC in the presence of IPE (11); the product was isolated and purified by preparative thin layer chromatography on Silica Gel HR layers developed in chloroform/methanol/ammonium hydroxide (65:35:5, v/v) (3). The purified product showed only a single spot on a thin layer chromatogram. Methods for isolation of *trans*-parinaric acid, the preparation of lipid dispersions and the fluorescence measurements were the same as described by Sklar et al. (12).

RESULTS AND DISCUSSION

Figure 1 shows a typical plot of the logarithmic fluorescence intensity of *trans*-parinaric acid as a function of the reciprocal temperature (K^{-1}) for an aqueous dispersion of DPP-IPE. The increase in the fluorescence intensity of *trans*-parinaric acid is an indicator of the phase transition onset of DPP-IPE. The phase-transition temperature of DPP-IPE, calculated as described elsewhere (12), is 45.5 C when derived from the heating curve and 43 C when derived from the cooling curve. These temperatures are 3-4 C higher than that of DPPC, which had phase-transition temperatures of 41.5 C (heating curve) and 40.3 C (cooling curve) under identical experimental conditions (13). The saturated phosphatidylethanolamines undergo a lipid phase transition some 20-30 C higher than the corresponding phosphatidylcholines (14-16).

Our earlier biochemical studies showed that significant amounts of phosphatidyl-IPE (8-9% of microsomal phospholipids) accumulate, at the expense of phosphatidylcholine, in L-M cells and livers of rats treated with IPE. Thus, the physical measurements reported in this communication indicate that the incorporation of N-isopropylethanolamine head group into

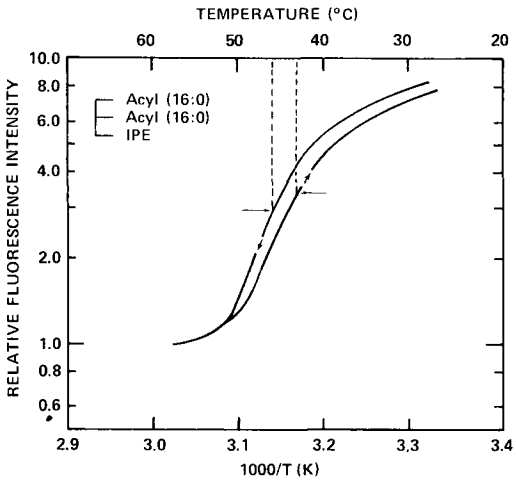

FIG. 1. The response of the fluorescence intensity of the *trans*-parinaric acid probe to the phase transitions of aqueous dispersions of dipalmitoyl phosphatidylisopropylethanolamine. The sample was cooled to ca. 10 C below the lipid transition temperature and then heated at a rate not exceeding 2 K/min. The concentration of DPP-IPE was 68 μM and the molar ratio of *trans*-parinaric acid to DPP-IPE = 1/124. The temperature at the midpoint of the transition for both heating and cooling curves are marked on the figure.

phospholipids could influence membrane fluidity and serve as a basis for future experimentation.

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Identification of Acidic Steroids in Feces of Monkeys Fed β-Sitosterol

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ABSTRACT

Vervet monkeys were fed a suspension of β-sitosterol in corn oil. Acidic steroids were separated from a 4-day pool of feces and subjected, after fractionation, to gas liquid chromatography and mass spectrometry. Evidence for the presence of derivatives of 27-carboxysitosterol, 27-carboxysitostanol and 7-hydroxy, 27-carboxysitostanol is adduced.

INTRODUCTION

In 1912, Ellis and Gardner (1) reported that rabbits could absorb plant sterols. Since then, ample evidence has accumulated to show that β-sitosterol is absorbed by rats (2-9), baboons (10), Vervet monkeys (10) and man (2,11-13). A neutral metabolic product of sitosterol, coprositostanol, was isolated by Rosenheim and Webster (14) from feces of rats fed β-sitosterol and brain powder. Conversion of sitosterol to acidic fecal steroids has been shown in non-human primates (10) and man (12). Mixed plant sterols can be converted to acidic products in rats (15). However, the nature of the acidic product(s) has not been elucidated. This communication relates to the identification of 2 products isolated from feces of Vervet monkeys *(Cercopithecus aethiops pygerethrus)*.

METHODS

Four Vervet monkeys were given a suspension of β-sitosterol in corn oil by stomach tube (300 mg/day for 5 days). Feces were collected during the feeding period, pooled and homogenized in water. Neutral and acidic steroids were separated (16) and the acidic steroids subjected to further fractionation. The bile acids were converted to the corresponding methyl esters with diazomethane and subjected to thin layer chromatography (TLC) on silica gel using benzene/acetone (60:40, v/v) (17). The bile acid methyl esters were converted to the corresponding trimethylsilyl ethers and analyzed by gas liquid chromatography-mass spectrometry (GLC-MS) on 1% SE-30 on 80/100 Gas Chrom Q, using 5α-cholestane as an internal standard. GLC-MS was done with a Hewlett-Packard 5981A spectrometer equipped with electron impact ionization.

RESULTS

Three of the peaks observed could be attributed to 27-carboxylic acids derived from sitosterol (sitostanoic acids). Two of these compounds are related to coprositostanol, one corresponding to the 3-trimethylsilyl ether, 27-methyl ester (Fig. 1, R = H) (compound 1–relative retention time = 1.63) and the other, to the 3,7-ditrimethylsilyl ether, 27-methyl ester (Fig. 1, R = OTMS) (compound 2–relative retention time = 3.50). The third compound corresponds to 27-carboxymethyl sitosterol 3-trimethylsilyl ether (Fig. 2) (compound 3–relative retention time = 2.24).

Fragments were observed for the various compounds showing the presence of an ethyl group which is characteristic of derivatives of β-sitosterol (18,19). In addition, loss of a side chain fragment containing 11 carbon atoms in a cholestanoic acid methyl ester derivative of β-sitosterol could be postulated (m/e 185). Loss

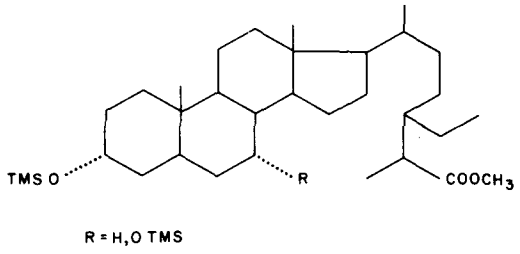

FIG. 1.

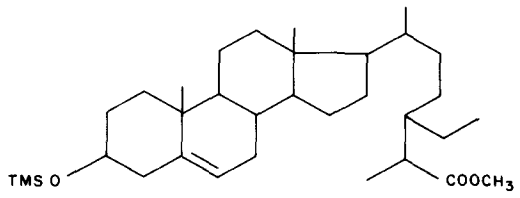

FIG. 2.

of a similar fragment from DL-24-methyl-3α, 7α-dihydroxy-5β-cholestan-26-oate (m/e 157) could be postulated (m/e 273 m-(18+157) (20). Specific ion fragments led us to propose the 3 structures shown. For compound 1, they were: monosubstitution of sterol ring, m/e 257, m-(90+side chain), fragments greater than the molecular weight of trimethylsily (TMS) ether derivative of lithocholate are m/e 498 and 484. A fragment suggesting side chain cleavage between C_{24} and C_{25} was observed at m/e 460. Other fragments were observed at m/e 435, 429 and 411. For compound 2, there were fragments of m/e 435, m-side chain; 411, m-(2×90+29); 255, m-(2×90+side chain). For compound 3, there were fragments of m/e 381, m-(90+59); 357, m-(C_{23}-C_{24} side chain scission +15); 255, m-(90+side chain). These data suggest the presence of cholestanoic acids derived from β-sitosterol with either monohydroxy or dihydroxy substitution.

DISCUSSION

Aringer et al. (21) have shown that rat liver mitochondria are capable of hydroxylating the side chain of cholesterol, campesterol and β-sitosterol. Two products, the 26- and 29-hydroxy derivatives of sitosterol, were obtained. It also has been shown (22) that rat or mouse liver mitochondria can oxidize [28-^{14}C]-ergosterol and [U-^{14}C]ergosterol to $^{14}CO_2$, indicating that mammalian systems can metabolize plant sterols in "normal" ways. Aringer and his colleagues (18,19) have demonstrated that β-sitosterol can be hydroxylated at the 7α and 12α positions. Subbiah (23) had suggested that the rat, when injected intravenously with sitosterol, might convert it into a 3β-hydroxy C_{29} acid similar to compound 1 (Fig. 1) proposed by us.

Our findings indicate that plant sterols can be oxidized to yield acidic products resembling conventional bile acids and suggest that this metabolic possibility should be considered in assessing the physiological action of plant sterols.

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Arachidonic Acid: Occurrence in the Reproductive Tract of the Male House Cricket (*Acheta domesticus*) and Field Cricket (*Gryllus* spp.)

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ABSTRACT

Reproductive tissues from male house and field crickets were investigated for the presence of arachidonic acid. Arachidonic acid was identified by retention behavior on 3 gas liquid chromatographic column systems–SE-30, OV 225, and Silar 10C–and by gas liquid/positive ion chemical ionization mass spectrometry. Arachidonic acid constituted 0.3 and 1.2% of total fatty acids in the house and field cricket, respectively. Freeze-dried male reproductive tissues from house and field cricket contained ca. 0.02 and 0.16% arachidonic acid and 8.2 and 8.6% total fatty acids. Other fatty acids found in reproductive tracts of both species of crickets were 16:0, 18:0, 18:1, 18:2, 20:0, 20:1 and 20:3.

INTRODUCTION

In general, insects metabolize fatty acids along pathways shown to be operative in vertebrates (1-4) and are able to synthesize the 16- and 18-carbon saturated and monoenoic acids from available metabolites. Many species of insects also require dietary sources of linoleic (18:2[n-6], octadeca-9,12-dienoic acid), or linolenic (18:3[n-3], octadeca-9,12,15-trienoic acid) acid as do vertebrates and this requirement suggests a function similar to that in higher animals. In mammals, linoleic and linolenic acids are essential for proper growth and health and are desaturated and elongated to polyenoic 20- and 22-carbon acids (5). One, though probably not the only, function of these fatty acids in animals is to serve as precursors of prostaglandins (6).

Data concerning the occurrence and function of these longer (20-22 C) chain fatty acids in insects are relatively scarce compared to similar data available on higher animals. The mosquito *(Culex pipiens)* requires a dietary source of arachidonic acid (eicosa-5,8,11,14-tetraenoic acid) (7), a requirement that can be met by other 20- and 22-carbon polyenoic acids (8). Blood-sucking insects in general require a blood meal for normal reproduction. Blood is a good source of arachidonic acid (9) and the requirement for a blood meal may well indicate a similar requirement for dietary arachidonic acid in other blood-sucking insects.

Gas liquid chromatographic (GLC) analysis of the fatty acids of the tobacco budworm *(Heliothis virescens)* (10) and a cockroach *(Periplaneta americana)* (11) revealed a fatty acid identified as arachidonic acid. Mosquito cell lines grown in tissue culture in the presence of calf serum contain 20- and 22-carbon polyenoic acids (12,13). Other data presupposing the presence of arachidonic or other polyenoic acids include the identification of prostaglandins in the salivary glands and reproductive organs of the tick (*Hyalomma anatolicum excavatum* Koch) (14) and house cricket *(Acheta domesticus)* (15-17), and a stimulatory effect of prostaglandin E_2 on oviposition of *A. domesticus* (17) and *Teleogryllus commodus* (18). Although the presence of prostaglandins presupposes the presence of arachidonic acid (or other 20-carbon polyenoic acid precursor), this acid has not been demonstrated in the house cricket nor was it detected in a study of the fatty acid composition of a related species *(Gryllus bimaculatus)* (19).

In the study reported here, we examined the reproductive tracts of male house crickets and field crickets for occurrence of arachidonic acid. Identification was based on retention behavior on 3 gas liquid chromatographic column systems and by gas chromatography/positive ion chemical ionization MS.

MATERIALS AND METHODS

House crickets were purchased from a commercial supplier and maintained in the laboratory on Purina mouse chow (17). Field crickets were captured from the native habitat near Athens, GA. Reproductive tracts (testes, seminal vesicles, accessory glands and ejaculatory duct) of males were removed (15) and maintained in the frozen state until time of

analysis. Tissues were lyophilized and methyl esters prepared from weighed samples of freeze-dried tissues (20). Methyl heneicosanoate was added as internal standard prior to the extraction-transesterification procedure. The identification of methyl arachidonate was based on GLC retention behavior on 3 column systems and on GC/MS data.

GLC data were obtained with a MicroTek 220 chromatograph equipped with dual flame ionization detectors and an electronic integrator. Glass columns (180 x 0.4 cm id), packed with 10% Silar 10C on 100/120 Gaschrom Q, and 3% OV 225 on 70/80 Chrom W (AW) (DMCS), were maintained at 180 C. Other operating conditions were as described elsewhere (20). GC/MS data were obtained with a Hewlett Packard 5985 mass spectrometer operated in the positive ion chemical ionization mode and fitted with a 3% SE-30 column. Column oven temperature was 180 C. Methane served as carrier gas (15 cc/min) and source of positive ions.

Methyl arachidonate, methyl heneiocosanoate and a GLC reference mixture containing methyl esters of 16:0, 18:0, 18:1, 18:2, 20:0, 20:1, 22:0 and 24:0 were obtained from Nu-Chek-Prep (Elysian, MN).

RESULTS AND DISCUSSION

GLC of the fatty acid methyl esters of the reproductive tissue of the male field and house crickets revealed several major and minor peaks, one of which exhibited an equivalent chain length (21) identical to that of methyl arachidonate on Silar 10C and OV 225 columns. GC/PCI mass spectral analysis of the 20-carbon esters revealed $(M+1)^+$ and $(M+1-2H)^+$ ions corresponding to 20:0, 20:1, 20:3 and 20:4 (Fig. 1). On the 3% SE-30 column, the 20:4 ester eluted just prior to 20:3. We conclude from the mass spectral data and from retention behavior on the 3 column systems–OV 225, Silar 10C, and SE-30–that the 20:4 component is arachidonic acid. This acid constituted 0.3 and 1.2% of total fatty acids in male house cricket and field cricket reproductive tissues, respectively. The fatty acids found by GLC analysis are listed in Table I.

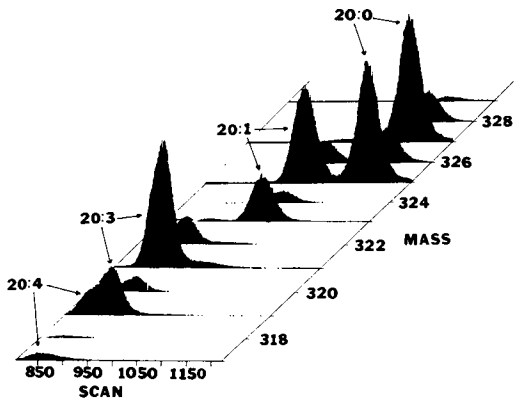

FIG. 1. Computer reconstructed GC/MS chromatogram of $(M+1)^+$ and $(M+1-2H)^+$ ions of 20 carbon fatty acid methyl esters obtained from male house cricket reproductive tissues.

The total fatty acid content of freeze-dried tissues–as determined by the internal standard procedure–was 8.2 and 8.6%, respectively, and on a dry weight basis the tissues contained ca. 0.025 and 0.158% arachidonic acid.

Failure to detect arachidonic and perhaps other 20- or 22-carbon polyenoic acids in insects may be a result of the low levels at which these compounds occur or of their concentration in phospholipids of specialized tissues. In vertebrates, these fatty acids are concentrated in phospholipids of nerve and other tissues and may not be detected during fatty acid analysis of total body lipids, particularly in the presence of large amounts of storage fat. It seems likely that this condition also may be true with insects. In order to detect and quantitate these fatty acids in insects, it may be necessary to isolate and analyze specialized tissues or to examine phospholipids and other lipid classes as was done with the cockroach (11). The low level of arachidonic acid found in the house and field cricket suggests that its

TABLE I

Fatty Acids of the Reproductive Tracts of Male House Crickets *(Acheta domesticus)* and Field Circkets (*Gryllus* spp.)

	Fatty acids (wt %)								
	16:0	18:0	18:1	18:2	20:0	20:1	20:3	20:4	other
A. domesticus	16.4	13.0	19.1	39.0	3.0	1.6	6.0	0.3	1.9
Gryllus spp.	19.3	8.5	22.2	33.3	1.2	1.5	6.2	1.2	6.6

major function in these species may be as a precursor of prostaglandin rather than as a structural component of membrane systems.

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Antioxidants in Neoplastic Cells: IV. Evidence that the Phenolic Antioxidant A274 Is 9-(4-Hydroxyphenyl)xanthene, an Artifact in Tissue Culture Media

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ABSTRACT

An extensively conjugated phenol (A274), recently shown to be present as a common trace constituent in randomly selected batches of commercial tissue culture media, was diazomethylated; the monomethyl derivative so prepared was shown to exhibit gas chromatographic and mass spectrometric properties identical to those observed for synthetically prepared 9-(4-methoxyphenyl)xanthene, but different from those of several isomeric compounds. The structure 9-(4-hydroxyphenyl)xanthene is thus proposed for A274.

INTRODUCTION

Murine neuroblastoma cells (C 1300, clone N18) grown in tissue culture acquire a striking resistance to spontaneous peroxidation during the same time that the logarithmically growing, spherical cells undergo cytodifferentiation into a nondividing, specialized form that possesses many of the properties classically associated with neurons (1). A semiquantitative assay method for soluble antioxidants developed as a part of that study (1) allowed the chromatographic isolation of an antioxidant ("A274") that was examined by high-resolution mass spectrometry (MS) and shown (2) to have a molecular weight (MW) of 274.1000 daltons, the most probable molecular formula being $C_{19}H_{14}O_2$; the base peak at m/z 181 indicated loss of the elements of $\cdot C_6H_4OH$, which, coupled with the fact that A274 readily formed monosubstitution products with acetylating or trimethylsilylating reagents but was only partially monomethylated by diazomethane, allowed postulation of the partial structure $C_{13}H_9O\text{-}C_6H_4OH$ for A274 (2).

We subsequently found that A274 can be isolated from serum-free batches of tissue culture medium with no cells having been present, and we determined that the antioxidant is introduced into the experiment as a contaminant of the tissue culture medium in at least some of the batches supplied by all commercial sources that we examined (3). This led us to conclude that the appearance of A274 in our cultured cells, in concert with the onset of cytodifferentiation, is an epiphenomenon deriving from the fact that the withdrawal of fetal bovine serum albumin from the medium preparations after the fourth day of culture not only precipitates the change in morphology, but also removes the binding mechanism that had rendered A274 unavailable to the cells during the first 4 days.

The presence of such a contaminant in a tissue culture medium potentially is a matter of serious concern to suppliers and to users alike, as the influence of this organic pollutant in such microenvironments is presently untested. Before any evaluation of the biological consequences of A274 can be considered practical, more definite information about its chemical structure is necessary. In this paper, we report the identity of the gas chromatographic and mass spectrometric properties of the mono-*O*-methyl derivative of A274 to those of 9-(4-methoxyphenyl)xanthene.

METHODS

A 99.5-g sample of Dulbecco's Modified Eagle's Medium (DM-324, KC Biological, Inc., Lenexa, KS; manufacturer's lot number 2406857) was extracted with 100 ml of water, 267 ml of ethanol, and 133 ml of chloroform according to the Bligh-Dyer method (4). As no significant biochemical background was expected, the diphasic mixture was simply diluted with 125 ml each of water and chloroform, and the chloroform layer was separated and washed 4 times with 400-ml portions of water. Concentration in vacuo using a rotary flash evaporator gave a residue that was redissolved in 20 ml of chloroform and concentrated to dryness in a stream of nitrogen. A portion of the solid residue was dissolved in 2 ml of pentane for analysis, and the remainder was treated overnight with diazomethane (1 mmol) in ether. MS revealed that A274 was essentially

unaffected, and a second portion of diazomethane (3 mmol) in ether was added and stirred for 3 hr. The solvent was removed by warming to 40 C and passing a stream of nitrogen over the solution, and the residue was extracted with 2 ml of pentane for analysis.

9-(4-Methoxyphenyl)xanthene was prepared by a Friedel-Crafts reaction of *p*-methoxybenzal chloride (ref. 5, 14 g) with diphenyl ether (12.5 g) in nitrobenzene (50 ml) under a nitrogen atmosphere; after gradual addition of aluminum chloride (20 g) at 50-60 C, the reaction was stirred at 25 C for 4 days. The reaction was quenched in water, and the ether-soluble products were analyzed without purification. Details of the synthesis of Scheme IV and the other compounds in this study will be published separately (6).

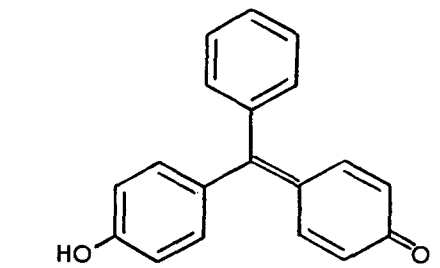
Positive-ion, electron-impact mass spectra were recorded using a Finnigan 4023 quadrupole mass spectrometer interfaced to a Model 2300 interactive minicomputer system, which controlled instrument operation and acquired data. The source was held at 250 C, and the electron energy was a nominal 70 eV. Samples were introduced through a Finnigan 9610 gas chromatograph fitted with a glass column packed with 3% OV-1 on 100-120 mesh Gas Chrom Q (4 mm id; length, 2 m); helium (21 ml/min) was used as the carrier gas; the injector, the transfer line, and the single-stage glass jet separator were held at 250 C; the column temperature was held at 60 C for 60 sec while the column effluent was diverted away from the separator, and then programmed from 60 C to 240 C at a rate of 10 C/min. Injection vol were 0.3 μl.

RESULTS AND DISCUSSION

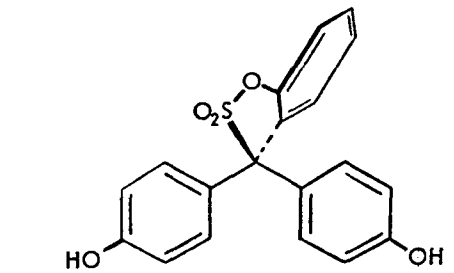
The electron-impact, positive-ion mass spectrum of the monomethyl ether of A274 exhibits 3 principal ions: a fairly abundant molecular ion (m/z 288), a rather strong ion corresponding to loss of a hydrogen atom from the molecular ion and the base peak (m/z 181). The abundance of m/z 288 (and the observed affinity of the neutral lipid [2] A274 for LH-20 Sephadex) suggests an extensively conjugated aromatic molecule, whereas the comparatively favored loss of 1 amu from m/z 288 indicates the attachment of a hydrogen atom at a site that is able to stabilize a positive charge (as, for example, an aldehyde or pyran derivative); the base peak (m/z 181) is the same as that observed (2) for A274, so the fragment lost may be presumed to be $\cdot C_6H_4OCH_3$, from which it follows that the site of etherification was a phenolic hydroxyl group. The absence of other fragments suggests that the ion m/z 181 is highly symmetrical, and the unreactivity of the second oxygen atom of A274 indicates that it must be present as either an ether, a carbonyl group, or possibly a tertiary alcohol having no α hydrogen atoms in the portion of the molecule having composition $C_{13}H_9O$.

A search of the formula index of *Chemical Abstracts* revealed that the triphenylmethane dye benzaurin (Scheme I), which has the same carbon skeleton as phenolsulfonphthalein (Scheme II) (a ubiquitous constituent in tissue culture experiments), has the desired molecular formula and functionalization of oxygen; the structure of Scheme I appears inconsistent with the observed pattern of fragmentation, however, and the actual mass spectrum of Scheme I was clearly different (R.M. Arneson and J.D. Wander, unpublished data) from that of A274.

Based on the analysis of Figure 1, one may envision A274 as a phenol nucleus attached to a tetrahedral carbon atom in a C_{13} moiety, of which the most likely possibilities would contain 2 symmetrically placed benzenoid rings. Such a structure essentially precludes the presence of a carbonyl group, as no symmetrical location is possible, but could be accounted for by a benzhydrol derivative, as for example, a 9-(hydroxyphenyl)-9-fluorenol (Scheme III). The mass spectra of crude preparations of the 3 isomeric monomethyl ethers of Scheme III exhibit prominent ions at m/z 181, but the presence of an abundant ion (m/z



SCHEME I



SCHEME II

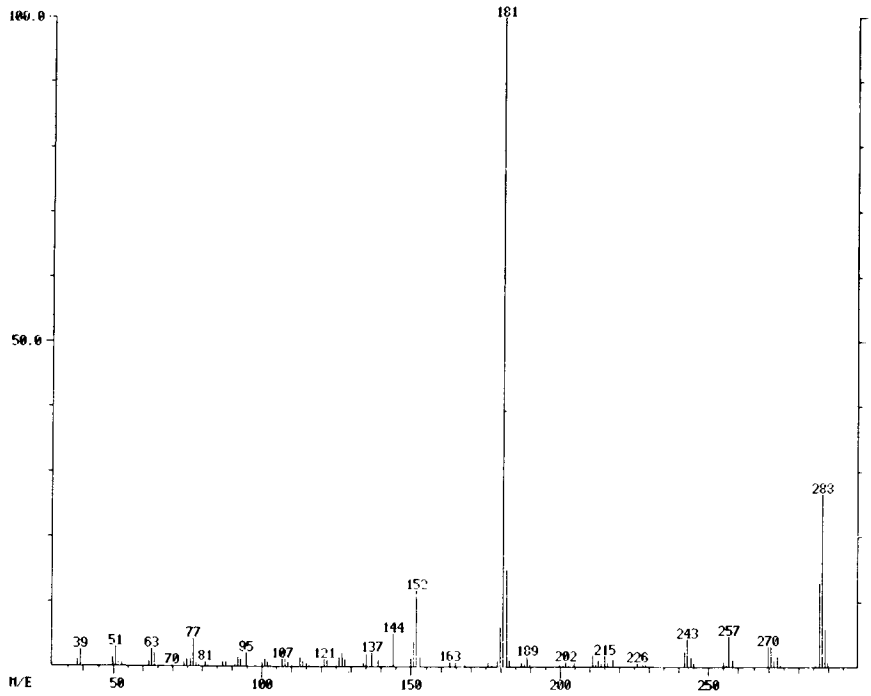
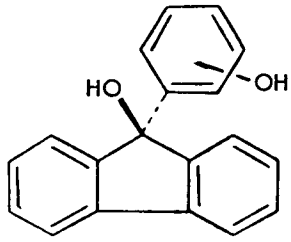
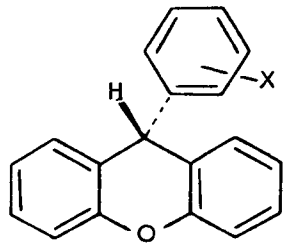


FIG. 1. 70-eV electron-impact, positive ion mass spectrum of the monomethyl derivative of A274.



SCHEME III



IV X = OH-p

V X = OH-m

VI X = OH-o

VII X = OCH_3-p

SCHEMES IV-VII

271) corresponding to loss of the tertiary hydroxyl group (6) eliminates III as a possible structure for A274.

The same essential structure can be preserved by placing a hydrogen atom at the quaternary center and inserting the nonphenolic oxygen atom as an ether bridge between the phenyl rings. Such a molecule would be predicted to undergo highly favored losses of either the hydrogen atom or the hydroxyphenyl group, as in Figure 1. Of the 2 available dibenzopyrans, the 9-(hydroxyphenyl)xanthenes (Schemes IV-VI) seemed the less likely to undergo competing reactions in the mass spectrometer, and the corresponding anisoles were prepared. The mass spectrum of 9-(4-methoxyphenyl)xanthene (Scheme VII, 9-[4-methoxyphenyl]-10-oxa-9,10-dihydroanthracene [7-10], presented as Figure 2, is essentially identical to that of Figure 1. Further support for the identity of Scheme VII and the monomethyl derivative of A274 comes from the fact that both molecules have the same gas chromatographic mobility (depicted as a reconstructed chromatogram at m/z 288, the molecular ion, in Figs. 3a and b); the exactness of this correspondence was demonstrated by mixing samples of VII and the monomethyl

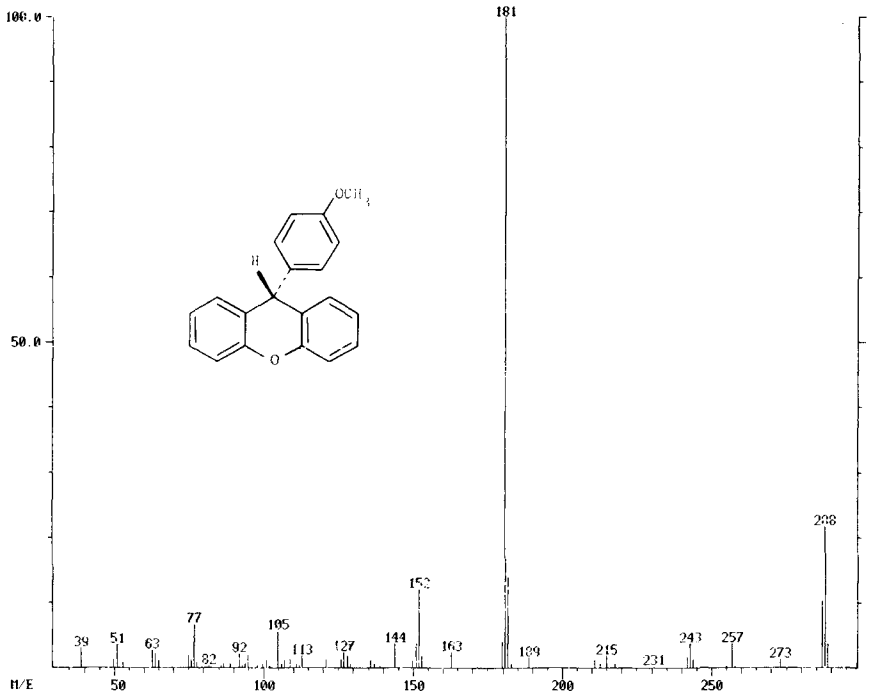


FIG. 2. 70-eV electron-impact, positive ion mass spectrum of 9-(4-methoxyphenyl)-xanthene (Scheme VII).

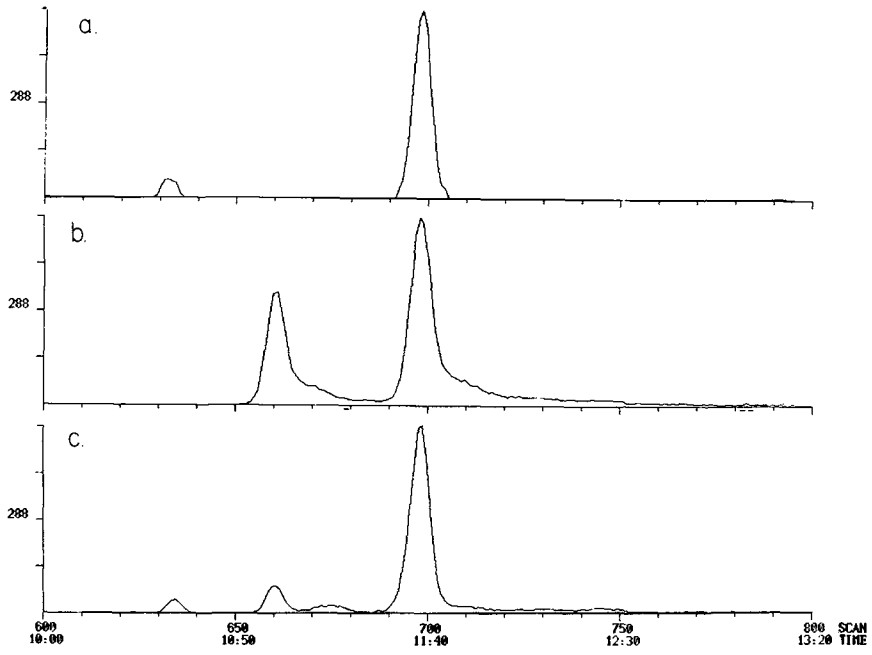


FIG. 3. Gas chromatographic analysis by recalling the ion current at m/z 288 (the molecular ion of the monomethyl derivative of A274 and of Scheme VII) of (a) the monomethyl derivative of A274, (b) a partially purified synthetic sample of 9-(4-methoxyphenyl)-xanthene (Scheme VII), and (c) a binary mixture of the 2 samples. The compounds giving the mass spectra recorded in Figs. 1 and 2 both occur at 11.6 min. The smaller peak at 11.0 min in b and c is the *ortho* isomer of Scheme VII, and the peak at 10.5 min in a and c is structurally unrelated to the compounds of interest; the presence of these contaminants provides a verification of relative retention times and a measure of the proportion in which a and b were mixed.

derivative of A274 and injecting the mixture under the same conditions to produce a peak having the same retention time, width and contour (Fig. 3c). The *ortho* isomer of VII also appears in Figure 3c, and the *meta* isomer elutes somewhat faster than the *ortho* (6).

Based on the evidence presented here, we propose that A274 is 9-(4-hydroxyphenyl)-xanthene (Scheme IV) (11,12). Less symmetrical, isomeric forms of IV also can be postulated, and, although most of those would be expected to show competing losses of a neutral, carbonyl-related fragment or fragments, this identification must be considered tentative at present.

Careful examination of the chromatogram of the initial extract of medium revealed a small amount (ca. 5%) of the monomethyl derivative of A274 prior to the methylation step. The absence of this species in earlier determinations results from its probable inactivity as an antioxidant and the fact that the earlier samples were generally introduced via the direct insertion probe after chromatography.

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Age-Strain Interrelations in Lipid Metabolism of Rats

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ABSTRACT

Various aspects of lipid metabolism were compared in Fisher 344 (F) and Sprague-Dawley (SD) rats aged 2, 6, 12, 18 and 24 months. The analyses included free and total cholesterol of serum and liver, LCAT, hepatic HMG-CoA reductase, cholesterol 7α-hydroxylase, fatty acid synthetase, acetyl CoA carboxylase and cholesterol synthesis from acetate or mevalonate. The body weight of SD rats increases with age whereas that of F rats plateaus at 9-12 months. Liver and aorta cholesterol levels were comparable for the 2 strains. Serum cholesterol varied but was usually lower in F rats. HMG-CoA reductase and cholesterol 7α-hydroxylase activities were not significantly different. Cholesterol synthesis from acetate was significantly higher only in 2-month-old F rats; synthesis from mevalonate was similar at each level. Acetyl CoA carboxylase and fatty acid synthetase activity were generally higher in F rats at every age level. The major difference between F and SD rats is in their pattern of weight gain with age. Differences in lipid metabolism are most marked between the young (2-month) rats.

INTRODUCTION

Examination of age-related changes in the lipid metabolism of experimental animals has been plagued with inconsistencies as a result of differences in the strain of animals used, age of "old" animals and a host of other uncontrolled variables. Carlson et al. (1) found that serum and liver cholesterol levels increased through 18 months of age in Sprague-Dawley rats but not at the same rate nor linearly in either tissue. Kritchevsky and Tepper (2) compared several strains of rats, all of which had increased levels of serum cholesterol with increasing age (1 to 3 months), but the pattern of the changes varied greatly. Dupont et al. (3) reported that serum cholesterol levels of CFE rats increased gradually from 3 to 12 months of age and then doubled between 12-18 months of age. We have observed little change from 2 to 18 months and a dramatic increase between 18 and 24 months of age in Charles River CD^{R}F rats (4). Liver cholesterol levels did not increase after 6 months of age in these rats. These studies compare the effects of aging on 2 strains of rats, Sprague-Dawley and Charles River CD^{R}F. The results permit comparison of the effects of aging within and between the 2 strains.

EXPERIMENTAL PROCEDURES

Male rats of Charles River CD^{R}F (Fisher 344, denoted F) and of Sprague-Dawley (SD) strains were purchased from either Charles River Breeding Laboratories, Inc., North Wilmington, MA or Harlan Industries, Inc., Indianapolis, IN. All animals were Caesarian-derived and barrier-reared and were fed diets (Wayne Lab Blox) of consistent composition. Six rats of 2, 6, 12, 18 and 24 months of age of each strain were used. All rats were killed ca. 6 hr after the beginning of the dark cycle to maximize levels of liver enzymes. Serum and liver samples were frozen and subsequently extracted with chloroform/methanol. Free and total cholesterol were analyzed by gas liquid chromatography (GLC) on 3% OV-17 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA), using 5α-cholestane (Applied Science Laboratories, State College, PA) as an internal standard (5). A Shimadzu 4B gas chromatograph equipped with a flame ionization detector was used. Serum lecithin-cholesterol acyl transferase (LCAT) was measured using the Stokke and Norum method (6).

Analysis of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and cholesterol 7α-hydroxylase activity in hepatic microsomal preparations was done as previously described (4,7-9) with some modifications. Cholesterol 7α-hydroxylase was measured using [4-^{14}C] cholesterol (New England Nuclear, Boston, MA), prepurified using thin layer chromatography (TLC) on Silica Gel G with ethyl acetate/hexane (8:2 v/v). The extraction method of Ackerman et al. (10) was used in the HMG-CoA reductase analysis. This involved saturation of the lactonized product (mevalonate) with equimolar mono- and dibasic potassium phosphate and extraction of mevalonate with chloroform. The extracts were backwashed with saturated equimolar mono- and dibasic potassium phosphate and placed in a scintillation vial. After evaporation of the chloroform, the residue was dissolved in 0.5 ml water and 10 ml Aquasol (New England

Nuclear, Boston, MA) added for liquid scintillation spectrometry.

Cholesterol biosynthesis from [1-^{14}C] acetate and [2-^{14}C] mevalonic acid (New England Nuclear, Boston, MA) using liver slices was done as described previously (4).

For the assay of fatty acid synthetase (FAS), 1 g of liver was suspended in 9 vol of 50 mM Tris-HCl (pH 7.5) buffer containing 15 M KCl, 4 mM $MgCl_2$, 4 mM 2-mercaptoethanol and 1 mM EDTA. The liver was minced with scissors, homogenized using a Potter-Elvehjem homogenizer and centrifuged at 20,000 x g for 10 min. The pellet was discarded and the supernatant was centrifuged at 100,000 x g for 45 min. The clear supernatant obtained was used for the enzyme assay. The reaction mixture contained potassium phosphate buffer (pH 7.0), 100 mM, 5 mM 2-mercaptoethanol, 3 mM EDTA, 50 μM malonyl CoA, 1.25 μM [1-^{14}C] acetyl CoA, 0.3 mM NADPH and enzyme solution in a final vol of 1 ml. Incubations were carried out for 5 min at 37 C. The reaction was stopped by addition of 0.03 ml of 60% perchloric acid. One ml of absolute ethanol was added, and the mixture was extracted 4 times with petroleum ether. The combined extracts were evaporated to dryness and assayed for radioactivity by liquid scintillation spectrometry (11). Aliquots of the high-speed supernatant were used for the acetyl CoA carboxylase (ACC) assay (12,13).

Analysis of variance was done using the *Statistical Package for the Social Sciences* (14). The means were compared using least significant differences ($p<0.05$) calculated from analysis of variance.

RESULTS AND DISCUSSION

Body weight increased more rapidly and to a higher level at 24 months in SD rats and was significantly higher at every age than in F rats (Table I). F rats reached a maximal weight at 12 months of age; by 24 months, weight had decreased significantly. In an earlier study, we had observed the maximal body weight of F rats at 12 months (4), but not the decrease at 18 and 24 months. SD rats appeared to be nearing maximal body weight at 24 months of age.

Increase in liver weight closely paralleled the body weight changes in SD rats, and the percentage of body weight represented by the liver did not change significantly (Table I). In F rats, liver weight remained relatively constant whereas body weight decreased, resulting in significant increases in liver as a percentage of body weight. Liver weight in proportion to body weight was higher in F than in SD rats at 6, 12, 18 and 24 months.

Total serum cholesterol increased gradually in F rats, with a maximum of 101.7 mg/dl reached at 24 months of age (Table II). Variability in total serum cholesterol levels increased with advancing age, as evidenced by the increase in standard error of the mean, especially at 24 months of age. This would seem to argue against the thesis that survivors in an experiment on aging are those animals with favorable physiology, because survivors seem to become more dissimilar with advancing age. A similar maximum (101.5 mg/dl) was observed in SD rats at 18 months of age. Serum LCAT activity was consistently higher in SD than in F rats. It increased with increasing age in both strains except for the 24-month-old SD rats, in which activity was significantly lower than at 18 months (Table II). The overall increase probably is in response to increased substrate (free cholesterol) which is a function of differences in both age and strain. A more thorough investigation of the causes for this increase, in light of the interdependence of LCAT activity with high density lipoprotein (HDL) cholesterol (15), is needed. Differences in LCAT activity and/or HDL cholesterol between these strains could provide a model for further study concerning their contribution to cholesterol metabolism.

Liver cholesterol levels (Table II) were significantly affected by both age and strain ($p<0.05$). Individual means indicate an increase at 18 and 24 months of age in both strains. Liver cholesterol levels were generally higher in SD than in F rats but significantly higher only at 6 months. Aortic cholesterol levels were significantly affected by age ($p<0.001$), but no strain difference was observed.

The actual amount of cholesterol present in the serum and liver of the rats was calculated from body and liver weights and serum and liver cholesterol levels. The results are expressed as mg of cholesterol. Age had a dramatic effect on the pool of serum plus liver cholesterol. The pool in 2-month-old F rats was 17.1 mg and it increased to 36.4 mg at 24 months. The pools in 2- and 24-month-old SD rats were 29.4 and 56.6 mg, respectively. Cholesterol pools in SD rats were higher (22-72%) at every age. Dupont et al. reported increases in serum cholesterol levels of aging (9 to 21 months) CFE rats but found no change in carcass (minus head, blood, gastrointestinal tract and liver) cholesterol concentration (16,17). Total carcass cholesterol increased with age as a result of increasing body weight. Their results (16,17) suggest a relative lack of change in the body pool with slow

TABLE I

Effect of Age on Body and Liver Weights of Fisher 344 (F) and Sprague-Dawley (SD) Rats (Mean ± SEM)

	Strain	Age (months) 2	6	12	18	24
Body weight (g)	F	297 ± 7*	405[a] ± 15*	440[a,b] ± 12*	434[a] ± 6*	392[a,c,d] ± 8*
	SD	413 ± 8	458 ± 10	614[a,b] ± 34	680[a,b,c] ± 29	701[a,b,c] ± 19
Liver weight (g)	F	9.3 ± 0.4*	11.6[a] ± 0.4	14.5[a,b] ± 0.7*	14.4[a,b] ± 0.4*	13.9[a,b] ± 0.3*
	SD	13.5 ± 0.4	14.6 ± 0.4	18.3[a,b] ± 1.2	20.3[a,b] ± 0.9	20.2[a,b] ± 1.7
Liver weight (% body wt.)	F	3.14 ± 0.10	2.88 ± 0.04*	3.29[b] ± 0.09*	3.32[b] ± 0.11	3.57[a,b,c] ± 0.11*
	SD	3.26 ± 0.10	3.25 ± 0.06	2.98 ± 0.14	3.00 ± 0.16	2.88 ± 0.21

[a-d]Letters indicate age used for comparison; (a, 2 months; b, 6 months; c, 12 months; d, 18 months) to indicate significant ($p<0.005$) difference. *Significant difference ($p<0.05$).

TABLE II

Serum and Tissue Cholesterol Levels of Fisher 344 (F) and Sprague-Dawley (SD) Rats at Various Ages (Mean ± SEM)

Variable	Strain	Age (months) 2	6	12	18	24
Serum cholesterol						
Total (mg/dl)	F	31.7 ± 1.9*	38.2 ± 2.5*	61.4[a,b] ± 5.2	67.8[a,b] ± 4.8*	101.7[a,b,c,d] ± 15.8
	SD	40.1 ± 2.5	48.8 ± 2.2	47.2 ± 5.0	101.5[a,b,c] ± 9.7	98.0[a,b,c] ± 22.3
Free (mg/dl)	F	9.3 ± 0.6*	11.0 ± 0.9	17.4[a,b] ± 1.0	18.3[a,b] ± 1.1	27.0[a,b,c,d] ± 3.6
	SD	12.6 ± 0.9	14.5 ± 0.4	14.0 ± 1.6	29.2[a,b,c] ± 3.1	29.2[a,b,c] ± 6.1
Lecithin-cholesterol acyl transferase (mol/hr)	F	0.63 ± 0.10*	0.78 ± 0.14*	0.82 ± 0.15	1.36[a,b,c] ± 0.22	1.79[a,b,c] ± 0.24
	SD	1.17 ± 0.12	1.36 ± 0.20	1.24 ± 0.19	3.08[a,b,c] ± 0.54	2.67[a,b,c] ± 0.63
Liver cholesterol (mg/g)	F	1.53 ± 0.06*	1.48 ± 0.03	1.44 ± 0.08	1.76[a,b,c] ± 0.07	1.76[a,b,c] ± 0.09
	SD	1.81 ± 0.10	1.67 ± 0.09	1.53[a] ± 0.06	1.72 ± 0.07	1.78 ± 0.09
Aortic cholesterol (mg/g)	F	1.20 ± 0.04	1.05 ± 0.07	1.12 ± 0.04	1.28[b,c] ± 0.06	1.47[a,b,c] ± 0.05
	SD	1.27 ± 0.03	1.23 ± 0.05	1.20 ± 0.03	1.23 ± 0.06	1.35[c] ± 0.06

[a-d]See legend to Table I.

TABLE III

Cholesterol Synthesis by Liver Slices from Fisher 344 (F) and Sprague-Dawley (SD) Rats of Various Ages (Mean ± SEM)

Substrate	Strain	Age (months) 2	6	12	18	24
[1-^{14}C] Acetate (dpm X 10^{-5}/mg cholesterol)	F	9.84 ± 3.72	4.41[a] ± 1.00	2.56[a] ± 0.29	3.02[a] ± 0.31	4.22[a] ± 0.86
	SD	4.93 ± 1.09	5.97 ± 1.73	3.80 ± 0.65	3.53 ± 0.91	2.79[b] ± 0.33
[2-^{14}C] Mevalonate (dpm X 10^{-5}/mg cholesterol)	F	8.81 ± 1.14	10.12 ± 1.21	9.11 ± 1.80	6.48[b,c] ± 1.02	4.87[b,c] ± 1.08
	SD	7.92 ± 1.29	11.19 ± 1.23	10.61[a] ± 1.59	6.56[b,c] ± 1.03	6.58[b,c] ± 0.97

[a-d]See legend to Table I.

Liver slices (0.5 g) were incubated under O_2 at 37 C for 3 hr in 5 ml phosphate buffer (pH 7.0) containing 0.6 mM $MgCl_2$, 30 mM nicotinamide and 0.5 μCi of the appropriate precursor. The reaction was stopped by addition of 5 ml of 15% alc KOH. Cholesterol was extracted from the saponified sample, isolated as the digitonide and assayed for mass and radioactivity.

TABLE IV

Liver Microsomal HMG-CoA Reductase and Cholesterol 7α-Hydroxylase in Fisher 344 (F) and Sprague-Dawley (SD) Rats of Various Ages (Mean ± SEM)

	Strain	Age (months) 2	6	12	18	24
3-Hydroxy-3-methylglutaryl Coenzyme A reductase (nmol/mg/30 min)	F	7.91 ± 0.79	9.92 ± 1.68	8.74 ± 0.88	11.13 ± 1.33	11.40 ± 2.53
	SD	7.22 ± 2.13	12.51 ± 2.65	9.81 ± 1.48	8.07 ± 0.99	10.76 ± 3.45
Cholesterol 7α-hydroxylase (nmol/mg/30 min)	F	13.94 ± 1.10	14.45 ± 1.45	12.98 ± 2.36	12.85 ± 1.92	17.25 ± 2.66
	SD	11.23 ± 1.72	11.01 ± 1.31	13.69 ± 2.04	13.47 ± 1.94	11.06 ± 1.51

TABLE V

Hepatic Acetyl CoA Carboxylase (ACC) and Fatty Acid Synthetase (FAS) in Fisher 344 (F) and Sprague-Dawley (SD) Rats (Mean ± SEM)

Age (months)	Enzyme*			
	ACC		FAS	
	F	SD	F	SD
2	0.149 ± 0.034*	0.122 ± 0.021	0.356 ± 0.067	0.300 ± 0.039
6	0.175 ± 0.012	0.178 ± 0.020	0.519 ± 0.117	0.554 ± 0.081
12	0.241 ± 0.024	0.178 ± 0.041	0.793 ± 0.146	0.699 ± 0.327
18	0.216 ± 0.024	0.191 ± 0.021	0.773 ± 0.178	0.676 ± 0.208
24	0.305 ± 0.024	0.193 ± 0.035	1.416 ± 0.321	0.614 ± 0.252

*Activity of both enzymes expressed as nmol product/min/mg protein ± SEM.

turnover rate. Their turnover studies indicated that, as CFE rats grew older, they exhibited increased retention of radioactive cholesterol in the carcass but not the liver.

Cholesterol synthesis and degradation by liver slices and by microsomal preparations were measured in an effort to explain the observed differences in tissue cholesterol concentrations. The levels of cholesterol synthesis by liver slices from both acetate and mevalonate are given in Table III. Synthesis from acetate dropped significantly between 2 and 6 months in F rats and remained at this low level through 24 months of age. We had observed this in earlier work with F rats (4). A decrease also was observed in SD rats, but it was much more gradual and only significantly lower ($p<0.05$) at 24 months of age. Synthesis from mevalonate, on the other hand, did not decrease until 18 months of age in both strains. Changes with age, in the case of mevalonate, were very similar in both strains, indicating similar age-related changes in postmevalonate cholesterol synthesis, but pointed out little differences between the strain which would explain the differences in rate and total accumulation of cholesterol with age.

The liver levels of microsomal HMG-CoA reductase, a control point in cholesterol synthesis (Table IV), were not significantly different in the 2 rat strains with advancing age. The decline observed in whole tissue cholesterol synthesis (liver slices) with age probably is regulated by changes in the amount of available enzyme, or in general cellular function, rather than by changes in enzyme activity.

Cholesterol 7α-hydroxylase activity in liver microsomal preparations (Table IV) indicated little change in bile acid synthesis rates. Decreased excretion of injected tritiated cholesterol observed in feces of aged rats, by Hruza and Zbuzkova (18), apparently is not a result of changes in synthesis rates. Changes in absorption of cholesterol or enterohepatic circulation of bile acids may be responsible for these observations. Further study of bile acid and cholesterol balance should help explain these differences.

At every age but 6 months, hepatic acetyl CoA carboxylase (ACC) and fatty acid synthetase (FAS) activities in F rats were greater than in SD (Table V). In SD rats, the activity of both enzymes increased sharply between 2 and 6 months of age and then reached a plateau. In F rats, activity rose steadily from 2 to 12 months. Activity at 18 months resembled that at 12 months, and then there was another sharp increase in activity in rats aged 24 months. The ratio of FAS to ACC activities increased with age in both strains. Ratios at 2, 6, 12, 18 and 24 months for F rats were 2.39, 2.97, 3.29, 3.57 and 4.64, and for SD rats were 2.46, 3.11, 3.93, 3.54, and 3.18. Fatty acid synthesis appears to increase in F rats throughout the life span but decreases in SD rats after 12 months of age, possibly in response to the amount of adipose tissue storage.

The differences between F 344 and SD rats provide a useful tool for comparative studies of lipid metabolism in aging. Both strains accumulate cholesterol in serum and liver with advancing age but at differing rates and to different levels. Examination of differences in cholesterol turnover in each strain may help to explain the observed variations in cholesterol levels. Elucidation of the underlying causes for these changes will aid in understanding this facet of the aging process.

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Diet and High Density Lipoproteins[1]

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ABSTRACT

The acute and subacute effects of different diets on serum high density lipoprotein (HDL) cholesterol concentration and/or HDL composition are described. In obese normolipemic, hypercholesterolemic (type II) and hypertriglyceridemic (type IV) patients, low HDL cholesterol values remained low during total starvation for 2 weeks. Prudent diets in patients with type II and IV hyperlipoproteinemia had no significant effects on HDL cholesterol within 3 weeks, whereas in normal individuals, a high carbohydrate diet given for 10 days caused significant decreases in HDL cholesterol with concomitant increases in HDL triglycerides as compared to a high-fat diet. The HDL triglyceride fatty acid composition changed in healthy volunteers during the day, depending on the type of ingested fat. The data demonstrate the possibility of acute manipulations of HDL in normal patients and the difficulties of normalizing low HDL cholesterol levels in patients by dietary means. Additional information on the function of HDL is desirable before the therapeutic manipulation of HDL cholesterol or other constituents of HDL can be evaluated regarding their effectiveness in the prevention of ischemic vascular disease.

Increasing epidemiological evidence suggests a protective role of high density lipoproteins (HDL) against atherosclerosis (1,2). The underlying mechanisms might be HDL-mediated cholesterol transport out of cells (3) and inhibition by HDL of low density lipoprotein (LDL) uptake by smooth muscle cells in tissue culture (4). Factors found to affect HDL cholesterol are race, sex, physical exercise, hormones and other drugs (5,6) as well as dietary factors. Since there is still insufficient information on the effects of dietary manipulation on HDL cholesterol and on HDL composition, we report such studies performed on a metabolic ward in normal subjects, patients with obesity and various types of hyperlipoproteinemia. "Diets" used were complete fasting, high carbohydrate diet, a fat-modified, low-cholesterol protocol and a formula diet with either corn or palm oil as fat constituents.

MATERIAL AND METHODS

Diets and Patients

Total starvation was extended for 14 days in 25 obese patients with normolipidemia, 11 obese patients with type II hypercholesterolemia and 13 obese patients with type IV hypertriglyceridemia. The age of each patient was between 20 and 50 years; obesity was defined as 30% above ideal weight. The classification of type II and IV hyperlipoproteinemia was made according to Fredrickson and Levy (7). During the total starvation regimen patients received a vitamin supply, 300 mg Allopurinol/day and fluid free of calories ad libitum.

The prudent diet was isocaloric and given for 3 wk to 14 patients with familial type II and to 11 patients with type IV hyperlipoproteinemia. The age of each patient was between 32 and 68 years. The average percentages for fat, carbohydrate and protein were 37, 48 and 15% respectively, with a ratio of polyunsaturated:saturated (P:S) fat of 1.5 and a cholesterol intake below 300 mg/day. Studies 1 and 2 on patients were done at a metabolic ward.

The high-carbohydrate (CH) diet in the normal volunteers (n = 12, age 20-27) consisted of 80% carbohydrate, 5% fat and 15% protein, and was compared to a high-fat diet (35% carbohydrate, 50% fat, 15% protein); each was given for 10 days.

In the acute experiments on postprandial HDL triglyceride fatty acids, the volunteers (n = 10, age 20-27) ingested equal portions of an isocaloric formula diet (40% fat, 45% carbohydrate and 15% protein) every 2 hr, the fat was either palm or corn oil.

Laboratory Methods

Blood was collected in sodium EDTA-tubes and the plasma was immediately analyzed. Ultracentrifugation was used to separate very low density lipoproteins (VLDL) at 1.006 g/ml followed by precipitation of LDL in the infranatant by heparin manganese chloride according to LRC procedures (8). Triglycerides (9) and cholesterol (10) were determined on a Technicon AA II autoanalyzer. Lipid analyses were quality controlled by the Center for Disease Control, Atlanta, GA. The precision coefficient (V_c) for cholesterol was 3.5 and 5.1 for triglyceride. The recovery of cholesterol from lipoproteins after fractionation with the

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ultracentrifuge was above 94% for normolipemic serum samples. From the HDL fraction, the triglyceride fatty acid composition was measured by gas liquid chromatography after preparation by thin layer chromatography (11). HDL were isolated from the d < 1.006 fraction by a second ultracentrifugation step at density 1.063 g/ml. Statistical analyses were made using the Friedman and Wilcoxon tests (12).

RESULTS

Total Starvation

HDL-cholesterol levels remained unchanged during 14 days of total starvation (Figs. 1-3). Adherence of the patients was good as checked by semiquantitative urine analysis of ketone bodies with an average weight loss of 450 g/day.

In normolipidemic, obese patients (Fig. 1), the LDL cholesterol levels decreased significantly ($p < 0.05$) during the 14 days of fasting, whereas HDL cholesterol remained unchanged.

In type II hypercholesterolemia, LDL and HDL cholesterol showed no significant changes, although the median of LDL cholesterol was lowered from 226 to 202 mg/dl.

In type IV hypertriglyceridemia, elevated triglyceride values (up to 600 mg/dl) were normalized within 1 wk (< 200 mg/dl). The LDL cholesterol, after a transient increase, fell significantly ($p < 0.05$) during the total starvation regimen; HDL cholesterol was low before and after fasting.

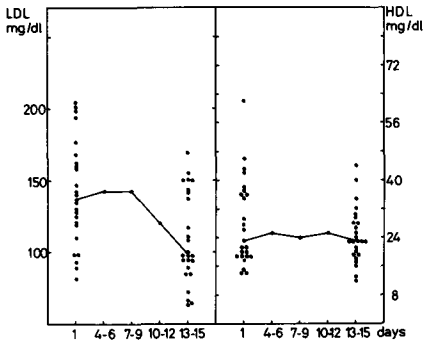

FIG. 1. Median of LDL and HDL cholesterol with total starvation in normolipidemia (n = 25).

Prudent Diet

HDL cholesterol levels in both types of hyperlipoproteinemia (Figs. 4 and 5) were unaffected by the prudent diet; the median of LDL cholesterol decreased significantly in type II hypercholesterolemia (Fig. 4) but was unchanged in type IV hypertriglyceridemia with a tendency to lower values after the prudent diet (Fig. 5). The weight of the patients was kept constant (± 2 kg in 3 wk).

High Carbohydrate Diet (Table I)

After 10 days, a significant increase in HDL triglycerides and a significant decrease in HDL cholesterol ($p < 0.05$) were observed. The increase in HDL triglycerides paralleled an increase in VLDL triglycerides, and with the decline of HDL cholesterol, a concomitant decrease of LDL cholesterol was noted.

HDL Triglyceride Fatty Acid Composition (Fig. 6)

Postprandial changes in HDL triglyceride fatty acid composition were evident, depending on the type of ingested fat. With corn oil (56% linoleic acid, 18:2, Fig. 6), there was a postprandial increase of 18:2 in HDL triglycerides, whereas the absolute amount of HDL triglycerides rose only slightly from 5.6 to 7 mg/dl and HDL cholesterol was unchanged

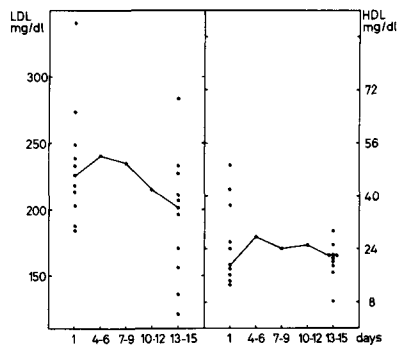

FIG. 2. Median of LDL and HDL cholesterol with total starvation in type II hyperlipoproteinemia (n = 11).

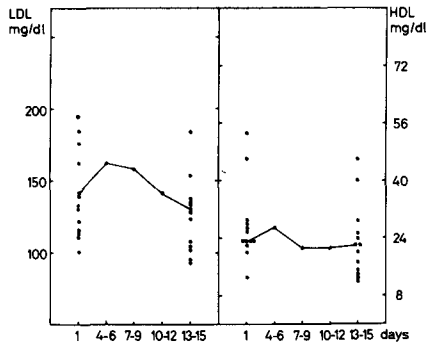

FIG. 3. Median of LDL and HDL cholesterol with total starvation in type IV hyperlipoproteinemia (n = 13).

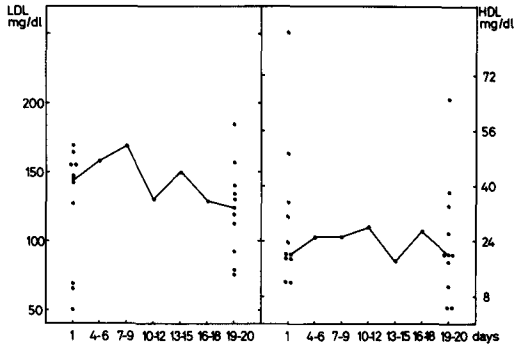

FIG. 5. Median of LDL and HDL cholesterol with prudent diet in type IV hyperlipoproteinemia (n = 11).

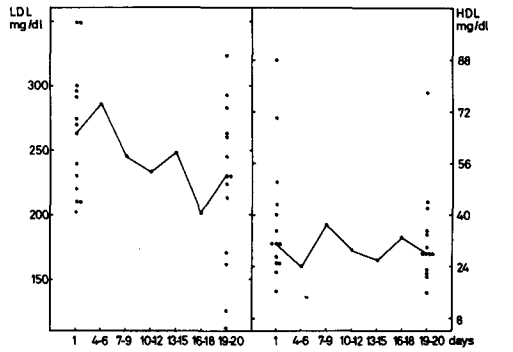

FIG. 4. Median of LDL and HDL cholesterol with prudent diet in type II hyperlipoproteinemia (n = 14).

(41.6 at 8 a.m. and 41.5 mg/dl at 8 p.m.). With palm oil (43% oleic acid, 40% palmitic acid), the fractional fatty acid changes of HDL triglycerides were less dependent on the type of ingested fat.

DISCUSSION

A high HDL cholesterol level is considered to possibly protect against the development of atherosclerosis (13). The logical conclusion would be to manipulate HDL cholesterol upward. This, from our findings, seems to be difficult, at least within 2-3 wk in patients with obesity or with type II and IV hyperlipidemias, where the HDL cholesterol levels usually are lower than in normal controls (1,2). In addition, we want to discuss 2 lines of evidence that a more differentiated point of view will be necessary to evaluate the usefulness of HDL cholesterol changes in the individual.

The correlations between lipoproteins, as suggested by epidemiological studies, cannot predict changes of the different lipoproteins following dietary manipulation. In epidemiological studies, plasma triglycerides or VLDL levels are inversely correlated to HDL cholesterol (14,15). Mechanisms for this relationship have been suggested (16-18). In our acute experiments with weight reduction in type IV hyperlipoproteinemic obese patients, a large decrease in serum triglycerides occurred without changing the HDL cholesterol level (Fig. 3). Even in long-term studies, no significant HDL alteration was found with weight reduction (19). Thus, VLDL triglyceride reduction does not necessarily induce increases of HDL cholesterol, as could be anticipated from the epidemiological findings. In fact, situations are known, e.g., alcohol (20) and estrogen intake (21,22), where VLDL triglycerides and HDL cholesterol both increase.

High LDL cholesterol and low HDL cholesterol could be identified as risk factors for coronary heart disease (CHD); in most epidemiological studies, LDL and HDL were negatively correlated (1) and both lipoproteins contrib-

TABLE I

Lipoprotein Triglycerides (G) and Cholesterol (CHOL) with High and Low Carbohydrate Diets

		Carbohydrate diet	
		Low	High
VLDL[a]	TG mg/dl	10.8 ± 10.7	41.4 ± 21.2 x[b]
	CHOL mg/dl	6.4 ± 5.9	5.7 ± 6.2
LDL	TG mg/dl	15.7 ± 10.3	23.8 ± 10.7 x
	CHOL mg/dl	119.6 ± 15.7	90.2 ± 25.3 x
HDL	TG mg/dl	3.0 ± 3.8	11.4 ± 3.8 x
	CHOL mg/dl	37.5 ± 9.3	26.5 ± 9.5 x

[a]VLDL = very low density lipoproteins; LDL = low density lipoproteins; HDL = high density lipoproteins.

[b]x $p < 0.05$; n = 12; mean ± SD.

uted to the risk profile for CHD. We observed, with a prudent diet in type II and IV hyperlipoproteinemia (Figs. 4 and 5), decreases in LDL cholesterol without changes of HDL cholesterol, as has been shown earlier for normal individuals (23,24) and patients with atherosclerosis (25). Recently, the failure of triglyceride lowering to correct low HDL cholesterol levels in type IV (26) and V (27) hyperlipoproteinemia was reported, whereas an increase of low HDL cholesterol levels after dietary treatment of type III hyperlipoproteinemia occurred (28). Thus, LDL and HDL seem to vary independently in most types of hyperlipoproteinemia, and polyunsaturated fatty acids appear to act mainly on LDL cholesterol. In a recent study with a very high P/S ratio of 4, LDL and HDL cholesterol decreased (29). At the same time, the fluidity of these HDL particles increased, rendering them more susceptible to catabolic sites. This leads to the second point of our discussion: that HDL function may be insufficiently explained by HDL cholesterol.

HDL is a molecule with important constituents other than cholesterol. These can vary independently and may influence HDL function.

The well known phenomenon of low-HDL cholesterol after high-carbohydate feeding (30-32) is accompanied by an increase in HDL triglycerides (Table I). Triglycerides and cholesteryl esters are located in the core of the HDL molecule; thus, an increase in HDL triglycerides could displace HDL cholesterol (33). It has been shown that, with a high-carbohydrate diet (18), HDL turnover is increased up to 4-fold concomitant with such changes. Conclusions about the significance of a low-HDL cholesterol in connection with increased turnover are not yet possible. Furthermore, the effects of carbohydrates on HDL might change with time, similar to plasma triglycerides which, after 6 months of such a diet, tend to normalize (34).

During the day, changes within the fatty acids of the HDL triglycerides were observed, depending on the type of ingested fat (Fig. 6) (11). Barter and Connor (35), who described this phenomenon in a study with radioactively labeled fatty acids, postulated the existence of a rapidly exchangeable HDL triglyceride pool, which is about 1/3 of HDL triglycerides. Nichols (14) reported as early as 1967 that the HDL subfractions can vary independently of each other, and that HDL_2 probably is the "Antiatherogenic" component (15,36). HDL apoproteins AI and AII and the apo AI/cholesterol ratio of HDL apparently are variable

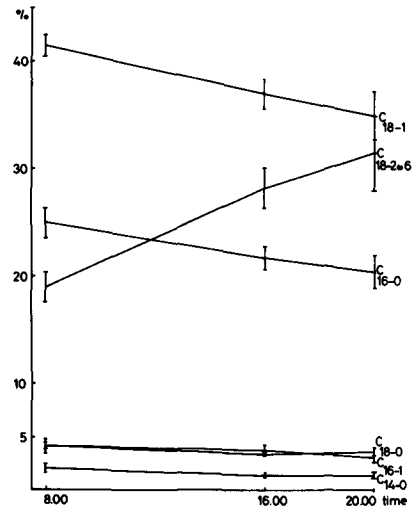

FIG. 6. HDL triglyceride fatty acid composition after corn oil diet ($p < 0.05$ for Δ 8.00-20.00 of C_{16-0}, C_{18-1} and C_{18-2}, n = 10).

(27-39). "New" HDL subclasses, such as a cholesterol-rich HDL-C, have been described with cholesterol feeding; this HDL-C behaves in tissue culture experiments like atherogenic LDL (40).

In conclusion, there are still many unanswered questions with regard to the meaning of changes in HDL cholesterol or other changes of the HDL molecule for the development of atherosclerosis. For reasons given in our paper, at present we see no major advantage in the formulation of LDL/HDL cholesterol ratios for evaluation of therapy. Such ratios would have been favorably influenced with our diets through decreases of LDL cholesterol, obscuring observations on HDL. Manipulation of HDL cholesterol with the diets used in patients with obesity and type II and IV hyperlipoproteinemias and low initial HDL cholesterol levels (2) probably is quite difficult compared to manipulation of LDL cholesterol or plasma triglycerides.

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Thiobarbituric Acid Reaction of Methyl Arachidonate Monohydroperoxide Isomers

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ABSTRACT

Methyl ester of monohydroperoxy eicosatetraenoic acid (MeHPETE) was prepared from methylene blue sensitized photooxidation products of methyl arachidonate. The thiobarbituric acid (TBA) value of MeHPETE was increased by adding ferrous sulfate to the reaction mixture. A linear relationship existed between the TBA value and the concentration of MeHPETE when ferrous sulfate was added. By using high performance liquid chromatography, MeHPETE was separated into 5 fractions whose isomeric compositions were determined by gas chromatography-mass spectrometry. The results of the TBA test for each fraction suggest that all of the MeHPETE isomers are positive to the TBA test. It is concluded that each isomer of HPETE formed by peroxidation of arachidonic acid in a biological system can yield TBA-reacting materials during the test reaction.

INTRODUCTION

The thiobarbituric acid (TBA) test has been widely used to measure the lipid peroxides in biological systems. The TBA-reacting materials from the peroxidation of polyunsaturated fatty acids (PUFA) is frequently referred to as malonaldehyde (MA). However, this volatile aldehyde seems to exist predominantly in a weakly bound state as a nonvolatile precursor and is released under the conditions of the TBA test (1-3). Accordingly, determination of the species of lipid peroxides responsible for coloring with TBA is necessary before the test reaction is interpreted. Five-membered monocyclic peroxides (1) or prostaglandin-like endoperoxides (2,3) have been regarded as nonvolatile MA precursors in peroxidized PUFA. It was also shown that acylic hydroperoxides prepared from β-linolenic acid result in a positive TBA test (4). Peroxidation of PUFA can yield several regioisomeric monohydroperoxides. For example, 6 isomers of hydroperoxy eicosatetraenoic acid (HPETE) are produced by free radical oxidation of arachidonic acid (5). Furthermore, it was suggested that regiospecific HPETE is formed by the reaction of lipoxygenase with arachidonic acid in platelet (6) and polymorphonuclear leukocytes (7). Recently, a coloring condition of the test for measuring lipohydroperoxides was developed to elevate the sensitivity of the test (8-10). Thus, it is of interest to clarify the reactivity of each HPETE regioisomer from the peroxidation of arachidonic acid.

We have determined the isomeric composition of methyl ester of hydroperoxy eicosatetraenoic acid (MeHPETE) produced from singlet oxygen oxidation (11) and hemeprotein-catalyzed peroxidation of methyl arachidonate (12). On the other hand, Porter et al. (5,13) succeeded in isolating HPETE isomers of biological importance from peroxidized arachidonic acid by using high performance liquid chromatography (HPLC). In this work, isomeric MeHPETE prepared from singlet oxygen oxidation was separated into several fractions by HPLC. The TBA value of each fraction was measured by adding metal salts which elevate the sensitivity of the test by accelerating the release of TBA-reacting materials (9,10). The results of the test reaction indicate that all of the regioisomers of HPETE produced in a biological system can act as nonvolatile precursors of TBA-reacting materials.

EXPERIMENTAL PROCEDURE

Materials

Methyl arachidonate (99+ %) was purchased from Sigma Chem., St. Louis, MO. Isomeric MeHPETE was prepared using methylene-blue-sensitized photooxidation of methyl arachidonate in ethanol solution according to the method described previously (11).

HPLC

HPLC was done on a column (250 × 4 mm) of Zorbax SIL (DuPont), which is a silica gel powder, 5 μm in particle size, with Shimadzu-DuPont liquid chromatography LC-2. A Shimadzu UVD-2 variable length UV detector was used to monitor the effluent at 205 nm. Solvent flow was maintained at 1.0 ml/min; the eluting solvent consisted of 1.0% isopropanol in hexane. For fractionation of MeHPETE isomers, 200 μl of hexane solution was injected several times. Each fraction separated on HPLC was evaporated in vacuo and kept at -20 C as ethanol solution until use in the experiments.

Determination of the Isomeric Compositions of the Fractions

Fractions were divided into 2 parts, one of which was subjected to GLC and gas chromatography-mass spectrometry (GC-MS) analysis for determination of the quantities and isomeric compositions of MeHPETE. The derivatization procedure and conditions of GC-MS were the same as described previously (11). Methyl heptadecanoate was used as the internal standard in GC analysis.

TBA Reaction

The other part of the fraction, 1.0 ml of ethanol solution, was placed in a screw-capped test tube. Then, 1.0 ml of 0.67% of TBA and 2.0 ml of 20% trichloroacetic acid were added to the solution. The mixture was heated in a boiling water bath for 30 min. Chloroform (2.0 ml) was added to the solution after cooling, and the mixture was centrifuged at 2,500 rpm for 5 min. Absorbance of the supernatant was measured at 532 nm. The TBA value was determined by a standard curve of tetraethoxypropane (14).

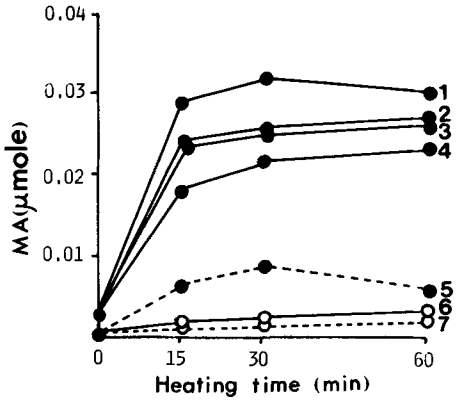

FIG. 1. Effect of iron catalysis on the TBA test of MePHETE. Each tube contained 0.18 μmol of MeHPETE or 0.22 μmol of methyl arachidonate. 1: MeHPETE plus 100 μmol of ferrous sulfate. 2: MeHPETE plus 10 μmol of ferrous sulfate. 3: MeHPETE plus 10 μmol of ferrous sulfate and 10 μmol of butyl hydroxy toluene. 4: MeHPETE plus 1.0 μmol of ferrous sulfate. 5: MeHPETE only. 6: Methyl arachidonate plus 10 μmol of ferrous sulfate. 7: Methyl arachidonate only.

RESULTS

Figure 1 shows the effects of the different concentrations of iron salt on the TBA reaction of MeHPETE. The color intensity increased as more iron salt was added. Thirty min of heating was long enough to determine the TBA value of MeHPETE. Unoxidized methyl arachidonate produced no significant coloring with and without the addition of iron salt, as compared to MeHPETE. Butyl hydroxytoluene, a free radical scavenger, had no effect on the coloring of MeHPETE when 10 μmol of ferrous sulfate was added to the solution. Accordingly, a secondary oxidation during the test reaction, if occurred, scarcely affected the TBA value of MeHPETE in the test solution. Thus, 10 μmol of ferrous sulfate was added to the reaction mixture in subsequent experiments.

A linear relationship was observed between the concentration of MeHPETE and the TBA value in the range of 0.1-0.4 μmol of MeHPETE (Fig. 2). Thus, this range of concentration of MeHPETE was subjected to TBA test reaction to determine the reactivities to TBA.

Figure 3 shows a typical pattern of MeHPETE on HPLC, including the 5 peaks which were fractionated. The isomeric composition of methyl hydroxy eicosanoate derived from each fraction of MeHPETE is shown in Table I. Each peak in HPLC was assigned to the regioisomeric MeHPETE as follows. 1: 15-isomer; 2: a mixture of 12- and 14-isomers; 3: 11-isomer; 4: a mixture of 8- and 9-isomers; and 5: a mixture of 5- and 6-isomers. The elution pattern of regioisomeric MeHPETE on HPLC agreed with that reported by Porter et al. (13).

The TBA value of each fraction is shown in Table II. All of the fractions were colored with TBA, and their TBA values were raised 3-5 times by adding ferrous sulfate. Fraction 1 (15-isomer) and fraction 5 (5- and 6-isomers) produced higher values than the other fractions.

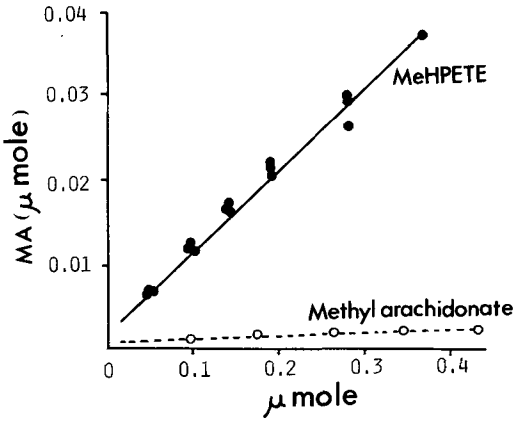

FIG. 2. The relationship between the TBA value and the concentration of MeHPETE or methyl arachidonate. Each tube contained 10 μmol of ferrous sulfate. Heating time was 30 min.

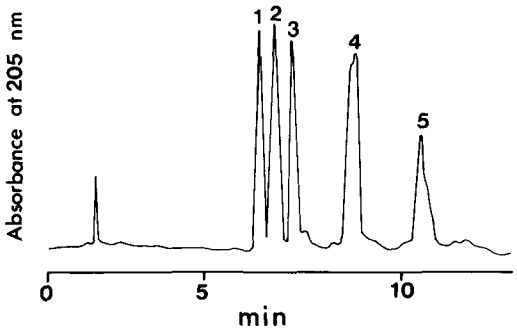

FIG. 3. HPLC of isomeric MeHPETE.

DISCUSSION

It has been suggested that iron ion affects the TBA assay of oxidized lipids (15,16). Ferrous ion catalyzes the decomposition of hydroperoxides, the primary products of fatty acid peroxidation (17). Thus, enhancement of the coloring with TBA by adding ferrous salt means the ferrous ion in the test reaction accelerates the decomposition of the hydroperoxy group. In previous papers (9,10), we suggested that the hydroperoxy group cannot be decomposed under the test conditions and that the addition of ferrous ion to the test solution is useful for elevating the sensitivity of monohydroperoxides of methyl linoleate and methyl linolenate to TBA. Several regioisomeric HPETE can be formed from enzymatic and nonenzymatic peroxidation of arachidonic acid, i.e., 5-, 6-, 8-, 9-, 11-, 12-, 14- and 15-isomers by singlet oxygen oxidation (13), 5-, 8-, 9-, 11-, 12- and 15-isomers by autocatalytic oxidation (5), 15-isomer by soybean lipoxygenase (18), 12-isomer in platelets (6), and 5- and 15-isomers in polymorphonuclear leukocytes (7, 19,20). Dahle et al. (1) suggested that peroxy radicals having a double bond at β-γ to the carbon can cyclize to form 5-membered cyclic peroxides which release MA during the test reaction of oxidized PUFA. Furthermore, Pryor et al. (2,3) pointed out that these peroxy radicals yield prostaglandin-like endoperoxides which can act as the precursor of MA in the TBA reaction. Also reported was that synthetic hydroperoxides can yield endoperoxides in the presence of a free radical initiator (21). Porter and Funk (22) detected prostaglandin-like compounds during the decomposition process of acyclic hydroperoxides which were prepared from enzymatic peroxidation of γ-linolenic acid and suggested that regioisomeric hydroperoxides with a double bond at β-γ to the carbon can cyclize to yield endoperoxides when a free radical initiator is added.

Iron ion can act as the free radical initiator producing peroxy, alkoxy and hydroxy radicals from the hydroperoxy group (17). Accordingly, it is probable that some regioisomers of MeHPETE, i.e., 6-, 8-, 9-, 11-, 12- and 14-isomers containing a double bond at β-γ to the carbon, can act as precursors of MA. However, Table II suggests that 15-isomer and perhaps 5-isomer which have no double bonds at β-γ to the carbon (5,13) give more TBA-reacting materials than the other isomers. The coloring of these isomers with TBA cannot be explained by the theory just mentioned. The results obtained from this work, therefore, indicate that the TBA test with lipid peroxides is a more complex reaction.

It was reported that 2,4-alkadienal and 2-alkenal produce red pigments in oxidized lipids with TBA (23,24). Lillard and Day (25), and Patton (26) suggested that MA can be formed through degradation of certain carbonyls such as alkadienal. These volatile carbonyls are generally known to be produced by chain scission of hydroperoxides of PUFA (27). It seems likely that each MeHPETE regioisomer yields carbonyls which contribute to coloring with TBA during the reaction. Another explanation of the coloring of 15- and 5-isomers with TBA is that cyclic peroxides can be produced from hydroperoxides with no double

TABLE I

GC-MS Analysis of Isomeric Methyl Monohydroxy Eicosanoate from Each Fraction of MeHPETE on HPLC

Fraction	Relative percent							
	5-OH	6-OH	8-OH	9-OH	11-OH	12-OH	14-OH	15-OH
1	–	–	–	–	–	2	5	93
2	–	–	–	–	2	60	30	8
3	–	–	–	–	87	4	–	9
4	–	–	52	45	1	2	–	–
5	73	22	–	5	–	–	–	–

TABLE II

The Reactivities of MeHPETE Regioisomers to TBA

Peak on HPLC	Isomer	TBA value (μmol MA/μmol MeHPETE)[a]	
		No addition	Plus $FeSO_4$
1	15-OOH	0.044 ± 0.007	0.221 ± 0.004
2	14-OOH 12-OOH	0.046 ± 0.008	0.121 ± 0.002
3	11-OOH	0.027 ± 0.003	0.111 ± 0.009
4	9-OOH 8-OOH	0.021 ± 0.002	0.081 ± 0.002
5	6-OOH 5-OOH	0.053 ± 0.006	0.223 ± 0.014
MeHPETE[b]		0.028 ± 0.004	0.118 ± 0.010

[a]Average from 4 experiments.
[b]Not separated by HPLC.

bonds at β-γ to the carbon.

From these results, it can be concluded that all of the HPETE isomers formed by peroxidation of arachidonic acid in a biological system can yield TBA-reacting materials when the TBA test is used for measuring lipid peroxides. The products formed by the decomposition of each isomeric HPETE will be reported in the future.

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Incorporation of *cis*-Octadecenoic Acids into the Rat Liver Mitochondrial Membrane Phospholipids and Adipose Tissue Triglycerides[1]

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ABSTRACT

The incorporation of the dietary *cis* 18:1(n-12) and *cis* 18:1(n-10) into liver mitochondrial membrane phospholipids and adipose tissue triglycerides was studied in 4 groups of rats fed diets containing 10 weight percent (wt %) of fat with the following contents of octadecenoic acids: 50% *cis* 18:1-(n-12) + 9% *cis* 18:1(n-9); 25% *cis* 18:1(n-12) + 32% *cis* 18:1(n-9); 50% *cis* 18:1(n-10) + 10% *cis* 18:1(n-9); or 54% *cis* 18:1(n-9). Dietary linoleic acid was 3 wt % in all 4 groups. In the mitochondrial membranes, the isomeric octadecenoic acids were primarily incorporated into the 1-position of phosphatidylcholines and phosphatidylethanolamines at the expense of saturated fatty acids. The maximal incorporations observed in the 1-position of phosphatidylethanolamines were 4.8% 18:1(n-12) and 8.9% 18:1(n-10). No effects on the contents of polyunsaturated fatty acids in the phospholipids were seen. In the adipose tissue, the isomeric octadecenoic acids were incorporated at a level of 13% *cis* 18:1(n-12) or 23% *cis* 18:1(n-10), paralleled by a reduction in the content of oleic acid.

INTRODUCTION

Positional isomeric *cis*, as well as *trans*, octadecenoic acids are present in partially hydrogenated oils (1) and also are found in margarines (2) used for human consumption. Small quantities of isomeric fatty acids have also been found in human depot fat (3), human milk (4), human cutaneous lipids (5), as well as in rat liver (6) and in bovine fat (7).

In most of the previously published nutritional reports on the metabolism of isomeric octadecenoic acids, partially hydrogenated fats have been used in the diets (8-11). These fats contain primarily *trans* octadecenoates and are, therefore, less suitable for investigating the possible biological effects of isomeric *cis* octadecenoates also present in the partially hydrogenated fats. Few papers have reported the effects of diets containing high levels of one positional isomeric *cis* octadecenoic acid alone or together with oleic acid. Sand et al. (12) demonstrated that petroselinic acid, *cis* 18:1-(n-12), when supplied at a level of 3% in the diet of rats, was incorporated into the carcass lipids and after 3 weeks represented 13% of all fatty acids. Mohrhauer et al. (13) found that dietary *cis* 18:1(n-12) retarded the conversion of linoleic acid to eicosatrienoic acid and arachidonic acid in the rat liver. Mounts (14) administered *cis* 18:1(n-10), *cis* 18:1(n-8), *cis* 18:1(n-7), or *cis* 18:1(n-6) together with *cis* 18:1(n-9) to laying hens and found that the positional isomeric fatty acids were primarily incorporated into the 1-position of the egg yolk phospholipids. In vitro, *cis* octadecenoic acids were taken up by liver mitochondria and incorporated into the phospholipids (15). The incorporation of *cis* octadecenoic acids increased as the position of the double bond was moved toward either end of the carbon chain, the *cis* 18:1(n-9) being incorporated at the lowest rate.

In this paper, we describe an experiment in which rats were fed 5 weight percent (wt %) *cis* 18:1(n-12) or *cis* 18:1(n-10). The incorporation of the isomeric fatty acids into the liver mitochondrial membrane phospholipids was determined in order to see whether the double bond position affected the total incorporation of the isomeric fatty acids as well as their distribution between the 1- and 2-positions. The fatty acid composition of the phospholipids also was analyzed to reveal any retardation in the formation of polyunsaturated fatty acids, thus affecting the unsaturation of the membrane phospholipids of rats fed high levels of isomeric fatty acids.

EXPERIMENTAL PROCEDURES

Animal Experiments

Twenty-four weanling male Wistar rats (specific pathogen-free) were divided into 4 groups; each group contained 6 rats with similar average weights. The rats received a diet of the following composition (wt %): casein (A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark), 20%; sucrose, 64%; vitamin mixture, 0.5%; salt mixture (including trace elements), 5.0%; choline chloride, 0.5%; and fat, 10%. The compositions of the vitamin mixture and the salt mixture were as previously described

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(16). The compositions of the dietary fats used for the 4 experimental groups were (wt % of the total diets): 6% refined parsley seed oil (PO) + 4% sunflower seed oil (SO); 3% PO + 3% olive oil (OO) + 4% SO; 5% ethyl-*cis*-8-octadecenoate (EO) + 5% SO; 6% OO + 4% SO. Refined parsley seed oil was prepared as follows: ground parsley seeds were extracted with hexane in a Soxhlet apparatus. The hexane extract was fractionated by low temperature crystallization at -10 C. The lower phase was extracted with methanol and further purified with activated carbon. The product was a colorless oil which was shown by thin layer chromatography (TLC) to contain only triglycerides. The oil that was analyzed by gas liquid chromatography (GLC) and ozonolysis (11) had the following composition: 16:0, 3.0%; 16:1, 0.4%; 18:0, 0.5%; 6 *c*-18:1, 83.2%; 9 *c*-18:1, 1.7%; 18:2, 10.2%; 18:3, 0.4%. The ethyl-*cis*-8-octadecenoate was prepared by Prof. F.D. Gunstone, University of St. Andrews, Scotland. The purity was better than 99.5% as analyzed by GLC, infrared spectroscopy and ozonolysis. The sunflower seed oil was "Solex" (Aarhus Oliefabrik A/S, Aarhus, Denmark). The fatty acid composition was: 16:0, 7.6%; 16:1, 0.1%; 18:0, 3.7%; 9 *c*-18:1, 20.6%; 18:2, 66.3%; 18:3, 1.3%. The olive oil was cold-pressed, Pharmacopea Nordica. The fatty acid composition was: 16:0, 8.9%; 16:1, 1.0%; 18:0, 2.8%; 9*c*-18:1, 75.9%; 18:2, 9.6%; 18:3, 1.1%.

The rats were caged in pairs at 25 C and a relative humidity of 45%. Diets and water were supplied ad libitum. The rats were examined and weighed weekly during the experimental period. After 3 weeks of experiment, the rats were anesthesized with diethyl ether and killed by heart puncture. The liver and a sample of the adipose tissue were immediately excised.

Preparation of Mitochondrial Membranes

The livers from each group of rats were divided into 3 pools of 2 livers each. Mitochondrial membranes were prepared as previously described (11). The protein content was determined by the micro-biuret method (17).

To estimate the purity of the mitochondria, the specific activities of marker enzymes in the prepared mitochondria were measured following 2 separate preparations of mitochondria (literature data for purified cellular fractions in parentheses): cytochrome oxidase, EC 1.9.3.1, for mitochondria (18), 1032 ± 101 nmol/min/mg protein (1340 ± 460 nmol/min/mg protein [19]); acid phosphatase, EC 3.1.3.2, for lysosomes (20), 35.1 ± 0.1 nmol/min/mg protein (950 ± 362 nmol/min/mg protein [19]); glucose-6-phosphatase, EC 3.1.3.9, for endoplasmic reticulum (21), 39.1 ± 4.6 nmol/min/mg protein (192 ± 54 nmol/min/mg protein [19]); and AMP'ase, EC 3.1.3.5, for cell membranes (22) 13.9 ± 8.5 nmol/min/mg protein (990 nmol/min/mg protein [23]). Based on the literature data, the composition of the prepared mitochondrial fraction was calculated (23): 77 ± 7% mitochondria, 3.7 ± 0% lysosomes, 20.4 ± 2.3% endoplasmatic reticulum, and 1.4 ± 0.9% cell membranes.

Liver Mitochondrial Lipids

The lipids of the mitochondrial membrane fractions were extracted according to Folch et al. (24). Phospholipids were separated by TLC on prewashed 0.5 mm plates of Silica Gel H using chloroform/methanol/water (100:40:6, v/v/v) as solvent, followed by rechromatography in diethyl ether/acetic acid (100:3, v/v) to improve the separation of cardiolipins and free fatty acids. The lipids were visualized with 2′,7′-dichlorofluorescein. The phospholipids were scraped off and methylated and the methyl esters were subjected to GLC (11). The contents of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) were determined (25) following TLC as already described.

To determine the distribution of fatty acids between the 1- and the 2-positions of PC and PE, the lipid extracts from each experimental group were pooled. PC and PE were separated by TLC as already described. The positions of the phospholipids were visualized by exposure to iodine vapor for a short time. The PC and PE spots were then scraped off and extracted with chloroform/methanol (2:1, v/v). The phospholipids were dissolved in diethyl ether/methanol (98:2, v/v) and incubated with 100 µl phospholipase A_2 solution (8 mg snake venom, *Crotalus atrox*, no. V-7000, Sigma Chemical Company, St. Louis, MO, dissolved in 2 ml 0.1 M sodium borate buffer containing 0.02 M calcium acetate, pH 7.2) for 2 hr at room temperature with moderate stirring (26). The resulting lysophospholipids and the free fatty acids were separated by TLC using chloroform/methanol/water (100:40:6, v/v/v), visualized with 2′,7′-dichloroflourescein, and were then methylated. A sample of the methyl esters was gas-chromatographed to determine the composition of the fatty acids in the 1- and 2-positions. From the remaining sample of the methyl esters, the methyl octadecenoates were separated by argentation TLC followed by preparative GLC (11). The position of the double bond in the octadecenoates was determined by ozonolysis followed by reductive cleavage of the ozonides and GLC of the cleavage products (11).

Adipose Tissue Lipids

The samples of adipose tissue were stored on Dry Ice. The tissue was ground with anhydrous sodium sulfate and extracted with chloroform. TLC showed that the extracts mainly contained triglycerides (95%). Fatty acid composition and the content of positional isomeric octadecenoic acids were analyzed as we already described.

RESULTS

Fatty Acids in the Dietary Fats

In order to make 4 dietary fats containing different octadecenoic acids but having similar contents of saturated, monoenoic and polyenoic fatty acids, refined parsley seed oil, sunflower seed oil, ethyl-*cis*-8-octadecenoate and olive oil were combined (Table I). It was thus possible to compare the incorporation of *cis* 18:1(n-12) and *cis* 18:1(n-10) under very similar dietary conditions. A control group receiving oleic acid as the only octadecenoic acid was included in the experiment to reveal possible effects of isomeric fatty acids on the metabolism of linoleic acid.

Phospholipids of Liver Mitochondrial Membranes

No significant differences in the contents of PC and PE were observed, with the exception that PE in the group fed PO + OO + SO was lower than the reference group (Table II).

Fatty Acids in the Phospholipids of Liver Mitochondrial Membranes

Isomeric fatty acids in the diets did not, within the experimental period of 3 weeks, influence the contents of polyunsaturated fatty acids in the mitochondrial phospholipids (Table II) of those rats fed sufficient linoleic acid. In the groups receiving isomeric fatty acids, the contents of octadecenoic acids in the phospholipids were slightly elevated compared to the control group fed 6% OO + 4% SO. This was paralleled by reduced levels of saturated fatty acids.

In PC, as well as in PE (Table III), *cis* 18:1-(n-12) and *cis* 18:1(n-10) were primarily esterified at the 1-position. The incorporation of *cis* 18:1(n-12) and *cis* 18:1(n-10) did not, in general, affect the contents of oleic or vaccenic acid.

Fatty Acids in the Total Lipids of Adipose Tissue

The adipose tissue (Table IV) contained more *cis* 18:1(n-12) and *cis* 18:1(n-10) than found in the mitochondrial phospholipids. The incorporation of *cis* 18:1(n-10) was considerably higher than that of *cis* 18:1(n-12). The incorporation of isomeric fatty acids was accompanied by reduction of the contents of oleic acid whereas the contents of saturated fatty acids were largely unaffected. Likewise, no effect on the linoleic acid content was found.

DISCUSSION

Previous experiments have established that, in normal rats fed unhydrogenated fats, the phospholipids of liver (9) as well as the triglycerides of adipose tissue (27) contain oleic

TABLE I

Fatty Acid Composition of Dietary Fats: Weight Percentages Determined by Gas Liquid Chromatography and Ozonolysis

	Dietary fat (%)			
	6% PO + 4% SO	3% PO + 3% OO + 4% SO	5% EO + 5% SO	6% OO + 4% SO
Fatty acids				
12:0	–	0.1	–	–
16:0	4.8	6.6	3.8	8.4
16:1	0.3	0.5	0.1	0.6
17:0	–	0.1	0.1	0.2
17:1	0.1	0.1	0.1	0.2
18:0	1.8	2.5	1.9	3.2
18:1(n-12)	49.9	25.0	–	–
18:1(n-10)	–	–	50.0	–
18:1(n-9)	9.3	31.5	10.3	53.8
18:2	32.6	32.4	33.1	32.2
18:3	0.8	1.0	1.7	1.2
20:0	0.2	0.3	0.1	0.3

PO: parsley seed oil; SO: sunflower seed oil; OO: olive oil; EO: ethyl-*cis*-8-octadecenoate.

TABLE II

Phosphatidylcholines and Phosphatidylethanolamines of Rat Liver Mitochondrial Membranes

	Dietary group[a]							
	6% PO + 4% SO		3% PO + 3% OO + 4% SO		5% EO + 5% SO		6% OO + 4% SO	
	PC	PE	PC	PE	PC	PE	PC	PE
μg P_i/mg protein[b]	4.0 ± 0.3[c]	2.3 ± 0.2	3.3 ± 0.2	2.2 ± 0.1[d]	3.2 ± 0.2	2.3 ± 0.1	3.9 ± 0.2	2.5 ± 0.1
Fatty acid[e]	(%)[f]							
16:0	18.6 ± 0.2[c]	14.8 ± 0.7	20.9 ± 1.0	16.1 ± 0.7	21.3 ± 0.5	14.1 ± 0.4	21.8 ± 0.5	16.7 ± 0.5
16:1	2.5 ± 0.4	0.8 ± 0.1	1.9 ± 0.1	0.7 ± 0.2	1.3 ± 0.3	0.6 ± 0.1	1.1 ± 0.1	0.5 ± 0.0
18:0	19.1 ± 0.3	23.3 ± 0.3	20.3 ± 0.3	26.5 ± 3.0	19.3 ± 1.5	25.3 ± 2.1	21.7 ± 1.0	25.4 ± 0.5
18:1	11.0 ± 0.3	9.2 ± 0.4	9.5 ± 1.2	7.1 ± 1.3	11.9 ± 1.6	10.0 ± 1.9	9.2 ± 0.2	6.6 ± 0.3
18:2	8.5 ± 0.4	3.7 ± 0.3	7.9 ± 0.6	3.5 ± 0.3	10.1 ± 0.1	5.3 ± 0.5	7.0 ± 0.2	2.8 ± 0.2
20:3	1.0 ± 0.2	0.4 ± 0.0	0.6 ± 0.3	0.2 ± 0.2	0.7 ± 0.3	0.2 ± 0.1	0.8 ± 0.0	0.2 ± 0.0
20:4	31.7 ± 0.6	32.8 ± 0.6	32.2 ± 0.5	31.4 ± 1.4	29.7 ± 1.0	31.0 ± 0.5	32.4 ± 0.2	32.6 ± 0.3
22:4	1.3 ± 0.2	2.9 ± 0.2	1.1 ± 0.1	2.3 ± 0.1	0.7 ± 0.1	2.2 ± 0.1	0.6 ± 0.1	1.7 ± 0.1
22:6	3.0 ± 0.2	9.1 ± 0.2	3.4 ± 0.4	9.5 ± 0.3	2.3 ± 0.3	8.0 ± 0.0	3.0 ± 0.1	10.0 ± 0.4
Σ Saturated	37.7	38.1	41.2	42.6	40.6	39.4	43.5	42.1
Σ Monounsaturated	13.5	10.0	11.4	7.8	13.2	10.6	10.3	7.1
Σ Polyunsaturated	45.7	48.9	45.2	46.9	43.9	46.7	43.8	47.3

[a]Averages of 3 different pools.

[b]μg Phosphorus/mg protein.

[c]SEM.

[d]Significantly different from reference group. $P<0.05$ by Student's t-test.

[e]Minor components omitted.

[f]Weight percentages determined by GLC.

acid and vaccenic acid as the only octadecenoic acids; oleic acid is primarily located in the 2-position and vaccenic acid in the 1-position of the phospholipids.

This experiment shows that, when *cis* 18:1-(n-12) or *cis* 18:1(n-10) is included in the dietary lipid at a level of 25-50 wt %, these fatty acids are incorporated into PC and PE of the liver mitochondrial membrane and TG of adipose tissue. The extent of incorporation is dependent on the double bond position of the octadecenoic acid, as the *cis* 18:1(n-10) is preferentially incorporated.

In PC and PE of the mitochondrial membranes, the *cis* 18:1(n-12) and *cis* 18:1(n-10) are primarily incorporated into the 1-position where they replace saturated fatty acids, whereas the contents of oleic acid and vaccenic acid are unaffected. It is therefore unlikely that the introduction of positional isomers into the mitochondrial phospholipids has caused a reduction of the membrane fluidity. In the 1-position of PE and PC, the percentage of *cis* 18:1(n-10) in the octadecenoates is higher than in the diet, whereas the percentage of *cis* 18:1(n-12) always is lower in the phospholipids than in the diet. A similar accumulation of *cis* 18:1(n-6) has previously been described by Wood (10). Both *cis* 18:1(n-10) and *cis* 18:1(n-12) are excluded from the 2-position. Apparently, 2 mechanisms of selection are operative: one that excludes isomeric fatty acids from the 2-position, and one that selects which isomers are incorporated into the 1-position. It is particularly interesting that the *cis* 18:1(n-10) is shown to accumulate in the mitochondrial phospholipid since the *cis* 18:1(n-8), which also has a double bond located in a neighboring position compared to oleic acid, has been shown to be nearly completely excluded from rat tissues (8,11).

Because there is almost no incorporation of positional isomers in the 2-position, it is reasonable that no effects are found on the contents of polyunsaturated fatty acids. Possibly, the interference of *cis* 18:1(n-12) with the chain elongation of linoleic acid demonstrated by Mohrhauer et al. (13) may be explained by the combination of a high level of dietary *cis* 18:1(n-12), up to 21.6 calcd %, and a low intake of linoleic acid, 0.2 calcd %.

In the 4 dietary groups in this experiment, the total contents of octadecenoates in triglycerides from depot fat are similar. The introduction of isomeric fatty acids into the adipose tissue is primarily compensated by a decrease in the oleic acid content, which can be of either exogenous or endogenous origin, whereas the vaccenic acid content is largely unaffected. This may indicate that the microsomal chain elongation of palmitic acid is decreased by the dietary regimen, whereas the Δ9-desaturation is not. The incorporation of *cis* 18:1(n-12) in the adipose tissue is much smaller than the incorporation of *cis* 18:1(n-10) when fed at the same dietary level. This may result from the action of lipoprotein lipase or the hormone-sensitive lipase in the adipose tissue whose substrate specificities with regard to positional isomers have not yet been reported. Alternatively, a

TABLE III

Positional Distribution of Octadecenoic Acids[a] in the Phosphatidylcholines and Phosphatidylethanolamines of Rat Liver Mitochondrial Membranes[b]

	Dietary group							
	6% PO + 4% SO		3% PO + 3% OO + 4% SO		5% EO + 5% SO		6% OO + 4% SO	
	Position							
	1	2	1	2	1	2	1	2
PC								
18:1(n-12)	4.7	0.7	2.5	0.3	–	–	–	–
18:1(n-10)	–	–	–	–	6.1	1.4	–	–
18:1(n-9)	4.4	6.2	5.1	6.1	2.0	5.7	3.6	8.3
18:1(n-7)	5.6	1.9	3.7	2.4	1.7	1.8	2.0	1.4
PE								
18:1(n-12)	4.8	0.2	1.6	0.1	–	–	–	–
18:1(n-10)	–	–	–	–	8.9	0.7	–	–
18:1(n-9)	8.2	1.2	8.7	1.5	5.0	1.2	6.0	2.1
18:1(n-7)	5.9	0.7	4.0	0.9	3.4	0.4	3.3	0.6

[a]Fatty acids are expressed as weight percentages of the total fatty acids recovered from the 1- or 2-position.
[b]Averages of 2 determinations on the combined pools of lipid extracts.

TABLE IV

Major Fatty Acids[a] of Rat Adipose Lipids: Weight Percentages Determined by Gas Liquid Chromatography and Ozonolysis

	Dietary group			
	6% PO + 4% SO	3% PO + 3% OO + 4% SO	5% EO + 5% SO	6% OO + 4% SO
Fatty acid	(%)			
14:0	1.2 ± 0.1[b]	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.1
16:0	20.2 ± 0.1	18.8 ± 0.8	16.4 ± 0.7	18.7 ± 0.5
16:1	7.9 ± 0.5	6.5 ± 0.5	5.9 ± 0.4	6.0 ± 0.5
18:0	1.6 ± 0.1	1.9 ± 0.1	1.7 ± 0.2	1.9 ± 0.1
18:1(n-12)	13.4 ± 0.8	5.3 ± 0.4	–	–
18:1(n-10)	–	–	23.3 ± 0.9	–
18:1(n-9)	26.9 ± 0.9	37.3 ± 0.5	21.1 ± 0.9	44.7 ± 0.2
18:1(n-7)	2.4 ± 0.1	2.1 ± 0.2	1.6 ± 0.1	2.2 ± 0.1
18:2	23.9 ± 0.6	23.7 ± 0.8	25.7 ± 1.0	22.9 ± 0.3

[a]Averages of 6 animals. Minor components less than 1% omitted.

[b]Standard error of the mean.

higher rate of β-oxidation of *cis* 18:1(n-12) in the liver may result in a smaller release of this acid in VLDL- and LDL-triglycerides. Interesting is that the incorporation of positional isomers into the adipose tissue does not affect the 18:2 content. This indicates that when sufficient levels of linoleic acid are fed, as in our experiment, no effects from the positional isomeric *cis* octadecenoates on the metabolism of polyunsaturated fatty acids can be expected. This agrees with the observation that, although positional isomers are fed at high dietary levels as in this experiment, and although they may accumulate in the octadecenoate fraction, the total incorporation of these fatty acids into liver mitochondrial phospholipids is modest.

It has been demonstrated that the intestinal absorption of free fatty acids is independent of double-bond geometry (28). It has also been shown that methyl oleate and triolein are equally well absorbed in the rat (29), and that ethyl oleate is hydrolyzed by rat pancreatic lipase (30). Therefore, it seems unlikely that the selective metabolism of *cis* octadecenoic acids demonstrated in this paper should be influenced by the use of an ethyl ester in one of the diets.

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Seasonal Changes in Lipid and Fatty Acid Composition of the Freshwater Mollusk, *Diplodom patagonicus*

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ABSTRACT

Diplodom patagonicus is a freshwater bivalve mollusk living in lakes of the patagonian Andes mountains in Argentina. Lipid composition and seasonal changes in the mollusk were studied in the natural habitat. In addition to common nonpolar and polar lipids, small amounts of alk-1-enyldiacylglycerol ethers and significant quantities of ceramide aminoethyl phosphonate were present. Total lipid content changed during the year, primarily because of decreased triacylglycerols in winter. The fatty acid composition of the lipids, remarkably different from that of marine bivalves, and even from other fresh water animals, was especially rich in the $\omega 6$ fatty acids, linoleic and arachidonic (ca. 25%), and poor in the $\omega 3$ acids, 20:5 and 22:6. The $\omega 6/\omega 3$ acid ratio was ca. 2, which is very high compared to marine bivalves. The fatty acid composition and the $\omega 6/\omega 3$ acid ratio were constant during the whole year, suggesting a very stable diet, rich in vegetal detritus and poor in diatoms. The influence of environmental temperature fluctuation with season on fatty acid composition also was negligible. Modest proportions of 22:2 nonmethylene-interrupted (NMI) acids were detected and confirmed by mass spectrometry. It was shown that 20:2 NMI acids were absent.

INTRODUCTION

Diplodom patagonicus (d'Orb) is a bivalve mollusk living in lakes of the patagonian Andes. We have localized dense colonies in Lake Nahuel Huapi at depths from 3 m to 30 m or more. This is a cold-water lake poor in organic nutrients, salts and plankton. Consequently, it was important to study the lipid composition of *D. patagonicus* in its habitat and compare it to that of similar marine bivalves.

In this work, we report the lipid and fatty acid compositions of *D. patagonicus* and the changes produced during the annual cycle.

EXPERIMENTAL PROCEDURES

Animals

Specimens of *D. patagonicus* were collected by skin divers at a depth of 4 m in a bay of Llao-Llao peninsula of Lake Nahuel Huapi, Río Negro province, Argentina. Samples of 10 mollusks, 6 to 7.5 cm long, were collected in April, July, October and December and analyzed in groups of 2.

Lipid Extraction and Analysis

Soft tissues were homogenized and the lipids extracted with chloroform/methanol (2:1, v/v) by the Folch procedure (1), were recovered and weighed. Polar and nonpolar lipids were fractionated by silicic acid absorption chromatography (2). Lipids of each fraction were separated by thin layer chromatography (TLC) on Silica Gel G using hexane/ethyl ether/acetic acid (80:20:1, v/v) for nonpolar lipids and chloroform/methanol/acetic acid/water (65:24:4:4, v/v) for polar lipids.

Lipids were identified by comparison of R_f with corresponding standards, by specific color reactions and also by additional bidimensional TLC (3,4). After lipid identification, routine analyses were performed by TLC on 20 x 40 cm plates. They were first developed with $CHCl_3/CH_3OH$/acetic acid/H_2O (65:25:4:4, v/v) and then with hexane/ethyl ether/acetic acid (80:20:1, v/v) in the same direction.

Quantitative estimation of the lipids was made by charring and densitometry (5).

Fatty Acid Analysis

An aliquot of the total lipids was saponified and, after extraction of the nonsaponifiables with petroleum ether, the fatty acids were esterified with 3 N HCl in methanol. The methyl esters were analyzed by gas liquid chromatography (GLC) in a Packard apparatus at 180 C. Two liquid phases were used: 15% EGSS-X on Chromosorb WPH 80/100 mesh and 15% EGSS-Y on Chromosorb WAW 80/100 mesh.

The chromatographic peaks were identified tentatively by comparison of the relative retention times ($R_{18:0}$) with those of standards

[1]Member of the Carrera del Investigador Científico of the CIC Buenos Aires Province, Argentina.

[2]Member of the Carrera del Investigador Científico of the CONICET, Argentina.

measured on both columns. In addition, identifications were checked by comparison of equivalent chain length of the esters using both columns as suggested by Christie (6), and also by hydrogenation followed by GLC of hydrogenated products (7). The number of double bonds was confirmed by TLC on Silica Gel H-$AgNO_3$, followed by GLC of the separated fractions (8).

In some cases, as specified later, the identities of the fatty acids were investigated by mass spectrometry in tandem with GLC on 15% DEGS coated on Chromosorb W. The methyl esters were injected in a Varian 1440 apparatus coupled to a Varian Mat. CH-7A mass spectrometer commanded by a Varian-Mat. data system 166 that receives information from teletype Tektronix 4010-1. The conditions used for the ion source were: temperature 200C, potential 70 eV, emission 1 mA; and for the ion current detector were: temperature 200 C, potential 22 eV, emission 600 μA. The scanning range was 50-550 mass units while the scan speed was 19 spectra/min.

RESULTS AND DISCUSSION

Lipid Composition

The lipid composition of *D. patagonicus*, shown in Table I, is the result of mono- and bidimensional TLC of neutral and polar lipids and of identification reactions described later. Triacylglycerols were the main components of the neutral lipids with a mean value of 20.6% of total lipid calculated from data obtained during an entire year.

To identify the less polar component of the chromatogram, this was recovered from the plate and chromatographed again with benzene/hexane (30:70, v/v) (9). Two spots were found with R_f values at ca. 0.5 and 1. The spot of R_f 0.5 gave a color reaction characteristic of sterols. When this fraction was saponified with 10% KOH for 45 min and the unsaponifiables chromatographed again with benzene/hexane (30:70, v/v), the spot with $R_f \cong 1$ remained unmodified. This was attributed to hydrocarbons, whereas the spot of R_f 0.5 disappeared, demonstrating that it corresponded to sterol esters. Also, this result verified the absence of waxes.

Free and esterified sterols also were recognized in the thin layer chromatograms of neutral lipids by color reaction with sulfuric acid/acetic acid (50:50, v/v). The spot with R_f 0.13, which maintained its position after saponification and gave a color reaction characteristic of sterols, was eluted from the silica gel and resolved by TLC with hexane/ethyl ether/methanol/acetic acid (90:50:5:2, v/v) into free sterols and diacylglycerols.

The existence of alk-1-enyl diacylglycerol ethers was recognized in the spot (R_f 0.67) found near the triacylglycerols, by reaction with 2,4-dinitrophenyl hydrazine. A spot with an R_f of 0.20, coincident with linoleylol, was attributed to aliphatic alcohols. Small amounts of pigments might be present in the monoacylglycerol spot and therefore they may be less than the values shown in Table I.

Phospholipids present in the mollusks are shown in Table I. In addition to the common phospholipids, a spot of R_f 0.40, a little higher than that of phosphatidylcholine (PC), was attributed to a ceramide-aminoethyl phosphonate since it gave positive reactions with ninhydrin and chlorobenzidine. This phospholipid has been recognized in other mollusks (10,11) and sea anemones (12).

TABLE I

Lipid Composition of *D. patagonicus*[a]

Nonpolar	Distribution (%)[b]	Polar	Distribution (%)
Hydrocarbons + esterified sterols	7.2 (4.6-10.9)	Phosphatidylethanolamine	19.6 (16.3-21.4)
Diacylglycerol ethers	5.5 (0 -11.1)	Ceramide aminoethyl phosphonate	21.1 (18.4-24.8)
Triacylglycerols	20.6 (12.0-27.0)	Phosphatidylcholine	
Fatty acids	1.0 (t- 4.0)		
Fatty alcohols	0.8 (t- 1.1)	Sphingomyelin	4.6 (2.9- 8.1)
Free sterols	14.2 (11.3-18.0)	Lysophosphatides	2.0 (t- 3.7)
Diacylglycerols			
Monoacylglycerols	3.4 (2.7- 6.0)		

[a]The lipids were separated by TLC on Silica Gel G; neutral lipids with hexane/ether/acetic acid (80:20:1, v/v) and polar lipids with $CHCl_3/CH_3OH$/acetic acid/H_2O (65:25:4:4, v/v).

[b]Results are the mean of 5 samples of 2 mollusks each, collected in April, July, October and December. Extreme values are in parentheses.

Seasonal Changes of the Lipids

The lipid content of *D. patagonicus* changes during the year; the lowest value (0.62% ± 0.05 SE, expressed with reference to wet weight of soft tissues) was found in winter (July) and the highest (1.45% ± 0.13 SE) in spring (end of October) (Fig. 1). This change includes the triacylglycerol content which decreases from 24.1% in autumn (April) to approximately half this value (12.6%) in winter (July). The highest value (27%) was found in spring.

The main function of triacylglycerols is energy storage and, as they generally increase with an enhanced food intake, these results would indicate that, in the spring, there is an increase in food consumption. In addition, an increased triacylglycerol in this period might indicate a prespawning conditioning of the animal, whereas the decrease in December might correspond to an expulsion of sexual elements.

The seasonal change in the phospholipids was very small (Fig. 1). These lipids are constituents of membranes and lipoproteins and, therefore, they are not subjected to the changes found with depot fats which are involved in storage and consumption of energy.

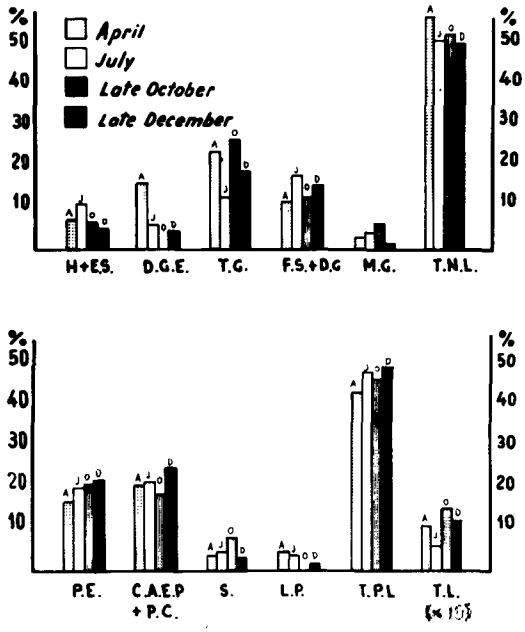

FIG. 1. Seasonal variation in lipid composition of *D. patagonicus*. H: hydrocarbons; E.S.: esterified sterol; D.G.E.: diacylglyceryl ethers; T.G.: triacylglycerols; F.S.: free sterols; D.G.: diacylglycerols; M.G.: monoacylglycerols; T.N.L.: total neutral lipids; P.E.: phosphatidylethanolamine; C.A.E.P.: ceramide aminoethylphosphonate; S: sphingomyelin; L.P.: lysophospholipids; T.P.L.: total polar lipids; T.L.: total lipids.

Fatty Acid Composition

The fatty acid composition of *D. patagonicus* is illustrated in Table II. The type of fatty acids found corresponds, in general, to an aquatic pattern since the longer chain ω3 acids, 20:5 and 22:6, are present, but the quantitative composition shows that palmitic, linoleic, eicosenoic and arachidonic acids are the main components. The high content of ω6 acids, linoleic and arachidonic (ca. 25% of all acids), and low amounts of ω3 acids found during the entire year are remarkable. The structure of both ω6 acids was determined by the GLC methods already indicated and, in addition, by mass spectrometry. This assignment of structure for 20:4ω6 was confirmed by comparing to the mass spectra of a standard sample.

Fatty acids of the ω6 family are typical of land animals feeding preferentially on linoleic-acid-containing plants. Therefore, *D. patagonicus* shows a characteristic fatty acid composition with a very high ω6/ω3 acid ratio that makes it different from even other freshwater animals and shows a certain similarity to land animals (13).

Paradis and Ackman (14) have shown that nonmethylene-interrupted (NMI) fatty acids, 20:2 and 22:2, may be found in mollusks. For this reason, a search for those fatty acids was made. In particular, the large peak attributed to a 20:1 methyl ester was investigated, since it could include NMI 20:2 acids. When the sample was chromatographed on EGSS-X, the retention time of this peak corresponded to 18:3ω3 and 20:1 methyl esters. Both components were resolved by GLC on EGSS-Y, showing that the 20:1 ester was the predominant fatty acid ester. In addition, the mass spectrum (Fig. 2) of the peak separated by GLC on 15% DEGS produced a molecular ion of 324, corresponding to 20:1 methyl ester, but no molecular ion of 322 could be detected. Therefore, we concluded that NMI 20:2 esters were not present in this peak.

The presence of NMI 22:2 acids also was investigated by mass spectrometry of the peak with $R_{18:0} = 4.18$ on the EGSS-X column. The mass spectrum (272) is shown in Figure 3. The chromatographic peak corresponding to spectrum 272 is tentatively identified as a 22:2 methyl ester. It shows a molecular ion (M^+) of 350 and a base peak of 82 (C_6H_{10}). The main fragmentation in the high range indicates the loss of 31 and 32 (methanol) mass units,

TABLE II

Seasonal Variation (%) in Fatty Acid Composition of *D. patagonicus*[a]

Fatty acid	Autumn (April)	Winter (July)	Spring (late Oct.)	Summer (late Dec.)
14:0	3.6 ± 0.3	7.0 ± 1.7	8.6 ± 0.5	7.0 ± 1.3
15:0 + X_1	1.9 ± 0.2	1.0 ± 0.4	trace	trace
16:0	17.6 ± 2.3	22.3 ± 0.5	27.5 ± 0.5	18.8 ± 0.6
16:1	3.8 ± 0.8	3.3 ± 0.2	5.8 ± 0.4	7.1 ± 0.2
17:0 + X_2	2.4 ± 0.2	—	trace	—
18:0	8.6 ± 0.9	6.2 ± 0.3	5.0 ± 0.3	6.5 ± 0.4
18:1	5.3 ± 0.4	5.0 ± 0.2	5.0 ± 0.2	7.4 ± 0.3
18:2ω6	8.8 ± 0.6	11.3 ± 0.4	9.8 ± 0.5	10.7 ± 0.3
18:3ω6	trace	trace	—	—
20:1 + 18:3ω3[b]	10.8 ± 1.7	12.6 ± 0.1	10.7 ± 0.2	11.1 ± 0.3
18:4ω3	1.2 ± 0.5	1.3 ± 0.2	1.3 ± 0.1	1.9 ± 0.1
20:3	0.5 ± 0.2	0.7 ± 0.0	trace	trace
20:4ω6 + 22:1[c]	17.2 ± 2.8	13.6 ± 1.0	13.7 ± 0.6	12.9 ± 0.9
22:2 + 20:4ω3[d] (NMI)	6.3 ± 0.5	5.9 ± 0.1	4.6 ± 0.2	5.2 ± 0.2
20:5ω3	2.7 ± 0.7	3.5 ± 0.3	2.5 ± 0.3	3.5 ± 0.2
X_3	1.6 ± 0.3	1.8 ± 0.1	1.0 ± 0.1	1.6 ± 0.1
22:4ω6	2.7 ± 0.3	2.1 ± 0.1	1.6 ± 0.1	2.4 ± 0.1
22:4ω3	2.7 ± 0.6	1.1 ± 0.1	1.2 ± 0.0	1.8 ± 0.2
22:5ω3	0.7 ± 0.3	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
22:6ω3	1.6 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	1.5 ± 0.1
Environmental temperature (C)	13	6	8	13.5

[a]The composition was calculated from gas liquid chromatogram on 15% EGSS-X (Chromosorb WHP) at 180 C. Results are the mean of 5 samples ± standard error.

[b]20:1 ca. 90% of total.

[c]20:4ω6 ca. 80% of total.

[d]22:2 ca. 80% of total.

producing ions at m/e 319 (M-31) and 318 (M-32), respectively. The lower mass region is heavily populated with hydrocarbon ions which are of little use for structural purposes. Because of double-bond migration at the molecular ion stage, the location of the double bonds cannot be firmly established.

As a tentative approach, ions at m/e 150 and 96 could indicate a Δ7,Δ14 structure whereas those ions at m/e 96 and 82 would point to a Δ7,Δ13 structure.

At present, because of the lack of standard spectra, it is not possible to determine the correct structure by this method.

The origin of this acid or acids is still open for dicussion and cannot be deduced from the current data. They might be derived from the diet, but we cannot discard the possibility that

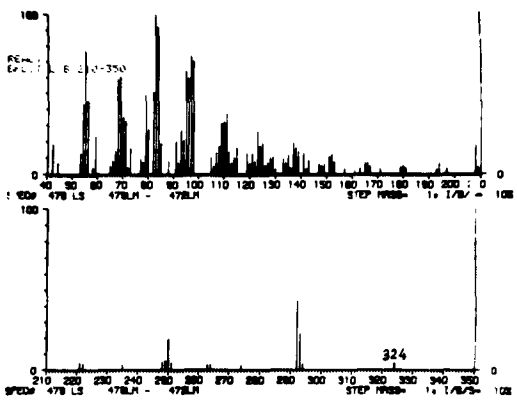

FIG. 2. Mass spectrum of gas liquid chromatographic peak identified as 20:1 acid methyl ester.

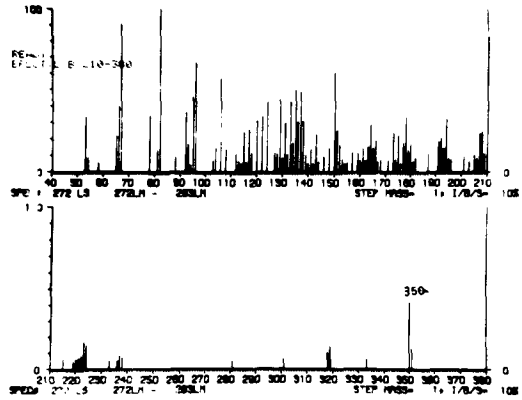

FIG. 3. Mass spectrum of gas liquid chromatographic peak identified as NMI 22:2 acid methyl ester.

they may be synthesized from traces of NMI 20:2 acid by elongation or from a 20:1 by desaturation and elongation.

Seasonal Changes of the Fatty Acid Composition

The fatty acid composition of *D. patagonicus* changes very little during the year (Table II). It would depend on 2 main factors: its food (amount and composition) and the cellular biosynthetic and catabolic activities. The 2 main food elements of filtering animals even as mollusks are plankton and plant detritus.

Generally, aquatic systems show phytoplanktonic "blooms," one of which generally occurs in spring, due to an increase in daylight, temperature and other factors provided there are enough nutrients for rapid cell multiplication of the algae. However, in Lake Nahuel Huapi, the temperature and concentration of nutrients is low and no phytoplanktonic "blooms" have been detected (S. Guarrera, private communications). Therefore, this observation and the stable seasonal fatty acid composition of *D. patagonicus* (Table II) would indicate a rather constant food composition and no seasonal change in biosynthetic activity.

Diatoms are the predominant phytoplanktonic species of Lake Nahuel Huapi and have been found in *D. patagonicus*. They generally are rich in 20:5ω3 acid (15). However, the fatty acid composition of *D. patagonicus* is very poor in both 20:5ω3 and 22:6ω3 acids. An increase of these acids would be expected in the mollusk if a "bloom" of diatoms had occurred, as has been demonstrated in other organisms (16,17). Therefore, it is probable that diatoms do not comprise the main food of *D. patagonicus*.

Linoleic and arachidonic acids constitute ca. 25% of all fatty acids during the entire year. Therefore, we may deduce that the diet probably is very rich in vegetation but that it is not necessarily of planktonic origin. This deduction is supported by the presence of abundant plant detritus in the habitat of *D. patagonicus*. The fatty acid composition of this detritus was very rich in linoleic acid (proportions commonly around 24%). Arachidonic acid probably could be biosynthesized in the mollusk from linoleic acid provided by the plants.

Seasonal changes of water temperature in a lake depend on, e.g., the depth, currents, winds, sunlight and hour. Temperature is quite variable in the surface water but constant in deep water. In the colony studied, it was different at depths of 4 m and 30 m. The samples analyzed were collected at 4 m and an extreme change of 7.5 C (6-13.5 C) was measured during the year. However, in spite of this difference, no special adaptation to this temperature change was shown in the fatty acid composition of the mollusk. We have found similar results in marine mollusks (11,16,17).

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The Effect of Different Proportions of Casein in Semipurified Diets on the Concentration of Serum Cholesterol and the Lipoprotein Composition in Rabbits

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ABSTRACT

The effect of different proportions of casein in semipurified diets on the concentration of serum cholesterol and the lipoprotein composition was studied in rabbits. Low-casein diets (10% w/w) resulted in serum cholesterol levels and growth rates that were lower than high-casein diets (40%). An intermediate proportion of casein (20%) produced intermediate concentrations of serum cholesterol, but only minor differences in food intake and weight gain, compared with the high-casein group. In the animals with the highest values of total serum cholesterol (the 40% casein group), most of the serum cholesterol was transported in the very low density lipoproteins, whereas with moderate hypercholesterolemia (the 20% casein group), the low density lipoproteins were the main carriers of cholesterol. Elevation in lipoprotein cholesterol was associated in all groups with an increased ratio of cholesterol to protein, suggesting the formation of particles relatively rich in cholesterol. When the rabbits on the diet containing 10% casein were subsequently transferred to the 40% casein diet, a steep increase in the level of serum cholesterol occurred. Conversely, switching the rabbits on the 40% casein diet to the 10% casein diet resulted in a decrease in the level of serum cholesterol.

INTRODUCTION

It has been well established that the protein source in the diet of rabbits plays an important role in the regulation of the concentration of serum cholesterol (1-3). The feeding of rabbits with semipurified diets containing casein results in hypercholesterolemia, whereas rabbits ingesting diets containing other proteins such as soybean protein are able to maintain low levels of serum cholesterol. The hypercholesterolemic properties of casein in rabbits have been found to be reproducible in other experimental animals such as swine (4), rats (5) and chickens (6). Previously, the focus has centered mainly upon the influence of the quality of the dietary protein source on the levels of serum cholesterol, whereas less research has studied the variation in quantity of the protein source. The objective of this study was to learn if increasing proportions of casein in the diet result in higher levels of serum cholesterol. Therefore, rabbits were fed semipurified diets containing 3 different levels of casein for a period of 4 weeks. In order to accentuate possible differences, the rabbits fed the high-casein diet were changed to the low-casein diet, whereas the animals on the low-casein diet were switched to the high-casein diet. The concentrations of cholesterol and protein in the different lipoprotein fractions of the rabbits fed the 3 levels of casein also are reported.

TABLE I

Composition of the Diets (g/100 g feed)

Maize starch	36.0
Maize oil	1.0
Coconut oil	13.8
Casein	20.0[a]
Saw dust	21.0
Vitamin premix	1.2
Mineral premix	1.0
$KHCO_3$	1.8
Ca_2HPO_4-$2H_2O$	2.9
NaCl	0.8
MgO	0.2
$MgCO_3$	0.3

[a]The proportion of casein in the high and low protein diets was varied at the expense of maize starch.

MATERIALS AND METHODS

Animals and Experimental Design

In the experiment, 21 male New Zealand white rabbits, aged 10 weeks and weighing ca. 1300 g, were used. The animals were housed individually in cages with wire mesh bases constructed of galvanized steel and were kept in a room with air conditioning and controlled lighting. On arrival, the rabbits were fed a commercial diet (Hope Farms, 3442 EH Woerden, The Netherlands) for 2 weeks to enable them to adapt to the new environment. The rabbits were divided into 3 groups of 6 animals and one group of 3 animals on the basis of their weight and levels of serum cholesterol. Subsequently, the 3 groups of 6 animals were changed, without further adaptation, to pelleted, semipurified diets containing 10, 20 and 40% (w/w) casein. The group consisting of 3

animals continued to receive the commercial diet. The composition of the semipurified diets (Table I) was similar to that described previously (3) and the difference in protein content was varied at the expense of maize starch. The individual food consumption was measured daily and body weights were determined 3 times a week. Food and water were offered ad libitum. However, the animals fed the commercial diet continuously were offered a restricted amount of 110 g/day over the first 10 days of the experiment and 130 g/day later on in order to obtain a similar growth rate as in the animals fed the semipurified diets. After feeding the semipurified diets for 4 weeks, the group receiving the 10% casein diet was changed to the 40% casein diet, whereas the rabbits on the diet with 40% casein were transferred to the 10% casein diet. The rabbits on the 20% casein diet continued to receive this diet. After the crossover, the animals were kept on the diets for another 3 weeks.

Sampling of Blood and Analytical Methods

Blood samples for the determination of total serum cholesterol were taken by incision from the marginal ear vein one week before the change to the semipurified diets and one day afterward. Subsequently, blood samples were collected twice a week. Lipoprotein analyses were done for the individual samples taken one week before and 4 weeks after the beginning of the experiment. The serum lipoproteins were isolated by density gradient ultracentrifugation, employing a modification of the method described by Redgrave et al. (7).

In an SW 50-1 cellulose nitrate centrifuge tube (Beckman Inc., Palo Alto, CA) was placed 1 ml serum. In order to prestain the lipoproteins, the serum was mixed carefully with a Sudan Black solution (0.1 ml) prepared as described by Narayan (8). The background density of the prestained serum was raised to 1.21 g/ml by adding 0.313 g solid KBr. Subsequently, the mixture was overlayered with equal volumes (2.1 ml) of salt solutions of densities 1.063 g/ml (11.42 g NaCl and 80.26 g KBr/ℓ) and 1.0063 g/ml (11.42 g NaCl/ℓ), respectively. All solutions contained 0.1 g/ℓ ethylenediaminetetraacetic acid (disodium salt). The samples were centrifuged for 16 hr at 234,000 g (avg.) at 20 C. The clearly visible lipoprotein bands were collected by tube slicing an analyzed for cholesterol and protein concentration. Cholesterol in whole serum and the lipoprotein fractions was measured according to the Röschlau et al. method (9), using the kit supplied by Boehringer Mannheim, Germany (Catalase Kit, cat. no. 123.087). The protein content of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were estimated by the method described by Markwell et al. (10), which is a modification of the Lowry et al. method (11). Since the high density lipoprotein (HDL) fractions were contaminated with serum albumin when analyzed by gradient polyacrylamide gel electrophoresis (3-12% slabgel, a modification of the method described by Masket et al. [12]), no determinations of protein were done in this lipoprotein class. Statistical analysis was performed using Student's one-tailed t-test as adapted by Snedecor and Cochran (13).

RESULTS

Food Consumption and Growth

The body weight, weight gain and food consumption throughout the experiment are presented in Table II. During the first period, the growth rate of the rabbits on the 20 and 40% casein diets was higher than the growth rate observed in the rabbits fed the 10% casein diet ($P < 0.01$). After the crossover, the highest weight gain was seen in the 40% casein group ($P < 0.001$), whereas similar growth rates occurred in the 20 and 10% groups. This higher growth rate, associated with a higher food intake, probably can be explained as a compensatory response to the poor growth on the low casein diet during the first period. In contrast to the first period, no differences in weight gain between the 10 and 20% groups were found in the second period of the experiment. The rabbits fed the restricted amount of commercial diet exhibited, during the first period, a growth rate which was higher than the growth rate of the animals fed the 10% casein diet ($P < 0.01$) and lower than that of the group fed the semipurified diet containing 20% casein ($P < 0.01$). Similarly, after the crossover, the rabbits fed the restricted amount of commercial diet exhibited a higher growth rate than the rabbits fed the 20% casein diet ($P < 0.05$), but lower than that of the rabbits fed the 40% casein diet ($P < 0.001$).

Serum Cholesterol

When the rabbits were changed from a commercial rabbit diet to semipurified diets containing casein, a rapid elevation in the concentration of serum cholesterol was observed in all 3 groups (Fig. 1). In the 10% casein group, the maximal level of serum cholesterol was reached after only 3 days on the experimental diet, whereas the concentrations of serum cholesterol in the 20% casein group reached a plateau after 14 days. The levels in

the rabbits fed 40% casein diets continued to increase. At the end of the first period, significantly higher concentrations of serum cholesterol were observed in the 40% group compared to the 20% group ($P < 0.05$) and in the 20% group compared to the 10% group ($P < 0.01$). When the rabbits on the diet containing 10% casein were transferred to the 40% casein diet, a significant increase in the concentration of serum cholesterol occurred ($P < 0.001$). Conversely, switching the group on the 40% casein diet to the 10% casein diet resulted in a decreased level of serum cholesterol ($P < 0.05$). The group receiving the 20% casein diet and the commercial diet remained at a constant level of serum cholesterol.

Cholesterol and Protein in the Lipoprotein Fractions

The cholesterol and protein concentrations and the ratios of cholesterol to protein in the different lipoprotein fractions immediately before the crossover are given in Table III. The increase in serum cholesterol was mainly reflected in the LDL and VLDL fractions, whereas in the HDL fractions, there were only relatively minor changes. An increase in the protein concentration of the VLDL and LDL fractions also was found but this was less pronounced than the increase of the cholesterol. Therefore, an elevation of the ratio of cholesterol to protein could be observed in the VLDL and LDL fractions of the rabbits fed the semipurified diets. This suggests that lipoprotein particles relatively rich in cholesterol were formed.

DISCUSSION

The growth of rabbits on semipurified diets containing casein is usually less than on commercial rabbit pellets (3,14). Hove and Herndon (15) reported that, in rabbits aged 4 weeks, higher growth rates could be obtained by increasing the proportion of casein in the diet in the range from 10 to 50%. In our study, during the first period, weight gain also was higher with the 20% casein diet than the 10% casein diet; however, a further increase in the proportion of dietary casein to 40% slightly, but not significantly, depressed the growth. Huff et al. (14), on the other hand, actually observed a loss of weight when feeding diets containing 54% casein compared to 27%. The reason for these contradictory results is unclear. The low growth rate on the 10% diet probably can be explained by a deficiency of amino acids, as the proportion of most of the essential amino acids present in the 10% casein diet is

TABLE II

Body Weight, Weight Gain and Food Consumption in Rabbits Fed Diets Containing Different Proportions of Casein

	Mean ± SEM[a]						
	First period (28 days)			Second period (21 days)			
	Initial body wt (g)	Wt gain (g/day)	Food intake (g/day)	Initial body wt (g)	Wt gain (g/day)	Food intake (g/day)	Final body wt (g)
10-40% Casein (6)[b,c]	1358 ± 38	10.7 ± 1.5**	63.0 ± 1.3**	1659 ± 71*	40.4 ± 1.9**	77.2 ± 2.8*	2507 ± 98
20% Casein (6)	1326 ± 36	29.3 ± 1.6	76.7 ± 1.3	2148 ± 51	10.5 ± 1.6	56.7 ± 1.7	2368 ± 45
40-10% Casein (6)[d]	1325 ± 50	25.0 ± 2.3	73.3 ± 1.0	2025 ± 102	11.3 ± 4.3	66.5 ± 1.7*	2265 ± 137
Commercial diet (3)	1330 ± 98	20.1 ± 0.9	110/130[e]	1895 ± 93	19.3 ± 2.9	130	2297 ± 105

[a]Differences between means of the 10-40% casein group and the 20% casein group and between means of the 40-10% casein group and the 20% casein group were analyzed for significance by Student's two-tailed t-test: *$P < 0.01$; **$P < 0.001$.

[b]Number of rabbits.

[c]The rabbits were fed 10% casein diets during the first period and 40% casein diets during the second period.

[d]The rabbits were fed 40% casein diets during the first period and 10% casein diets during the second period.

[e]The rabbits were fed 110 g of pellets during the first period of 10 days; subsequently, 130 g was administered; these amounts were finished by all the rabbits every day.

lower than that recommended for rabbits (16).

The rabbits fed the commercial diet consumed a much greater amount of food than the animals fed the semipurified diets, although the growth rate in some cases was lower. However, the composition of the commercial diet differs in many aspects from that of the semipurified diets. The semipurified diets had a higher caloric density (3,400 kcal/kg feed) than the commercial diet (2,800 kcal/kg feed) and the difference can be partly attributed to the high fat content of the semipurified diets. Differences in the availability of the energy also may be important.

It has been well established in rabbits that the feeding of semipurified diets containing casein results in hypercholesterolemia, whereas this effect is not observed with diets containing soy protein (1,2). The results of our study additionally show that increasing the proportions of casein in the diet results in higher levels of serum cholesterol. Huff et al. (14) reported that doubling the proportion of soybean protein from 27 to 54%, at the expense of the carbohydrate source (dextrose), had no significant effect on the concentration of serum cholesterol. Furthermore, Hamilton and Carroll (17) observed that the replacement of dextrose in a semipurified diet by maize starch, as used in our diets, did not result in changes in the levels of serum cholesterol. Therefore, it is suggested that the differences in the levels of serum cholesterol observed in our rabbits fed diets containing different proportions of casein can be attributed to the changes in the proportion of casein rather than to changes in the proportions of either total protein or of maize starch.

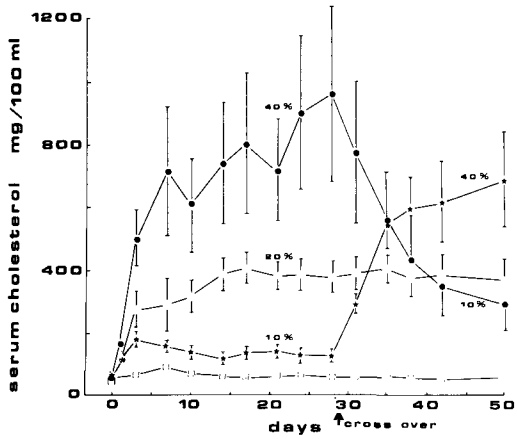

FIG. 1. Serum cholesterol concentrations in rabbits fed semipurified diets containing different proportions of casein. ★—★ = group receiving diet containing 10% casein before the cross-over and 40% casein after the cross-over; ●—●, 40 and 10%, respectively; ○—○ = 20% throughout the experiment; □—□ = commercial diet. The vertical bars represent the standard error of the mean at each time point.

The results obtained in our study are in agreement with those reported by Huff et al.

TABLE III

Cholesterol and Protein Concentration and Cholesterol:Protein Ratio in Serum Lipoproteins in Rabbits Fed Diets Containing Different Proportions of Casein

	Mean ± SEM			
	Initial (18)[a,b]	Casein 10% (6)[c]	Casein 20% (6)[c]	Casein 40% (6)[c]
Cholesterol[d]				
VLDL	17.8 ± 3.4	40.7 ± 5.4*	119.1 ± 33.7	542.2 ± 197.1*
LDL	14.0 ± 2.0	30.9 ± 13.7**	146.8 ± 15.3	210.0 ± 57.4
HDL	18.4 ± 1.2	15.1 ± 2.8***	60.2 ± 5.5	44.7 ± 11.5
Protein[d]				
VLDL	16.0 ± 2.9	26.8 ± 5.2	46.1 ± 14.9	137.2 ± 41.9*
LDL	18.9 ± 2.3	22.0 ± 4.1**	82.9 ± 8.6	116.2 ± 27.9
Cholesterol: protein ratio[e]				
VLDL	1.10 ± 0.05	1.77 ± 0.33^{+}	2.73 ± 0.29^{+++}	3.61 ± 0.47^{+++}
LDL	0.75 ± 0.05	1.38 ± 0.41	1.79 ± 0.10^{+++}	1.76 ± 0.08^{+++}

[a]Number of animals in parentheses.

[b]These figures represent the initial values of all the animals before the change-over to the semipurified diets.

[c]Values after feeding the diets for 28 days.

[d]Concentrations expressed in mg/100 ml. Comparison by Student's t-test of the increases in concentration from the initial values of the 10 and 40% groups with the 20% casein group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[e]Comparison by Student's paired t-test of the differences from the initial values within each group: $^{+}P < 0.05$; $^{+++}P < 0.001$.

(14). However, in their study, doubling the amount of casein in the diets resulted in higher concentrations of serum cholesterol, which was associated with loss of weight. It is known that, in rabbits, hypercholesterolemia occurs during periods of starvation (18,19). In our study, no loss of weight was observed. Furthermore, by feeding a group of rabbits a restricted amount of commercial diet, it was demonstrated that no consistent relationship existed between the growth rates and the levels of serum cholesterol. During the first period of the experiment, the rabbits fed the commercial diet exhibited a lower level of serum cholesterol and a lower growth rate than the group fed the 20% casein diet. However, after the crossover, the group fed the commercial diet also had lower concentrations of serum cholesterol but this was associated with a higher growth rate. Therefore, it is unlikely that the differences in serum cholesterol levels can be attributed to differences in growth rate rather than to differences in the diets per se.

It was observed over 50 years ago that the atherogenic properties of a particular protein could be enhanced by the incorporation of a higher proportion of the protein in the diet of rabbits. Newburgh and Clarkson (20) found that rabbits consuming a diet containing 36% protein derived from lean beef muscle developed atherosclerosis sooner than did rabbits receiving 27% of the protein in their diet. Similar results have been obtained in rats. Nath et al. (21) showed that, in rats fed casein diets, the levels of serum cholesterol were higher when the diet contained 70% casein compared to 40%. They found the highest levels of serum cholesterol in rats on 6% casein diets. However, on this low protein diet, weight loss also was observed, which might have contributed to the elevation of the levels of serum cholesterol. Hevia et al. (22) also reported that, in rats, the feeding of diets containing casein in increasing proportions (7.5, 15 and 30%) resulted in higher levels of serum cholesterol.

Other authors (5,14) have provided strong evidence that the amino acid composition of the protein under investigation plays an important role in the etiology of hypercholesterolemia. Hermus (3) indicated that the amino acids present in a 20% casein diet meet the tentative requirements of the rabbit. Therefore, an amino acid imbalance might be more relevant than an absolute deficiency of some particular amino acids. The data obtained from our study underscore this hypothesis, since increasing the proportion of casein in the diets resulted in further elevation of the serum cholesterol levels and not the reverse.

Higher proportions of casein in the diets resulted in higher levels of serum cholesterol, which was mainly attributable to increased cholesterol in the VLDL and LDL fractions. A relatively minor elevation of the HDL cholesterol occurred, whereas in the rabbits fed 10% casein diets no significant changes at all were detectable in this lipoprotein fraction. At moderate levels of serum cholesterol, the LDL was the major carrier of cholesterol (20% casein group), but at higher concentrations of serum cholesterol (40% casein group) most of the cholesterol was transported by the VLDL. These findings agree with those reported by other authors (23,24).

Hypercholesterolemia in rabbits caused by feeding semipurified diets containing casein resulted in the formation of lipoprotein particles with a high ratio of cholesterol to protein, suggesting the appearance of cholesterol-rich lipoprotein particles. Similar observations have been reported in rabbits made hypercholesterolemic by feeding cholesterol (25) or by starvation (19).

In conclusion, the results of this study show that, in rabbits, the hypercholesterolemic properties of semipurified diets containing casein can be enhanced by increasing the proportion of dietary casein. This finding might be very useful in studies on the mechanism underlying the hypercholesterolemic action of casein. Using diets with higher proportions of casein, more pronounced and more rapid elevations in serum cholesterol levels can be produced, which might help elucidate the mechanism of casein-induced hypercholesterolemia.

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Effect of Phagocytosis and Ionophores on Release and Metabolism of Arachidonic Acid from Human Neutrophils

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ABSTRACT

Challenge of human neutrophils prelabeled with [^{3}H]arachidonate and [^{14}C]palmitate or [^{14}C]-stearate with opsonized zymosan or the Ca^{2+} ionophores A23187 or Ionomycin caused the release of [^{3}H], but not [^{14}C], fatty acid. With the ionophores, but not zymosan, considerable conversion of the [^{3}H]arachidonate to hydroxyeicosatetraenoates occurred. Although various isomers were recovered, the 5-hydroxyeicosatetraenoate appeared to be the major product. In these experiments, no [^{14}C] products were detected such as lysophospholipid, diglyceride or monoglyceride. Although no definitive statement can be made about the mechanism of release of arachidonate, our data are most easily interpreted as the result of the action of a phospholipase A_2.

INTRODUCTION

There has been recent interest in the capacity of neutrophils to release arachidonic acid (20:4) from membranous lipid and to convert the 20:4 to bioactive molecules such as leukotrienes (1, 2) or the hydroxy derivatives of 20:4, the hydroxyeicosatetraenoates (HETE) (3). That a phospholipase A_2 might be involved in this process was first indicated by Kaplan et al. (4) who demonstrated that the phospholipase A_2 of rabbit neutrophils was inhibited by indomethacin. Arachidonate metabolites, including HETE, produced by neutrophils, have been shown to stimulate neutrophils to aggregate (5) and to increase hexose transport (6). We reported earlier (7) that challenge by opsonized zymosan caused the release of 20:4 from human neutrophil phospholipids, mainly phosphatidylcholine and phosphatidylinositol. Rubin et al. (8) showed that the Ca^{2+} ionophore A23187 and the formylated chemotactic peptide f-Met-Leu-Phe increased the incorporation of 20:4 into cellular phosphatidylinositol, indicating a deacylating-reacylating phospholipid cycle.

Two possible pathways for the release of 20:4 have been described in platelets: a direct release by phospholipase A_2 (9) and an indirect release by phospholipase C which produces diglyceride, followed by the action of a lipase that then releases 20:4 (10, 11). In order to investigate these 2 possibilities, we employed [^{14}C]palmitic (16:0) or [^{14}C]stearic (18:0) and [^{3}H] 20:4 as precursors for positions 1 and 2 of phospholipids, respectively. We compared the effect of the Ca^{2+} ionophores A23187 and Ionomycin to the effect of opsonized zymosan on the release of 20:4 from human neutrophils and its conversion to oxidative metabolites. If the phospholipase A_2 pathway were operative, we would expect to recover radiolabeled lysolipid whereas the phospholipase C pathway should produce double-labeled diglyceride and [^{14}C] monoglyceride.

EXPERIMENTAL PROCEDURES

Materials

[5,6,8,9,11,12,14,15-^{3}H] Arachidonic acid (5 Ci/mmol), [1-^{14}C]palmitic acid (57 mCi/mmol) and [1-^{14}C] stearic acid (56.5 mCi/mmol) were purchased from Amersham/Searle, Des Plaines, IL; zymosan A was obtained from Sigma, St. Louis, MO. A23187 and Ionomycin were kindly supplied by Dr. R. L. Hamill, Eli Lilly Co., Indianapolis, IN, and Dr. W.-C. Liu, Squibb Institute for Medical Research, Nutley, NJ, respectively. Plasma gel was obtained from the HTI Corp., Buffalo, NY, and lymphoprep was obtained from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY. Lipid standards were purchased from the Serdary Research Laboratories, London, Ontario, Canada. All other chemicals were the highest grade available from Fisher Scientific Co., Atlanta, GA.

Preparation of HETE. A solution of arachidonic acid in 60 ml of chloroform was placed in a 500-ml, round-bottomed flask, the solvent removed in vacuo, and dry air pumped over the flask for 2 days. The resulting hydroperoxides were taken up in methanol, reduced with sodium borohydride and purified by high pressure liquid chromatography (HPLC). This separation of the HETE was achieved with a 30 cm × 7.8 mm Waters Associates μ-Porasil

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packed column. The solvent used for elution was acetic acid/2-propanol/hexane (1:16:983, v/v). Six components, each showing a strong absorption at 235 nm, were isolated by HPLC from the $NaBH_4$-reduced, autoxidized arachidonate. Under HPLC conditions similar to our own, the HETE isomers are reported to elute in the order 12, 15, 11, 9, 8, and 5-HETE (12). We did not perform structural determinations on the components of autoxidation but did demonstrate that the second component eluted with authentic 15-HETE, prepared by the oxidation of arachidonic acid using soybean lipoxygenase (EC 1.13.11.12).

Methods

Human polymorphonuclear leukocytes were isolated from peripheral blood of normal volunteers by sedimentation of erythrocytes with plasma gel followed by purification over lymphoprep as previously described (7, 13). This procedure yielded a cell population which consisted of >95% neutrophils.

Zymosan was opsonized with normal serum as previously described (7). Stock solutions of A23187 (4×10^{-2} M) and Ionomycin (6.7×10^{-3} M) in dimethyl sulfoxide were diluted appropriately to 4 μM with phosphate buffered saline. Aliquots (0.50-ml) of the ionophores were added to 0.50 ml of radiolabeled cells in phosphate buffered saline to give a final ionophore concentration of 2 μM, a concentration known to be nondeleterious to the cells.

The neutrophils, 3.5×10^7 cells in 1.0 ml phosphate buffered saline, were incubated with [^{3}H] arachidonate and [^{14}C] palmitate or [^{14}C] stearate as previously described (7). The radiolabeled fatty acids were dissolved in 100% ethanol and added to the cells. About $3-5 \times 10^5$ cpm [^{3}H] and $1-2 \times 10^5$ cpm [^{14}C] were added, constituting a final ethanol concentration of 0.5% or less. Following a 2-hr incubation (37 C), the cells were centrifuged for 4 min at 250 x g, washed once with phosphate-buffered saline and resuspended to 0.50 ml in phosphate buffered saline. Stimuli (opsonized zymosan, ionophores) were prewarmed (37 C) before addition to cells. The tubes containing cells and stimuli were incubated at 37 C in a shaker bath for 5 min. The incubations were stopped by addition of 3.0 ml chloroform/methanol (1:2, v/v).

Lipids were extracted by a modification of the Bligh and Dyer method (14). Briefly, 1.0 ml of water and 2.0 ml of chloroform were added to the tubes. The lower chloroform phase was removed from the upper water/methanol layer (4.0 ml). Aliquots of both layers were counted. The water/methanol layer was acidified by addition of 0.20 ml of 90% formic acid. Extraction of the acidified water/methanol layer was performed by addition of 3.0 ml chloroform/methanol (5:1, v/v). The phases were separated by centrifugation and the chloroform layer removed. The initial chloroform extract and the acidified chloroform extract were chromatographed separately on Silica Gel H. The neutral lipids were separated by a hexane/ether/ formic acid system (90:60:6, v/v; system I) and phospholipids separated by a one-dimensional chloroform/methanol/acetic acid/water system (75:50:10:6, v/v; system II). In some experiments, a 2-dimensional system was used for phospholipid separation in order to separate phosphatidylserine from phosphatidylinositol, which were not resolved in the 1-dimensional system. This 2-dimensional system was as follows: first system, chloroform/methanol/acetone/acetic acid/water (60:12:24:18:6, v/v); second system, chloroform/methanol/ammonium hydroxide (98:2:1, v/v). HETE separation was done by a 1-dimensional system consisting of hexane/isopropanol/acetic acid (92:10:0.6, v/v; system III). In order to separate diglycerides from HETE, we used the following 2-dimensional system: first system, chloroform/methanol/acetic acid (98:2:1); second system, chloroform/methanol/ammonium hydroxide (98:2:1; system IV). Appropriate standards were used in all the separation systems described. The compounds were visualized by iodine, scraped into vials and counted in a toluene/Triton X-100/ water solvent (2:1:0.25, v/v) containing 0.4% Omnifluor. The percentage of each radiolabeled compound was determined from the total radioactivity recovered. Results are presented as a representative experiment of 3 separate experiments; for each lipid compound, the SEM <10% of the mean. Each experiment included duplicate samples.

RESULTS

In experiments not reported, we found that [^{14}C] 16:0 and [^{3}H] 20:4 were rapidly incorporated into cellular lipids; however, most was initially incorporated into triglyceride. After 2 hr of incubation, sufficient radiolabeled fatty acid had been incorporated into phospholipids to do the studies planned (Table I, phosphate-buffered saline control). Challenge with either opsonized zymosan or Ionomycin for 5 min caused a release of [^{3}H] 20:4, but not [^{14}C]-16:0, from the phospholipids (Table 1, top). Ionomycin caused increases in the free [^{3}H] 20:4 and the diglyceride-HETE area of the plate (these 2 classes of compounds were not

TABLE I

Distribution of [^{3}H] Arachidonate and [^{14}C] Palmitate Following Five-Min Challenge

Compound	Addition					
	Phosphate-buffered saline		Opsonized zymosan		Ionomycin (2 μM)	
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
Neutral lipid system (I)						
Triglyceride	44.0	63.6	42.0	62.5	47.0	64.5
Free fatty acid	1.9	.6	10.2	1.7	7.8	1.9
Diglyceride	2.7	2.8	3.6	3.5	6.0	4.4
Hydroxyeicosatetraenoate	.9	.2	.9	.2	6.0	.2
Monoglyceride	.7	3.4	1.7	3.3	2.2	3.2
Phospholipid	49.0	28.0	42.0	28.0	30.0	25.0
Phospholipid system (II)						
Neutral lipid	46.5	67.0	54.0	67.0	65.0	67.0
Phosphatidylethanolamine	9.3	1.8	10.8	3.0	8.7	2.8
Phosphatidylinositol	25.5	1.0	19.7	1.6	12.7	2.1
Phosphatidylcholine	18.5	27.0	14.4	26.0	12.7	25.0
Sphingomyelin/lysophosphatidylcholine	.2	1.2	.2	1.2	.6	2.0

The lipids were separated by the designated chromatographic systems and the percentage distribution was calculated as the percentage of the radioactivity in each compound divided by the total radioactivity recovered ([^{3}H] = 1.4 × 10^5 cpm; [^{14}C] = 6 × 10^4 cpm).

well separated in this chromatographic system.) Opsonized zymosan, on the other hand, only increased the amount of [^{3}H] 20:4. In neither case was any double-labeled diglyceride recovered. The major phospholipids which lose [^{3}H] 20:4 are phosphatidylinositol and, secondarily, phosphatidylcholine (Table I, bottom). In separate experiments using 2-dimensional chromatography, we found little, if any, label in phosphatidylserine. No lysophosphatidylcholine, the product of phospholipase A_2 activity, was recovered on the thin layer chromatography (TLC) plate. This pattern of distribution of the release and metabolism of 20:4 did not change with time; at a 1-min challenge (the earliest time release could be detected), the ratio of products was the same as that at 5 min, the time of optimal release.

As shown in Table I, very little [^{14}C] 16:0 was incorporated into phosphatidylinositol, the major source for free [^{3}H] 20:4. We therefore replaced [^{14}C] 16:0 with [^{14}C] 18:0, which predominates in phosphatidylinositol, at least in liver (15). Table II shows that Ca. 1/6 of the [^{14}C] 18:0 is incorporated into the phosphatidylinositol fraction; this, however, was not released upon stimulus by opsonized zymosan or the ionophore A23187 (ionomycin gave similar results). Again, no [^{14}C] lysophosphatidylcholine was recovered nor was there any evidence that [^{14}C] lysophosphatidylinositol was formed. Extraction of the water/methanol layer for identification of radiolabeled lysophospholipid by-products was determined to be 80% efficient for both lysophosphatidylinositol and lysophosphatidylcholine. Table III shows that the amount of [^{14}C] label recovered in the water-methanol layer, the expected partitioning of lysophosphatidylinositol was about the same as the phosphate-buffered saline control. Most striking, however, was the 10-fold increase in the amount of [^{3}H]-labeled compounds recovered in the water/methanol layer of the ionophore, but not opsonized zymosan-challenged cells. This amounted to Ca. 15% of the total amount of [^{3}H] recovered. Because most extraction procedures for the HETE and leukotrienes involve acidification prior to extraction (1, 2, 9, 10), the water/methanol layer was acidified with 0.2 ml of formic acid and reextracted with chloroform. This treatment extracted most of the ^{3}H- but not ^{14}C-labeled material into the chloroform layer. A sample of this was then chromatographed to determine the nature of the material extracted. In order to clearly separate diglyceride, HETE, and 20:4, we used 2 TLC systems—one that separated diglyceride and HETE from 20:4 and a 2-dimensional system that separated diglyceride from 20:4 and HETE (Table IV). The results of these chromatographic separations indicate that the major products recovered in the acidified chloroform layer were the HETE; only a few percent of the [^{3}H] recovered cochromatographed with standard diglyceride or 20:4 in both systems. Further, it appears that the major HETE recovered in the acidified chloroform layer were the 5, 11, 12 and 15 isomers. Chromatography of the original, nonacidified chloroform solution in the single-

TABLE II

Distribution of [^{3}H] Arachidonate and [^{14}C] Stearate Following Five-Min Challenge

Compound	Addition: Phosphate-buffered saline [^{3}H]	Phosphate-buffered saline [^{14}C]	Opsonized zymosan [^{3}H]	Opsonized zymosan [^{14}C]	A23187 (2 μM) [^{3}H]	A23187 (2 μM) [^{14}C]
Neutral lipid system (I)						
Triglyceride	41.0	57.0	43.0	62.0	44.0	59.0
Free fatty acid	2.9	1.8	9.0	3.0	8.5	3.7
Diglyceride	3.8	2.0	4.9	2.8	5.9	2.9
Hydroxyeicosatetraenoate	.8	1.5	1.4	2.3	5.0	1.6
Monoglyceride	.5	.2	.7	.2	2.5	.2
Phospholipid	51.0	37.0	40.0	31.0	34.0	33.0
Phospholipid system (II)						
Neutral lipid	45.0	59.0	53.0	61.0	64.0	62.0
Phosphatidylethanolamine	11.5	7.5	14.2	8.6	10.3	7.2
Phosphatidylinositol	25.5	16.0	20.0	13.6	14.8	14.0
Phosphatidylcholine	16.0	16.5	12.2	15.7	10.4	16.0
Sphingomyelin/lysophosphatidylcholine	.1	.4	.1	.5	.4	.5

The calculations are the same as described for Table I ([^{3}H] = 1 X 10^5 cpm; [^{14}C] = 3 X 10^4 cpm).

TABLE III

Recovery of Radioactivity in Water/Methanol Phase

Sample addition	Total counts/min: Aqueous layer (water/methanol) [^{3}H]	[^{14}C]	[^{3}H]/[^{14}C]
Phosphate-buffered saline	2,239	8,126	.27
Opsonized zymosan	2,762	6,546	.42
A23187 (2 μM)	25,068	9,032	2.77

An aliquot (1.0 ml) of the water/methanol phase was counted after the initial extraction with chloroform. Following acidification, the samples were reextracted for chromatography in systems III and IV (Table IV).

dimensional system revealed that 80% of the HETE in the initial extract was the 5-HETE (data not shown); this, therefore, amounts to roughly 8–10% of the [^{3}H] recovered in the initial extract. Combining the results of the 2 extractions indicates that 20–25% of the [^{3}H] 20:4 was released and that 2/3 of this was further metabolized to HETE when ionophores were used. 5-HETE appeared to be the major isomer formed, although significant amounts of the others were detected.

DISCUSSION

From these experiments, we conclude that human neutrophils released 20:4 from cellular phospholipid when challenged with opsonized zymosan or with the Ca^{2+} ionophores A23187 or Ionomycin. The major phospholipids degraded were phosphatidylinositol and phosphatidylcholine. Of the 2 possible pathways thus described that could account for the release of 20:4, we favor that involving a phospholipase A_2, even though we were unable to demonstrate the formation of a lysophospholipid. The reason for this tentative conclusion is that any pathway involving the phospholipase C should produce a double labeled diglyceride and a [^{14}C] monoglyceride. Neither product was recovered at any time of incubation. Although the same argument could be used against a phospholipase-A_2-mediated reaction, it is possible that a rapid reacylation of the lyso derivatives, such as described in rabbit peritoneal neutrophils (16), could occur, thereby protecting the cell from the lytic action of the lysolipids. The alternate proposal, i.e., degradation of phospholipid via a phospholipase C, would necessitate the complete degradation of the phospholipid and probable redistribution of the [^{14}C] fatty acids. This was not found. However, more work will be necessary before the

TABLE IV

Distribution of [^{3}H] Products from Acidified Chloroform Extraction

Compound	Percentage
One-dimensional system (III)	
Triglyceride	1.6
Fatty acid	3.6
Diglyceride and 11,12,15-hydroxyeicosatetraenoate	37.2
8,9-Hydroxyeicosatetraenoate	11.9
5-Hydroxyeicosatetraenoate	8.7
Unknown	8.6
Monoglyceride	11.9
Prostaglandin E_2 and thromboxane A_2, B_2	5.2
Phospholipid	7.9
Two-dimensional system (IV)	
Triglyceride	.8
1,3-Diglyceride	4.5
1,2-Diglyceride	2.9
Fatty acid and hydroxyeicosatetraenoate	68.9
Monoglyceride	7.1
Phospholipid and prostaglandin F_2	15.7

The distribution of radioactivity is calculated as described for Table I. The material extracted in the experiment described in Table III ([^{3}H] = 25,000 cpm; no [^{14}C]) was divided equally between the 2 systems.

precise mechanism of 20:4 turnover can be defined.

The major products of the metabolism of 20:4 were the HETE; little, if any, of the products of the cyclooxygenase pathway were detected. We cannot rule out the possibility that leukotrienes were produced in our experiments. We would not necessarily expect to obtain the same compounds found by Borgeat and Samuelsson (1, 2), however, since our experimental conditions were quite different. The major differences were that they did not prelabel their cells but added the 20:4 with the ionophore and they used high levels of 20:4 (133 μM) that we have found to be toxic to neutrophils (unpublished observations).

The differing effects of the various stimuli are especially intriguing: while stimulation by opsonized zymosan results in accumulation of free 20:4, stimulation of ionophores apparently results in the further conversion of this 20:4 to the various HETE. The significance of this difference is currently under investigation.

ACKNOWLEDGMENTS

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Sterol Ester Hydrolase in *Fusarium oxysporum*[1]

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ABSTRACT

Two electrophoretically different forms of sterol ester hydrolase (EC 3.1.1.13) were obtained from the cytoplasmic extract of the mycelia of *Fusarium oxysporum*. The entities, estimated at 60,000 (I) and 15,000 (II) molecular weights, were obtained in Sephadex G100 column chromatography of the ammonium sulfate precipitate from the cytoplasmic extract. A third form III, 75,000 MW, was obtained from the culture filtrate. The activity of the enzyme was increased by Triton X-100 and was not inhibited by *p*-chloromercuribenzoate (PCMB), a sulfhydryl reagent. The enzymes I and II were inhibited differentially by NaCl. The optimal activities of forms I, II and III occurred at pH 4.8, pH 8.0 and pH 7.0, respectively. The apparent Km values of 7.7×10^{-5}, 8.3×10^{-5} and 10.5×10^{-5}, respectively, indicate a similar order of affinity for cholesteryl oleate at pH 7.1. The rate of hydrolysis of cholesteryl esters were in the order: linoleate > oleate > valerate > butyrate > acetate. Cholesteryl benzoate and palmitate were not hydrolyzed. The properties of the microbial enzyme are discussed in relation.

INTRODUCTION

Sterol ester hydrolase (esterase), EC 3.1.1.13, which catalyzes the hydrolysis and synthesis of fatty acid esters of sterols, has been examined extensively in a number of mammalian tissues primarily because of the involvement of sterol esters in atherosclerosis (1). There have been relatively few studies of the microbial enzyme (2-7) in spite of the widespread distribution and variety of sterols and fatty acids which occur particularly in the fungi. Steroids in fungi are the precursors of a number of hormones which influence growth, sexuality and the regulation of nucleic acid activity (8-10). Sterols have also been implicated in the survival and temperature tolerance of these organisms (11).

Lower growth temperatures appear to facilitate the production of more highly unsaturated fatty acids in the fungi (12-14). Miller and de la Roche (13) have demonstrated that the sterol and sterol ester contents of membranes of *Fusarium oxysporum* f. sp. *lycopersici*, are affected by temperature. These data suggest, therefore, an active sterol-fatty acid ester metabolism in *F. oxysporum*.

This study examines in *F. oxysporum*, the properties of sterol ester hydrolase, one of the principal enzyme systems in the metabolism of sterol esters.

MATERIALS AND METHODS

Materials

Labeled cholesteryl oleate [oleate-1-^{14}C] 0.25 mCi was purchased from New England Nuclear Co. (Boston, MA); unlabeled cholesteryl esters, cholesterol, lecithin and sodium taurocholate were obtained from Supelco, Inc. (Bellefonte, PA). All other reagents were analytical grade. A microbial cholesterol esterase preparation was obtained from Boehringer Mannheim (Germany). Because of the patent, the source of the enzyme could not be obtained from the manufacturer.

Assay of Sterol Ester Hydrolase

The procedure essentially as described by Taketani et al. (2) was used for assaying the enzyme. Triton X-100 was used at 0.10% rather than at 0.3% in the assay as described by Taketani. Radioactive cholesteryl oleate was used as substrate at pH 7.1 unless mentioned otherwise. The chromatographic procedure, however, was modified as follows: After the chloroform extract of the reaction mixture was dried under N_2, aliquots in chloroform were applied quantitatively onto 20 x 20 cm Silica Gel 60 H thin layer plates (E.M. Reagent, E. Merck, Darmstadt, Germany). Standard cholesteyl oleate (25 μg) also was applied to the plates. The plates were developed by ascending chromatography in *n*-hexane/ethyl ether/acetic acid (70:30:1 by vol). The standards, sprayed with an anisaldehyde reagent consisting of acetic acid/sulfuric acid/*p*-anisaldehyde (50:1:0.5 by vol) located cholesteryl oleate, cholesterol and oleic acid at R_f 0.5, 0.15 and 0.33, respectively. The radioactivity of the samples was measured on a Beckman LS 8000 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA).

One unit of cholesteryl ester hydrolase activity is defined as the amount of enzyme producing 1 μmol of free cholesterol or free oleic acid/min at 37 C. All hydrolase activity expressed in this study must be regarded as the net activity for the reasons described by Brock-

[1]Contribution no. 1098.

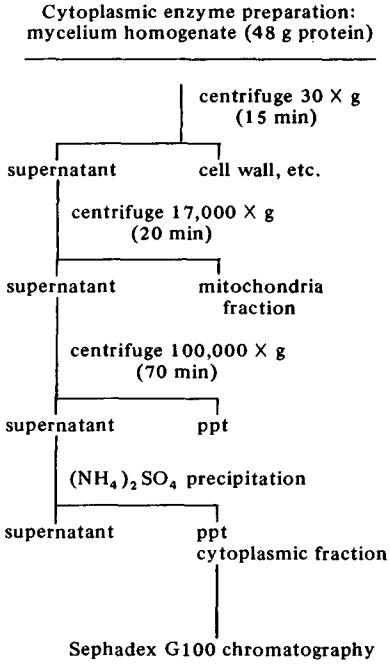
erhoff and Jensen (1). The same amount of enzyme, based on cholesteryl oleate activity, was used in all experiments where specifically indicated.

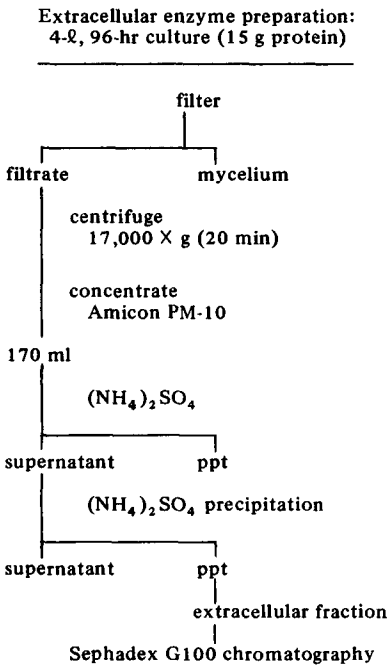
Assay for Substrate Specificity

Free cholesterol was measured colorimetrically by the method of Engelbrecht et al. (15). Ten μmol of the various ester substrates was incubated and the released cholesterol was extracted, separated and measured.

Extraction and Partial Purification of Cholesteryl Ester Hydrolase

The fungus *F. oxysporum* (DAOM 143566 is maintained at the Mycological Herbarium, Agriculture Canada, Ottawa, K1A OC6) was grown in 1-ℓ quantities of a modified Fries medium (16) at 30 C in shake cultures. After 40 hr incubation, the mycelia were filtered and washed. All extraction and purification procedures were performed at 0-4 C. For the cytoplasmic fraction, the filter-dried mycelia (134 g) were homogenized in 200 ml Tris buffer pH 7.4, 0.1 M with glass beads (268 g, 16-220 size, VirTis Co. Inc., Gardiner, NY) at high speed in the Omni Mixer (Servall Inc., Norwalk, CT) for 10 min. The fractions were obtained by differential centrifugation and by ammonium sulfate precipitation as illustrated in the following flow charts.





Crystalline, enzyme-grade $(NH_4)_2SO_4$ was added slowly to the gently stirred cytoplasmic and extracellular fractions to achieve 100% saturation. Only precipitates obtained between 75 and 100% saturation in the mycelium preparation and 35% saturation in the extracellular preparation demonstrated sterol ester hydrolase activity. The resulting precipitates were chromatographed on a Sephadex G100 column. Components in the chromatographic fractions were separated by polyacrylamide gel disc electrophoresis at 4 C using Tris-glycine buffer at pH 8.3 and a 10.5% acrylamide gels in 75 X 5 mm glass tubes at 3 mA/gel with bromophenol blue as the tracking dye. Electrophoretic samples contained 1-5% protein, which was determined by the method of Lowry et al. (17).

Cholesteryl ester hydrolase activity on the gels was detected by the Szécsi et al. method (18).

Molecular Weight Estimation

The molecular weight of the enzyme was estimated by column chromatography on a Sephadex G100 column as described by Ackers (19) and by sucrose density gradient centrifugation according to Martin and Ames (20) and Hyun et al. (21).

The molecular weight markers used for comparison with the enzyme were myoglobin (16,890 MW), ovalbumin (45,000 MW), bovine serum albumin (B.S.A. 68,000 MW), gamma

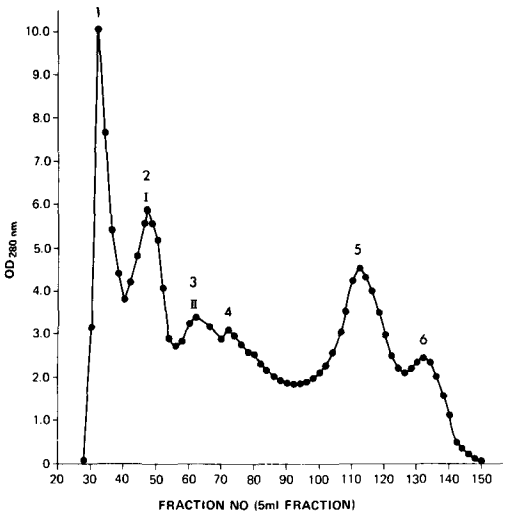


FIG. 1. Sephadex G100 column chromatography of the $(NH_4)_2SO_4$ (75-100% saturation) precipitate from the cytoplasmic fraction of *F. oxysporum* mycelia. Peaks II (fractions 42-56) and III (fractions 59-69) constitute the 2 active sterol ester hydrolase preparations. Column: 2.6 X 100 cm; eluent: sodium phosphate buffer 0.1 M, pH 7.1; V_t = 450 ml, V_o = 155 ml.

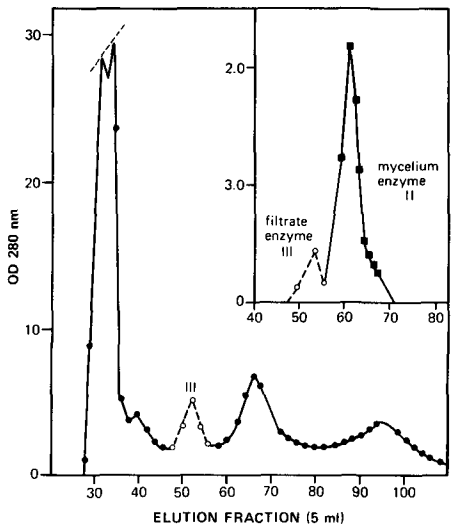


FIG. 2. Sephadex G100 column chromatography of the $(NH_4)_2SO_4$ precipitate from the extracellular culture liquid, and cochromatography of forms II and III (inset).

globulin (153,100 MW), and aldolase (160,000 MW).

Protein in all fractions was determined by the method of Lowry et al. (17) on the Technicon autoanalyzer (Technicon Corporation, Ardsley, NY).

Kinetic Experiments

The apparent Km and V_{max} were determined by varying the concentration of cholesteryl oleate as substrate while maintaining the other components at concentrations which did not limit the enzyme activity. The kinetic parameters where deduced from double reciprocal plots according to Lineweaver and Burk (22).

RESULTS

Figure 1 illustrates the elution pattern of the crude cytoplasmic extract on a Sephadex G100 column. Sterol ester hydrolase activities were obtained only in peak I (fractions 42-56) and peak II (fractions 59-69).

Figure 2 shows the elution pattern of the concentrated culture filtrate on the Sephadex gel column and the location of the form III of the hydrolase. Because of the molecular weight proximity of the cytoplasmic form II and this extracellular form III, the 2 active fractions were combined and rechromatographed (Fig. 2, inset) to ascertain their chromatographic difference. The data indicate that the forms are, indeed, different chromatographically as form III emerged ahead of form II. The molecular weights of the 3 forms I, II and III based on their elution patterns and by sucrose density

TABLE I

Purification of Sterol Ester Hydrolase

Purification step	Total activity (units)		Specific activity[a]		Purification	
	cytoplasmic	extracellular	cytoplasmic	extracellular	cytoplasmic	extracellular
Crude homogenate	30.8	75	0.0074	0.05	1	1
Ammonium sulfate precipitation	29.0	65	0.0063	0.42	1	8.4
Sephadex G100	21.6(I), 4.1(II)	50	0.148(I), 0.128(II)	5.0	23(I), 20(II)	100

[a]Enzyme units/mg protein.

gradient centrifugation relative to those of known molecular markers are 60,000, 15,000 and 75,000, respectively. Table I shows the purification of the cytoplasmic and extracellular enzymes.

Sterol Ester Hydrolase Activity

Although all concentrations of Triton X-100, ranging from 0.1-1.0%, increased the rate of hydrolysis of the sterol esters, the concentration obtained for the optimal activity was at 0.10% (Table II).

The crude enzyme extract hydrolyzed 4% of the cholesteryl oleate substrate. However, after the extract had been dialyzed against distilled water for 12 hr, the activity increased 9-fold. When the dialysate was recombined with the extract, the original, reduced activity was obtained suggesting a factor in the dialysate which modified the enzyme activity. Dialysis of the Sephadex G100 chromatographic fractions increased the enzyme activity similarly, again suggesting a modifying factor—possibly a component in the sodium phosphate elution buffer.

The Effect of *p*-Chloromercuribenzoate (PCMB) on Sterol Ester Hydrolase Activity

Concentrations of PCMB ranging from 0.5 mM to 10 mM had no significant effect on the hydrolysis of the cholesteryl oleate substrate either by the cytoplasmic or extracellular enzyme preparations.

The Effect of [Na^+] on Sterol Ester Hydrolase Activity

Figure 3 illustrates that, while the hydrolytic activity of forms I and II were markedly inhibited, form III of the enzyme was unaffected by [Na^+] up to 100 mM concentration. Inhibition of the 3 forms between pH 4.5 and pH 8 demonstrated a similar pattern.

pH Optima for Sterol Ester Hydrolase Activity

Figure 4 illustrates the distinctive effect of pH on the activities of the 3 enzyme fractions. The activity of enzyme I increased sharply as the pH was increased from 3 to 3.5 and showed a relatively broad maximum in the range pH 3.5 to 5.5. Above pH 5.5, a gradual decrease in activity was observed. In contrast, enzyme II and the extracellular enzyme III both had relatively narrow optima at pH 8.0 and pH 7.0, respectively.

Polyacrylamide Gel Disc Electrophoresis

Figure 5 illustrates the electrophoretic separation of the hydrolase enzymes from the cyto-

TABLE II

The Effect of Triton X-100 on the Hydrolysis of Cholesterol Oleate[a]

Amount of Triton X-100 (%)	Hydrolysis of cholesteryl oleate (%)
0	6.0
0.1	29.0
0.3	26.0
0.5	20.0
1.0	11.0

[a]Volume of reaction mixture 1.2 ml. Amount of enzyme 0.53 units (assay described in Methods).

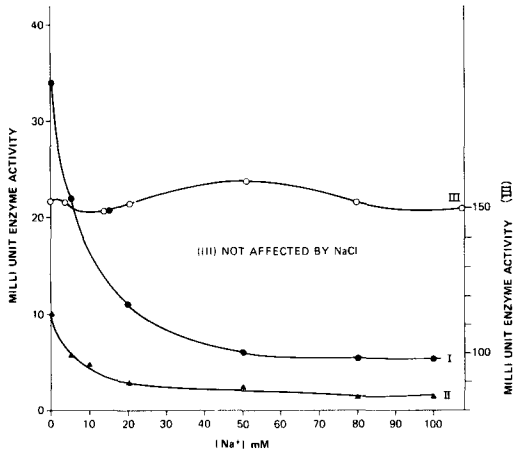


FIG. 3. The effect of sodium chloride on the activities of the sterol ester hydrolases I and II assayed at pH 4.5 and pH 8, respectively.

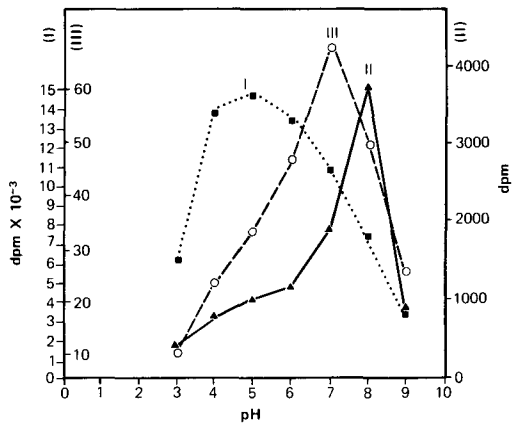


FIG. 4. The effect of pH on the activities of the sterol ester hydrolases I, II and III.

plasmic (I and II) and extracellular (III) preparations obtained from Sephadex G100 column chromatography. The 3 forms of the enzyme system migrated at distinctly different rates in electrophoresis. Forms I and III, which have close molecular weights 60,000 and 75,000, respectively, separated definitively when their combined fractions were subjected to electrophoresis. The faster migration of the heavier form III suggests greater ionic charges on this protein. The enzyme activities were destroyed by sodium dodecyl sulfate, making SDS-PAGE impractical.

Specificity for Fatty Acid Esters of Cholesterol

Table III shows the rates of hydrolysis of a number of fatty acid esters of cholesterol relative to the hydrolysis of cholesterol oleate. The 3 enzyme forms demonstrated a similar pattern of hydrolysis. While the benzoate and palmitate esters were not hydrolyzed, cholesteryl linoleate was hydrolyzed 2.5-fold faster than the oleate ester. The valerate, butyrate and acetate esters were hydrolyzed more slowly than the oleate substrate.

Kinetic Studies on Enzyme Preparations I, II and III

The apparent Km values for the enzyme preparations I and II are 7.7×10^{-5} and 8.3×10^{-5}, respectively, with cholesteryl oleate as substrate at pH 7.1 (Fig. 6). The inhibition of both enzymes by sodium chloride is noncompetitive as indicated by the Lineweaver-Burk plots for each system. The curves also indicate that enzyme preparation I is more sensitive to the inhibitory effect of sodium chloride than enzyme preparation II although activity of II is 5-fold less than enzyme preparation I. The Km value for the extracellular enzyme III is 10.5×10^{-5} with cholesteryl oleate. This form of the enzyme was unaffected by sodium chloride up to 100 mM concentrations of $[Na^+]$.

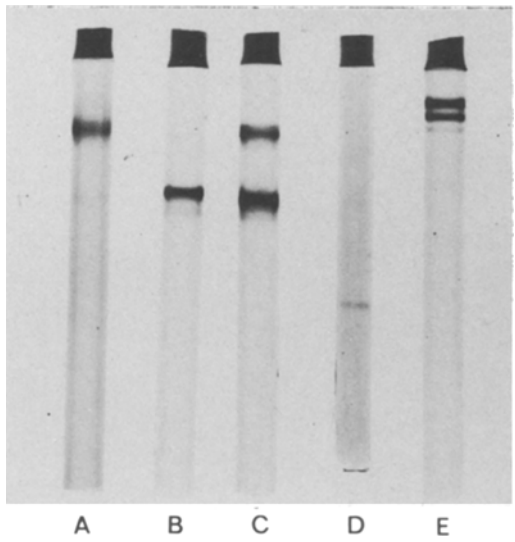


FIG. 5. Acrylamide gel disc electrophoresis of the Sephadex G100 chromatographic fractions of the cytoplasmic and extracellular preparations. The enzyme bands were located according to Szécsi et al. (18) (see Materials and Methods, Figs. 1, 2). (a) Cytoplasmic enzyme I, (b) extracellular enzyme III, (c) enzymes I and III, (d) cytoplasmic enzyme II, (e) commercial microbial enzyme (Boehringer Mannheim Canada Ltd., Quebec).

DISCUSSION

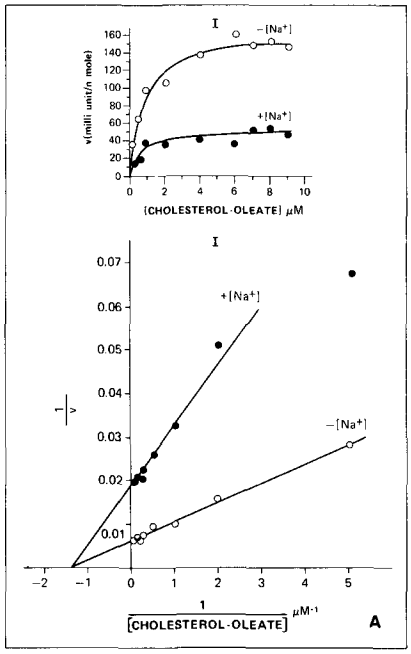
Sterol ester hydrolase from both microbial and animal sources have demonstrated increased activity in the presence of Triton X-100 (2,3,23). The concentration for optimal activity

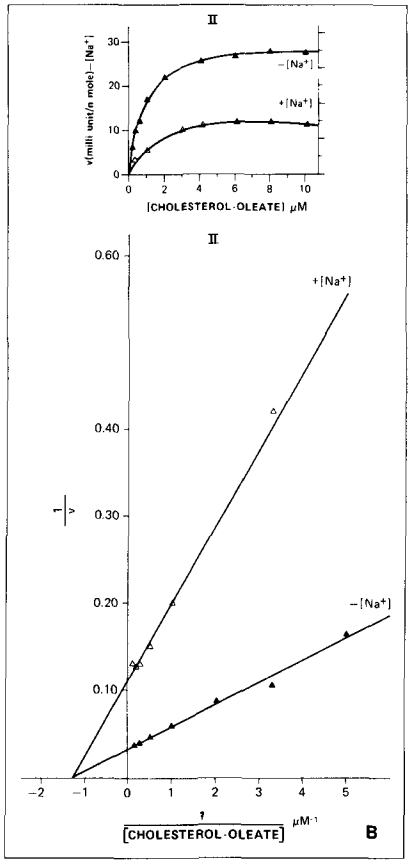
TABLE III

Relative Rates of Enzymic Hydrolysis of Cholesteryl Esters by Cholesteryl Ester Hydrolase Form II[a]

Substrate Cholesteryl ester (10 mM)	Amount (mg)	Cholesterol released (mg)	Cholesteryl ester (residue) (mg)	Total recovery (mg)	Hydrolysis (%)	Hydrolysis relative to oleate (100)
Linoleate	6.6	4.68	1.34	6.02	78.0	252
Oleate	6.4	1.9	4.23	6.13	31.0	100
Valerate	4.8	0.59	3.02	4.51	13.0	42
Butyrate	4.6	0.13	4.27	4.40	3.0	10
Acetate	4.4	0.06	3.95	4.01	1.6	5.2
Benzoate	5.0	0	4.82	4.82	0	0
Palmitate	6.2	0	5.85	5.85	0	0

[a]The rates of hydrolysis of these substrates by forms I and III are not significantly different from those shown here.





ranged from 0.1-0.3% of the assay mixture. It has also been shown that, at concentrations of the compound over 1%, the hydrolase activity was reduced (21). The published data suggest further that the surfactant might also affect the quaternary structure and hence, the hydrolytic function of the enzyme molecule itself. Interesting is that the hydrolases of rat liver (24) and red blood cells (25) are both inhibited by Triton X-100 at concentrations which stimulated other hydrolase activities.

The enzyme has been isolated and purified previously by various methods (1). In this study, the enzyme activity was obtained in the precipitate produced between 75 and 100% ammonium sulfate saturation, suggesting inherent differences in the esterase proteins from that obtained by Hernandez and Chaikoff (26). Sephadex G-75 chromatography of the culture broth of *F. oxysporum* (strain IGH-2) exhibited one active hydrolase peak at the solvent front (3). However, no estimate was made of the molecular weight of the active protein in that study. Chromatography of other enzyme preparations (6,27) also produced single, active peaks. No reports have been obtained showing the electrophoretic separation of microbial sterol ester hydrolase preparations. Animal preparations, however, have always produced single enzyme bands in electrophoresis (21,28). The cytoplasmic preparation from *F. oxysporum* contained 2 distinct enzyme entities I and II whereas the extracellular preparation contained one hydrolase, from III. Because the enzyme preparations obtained form the G100 Sephadex chromatography were not homoge-

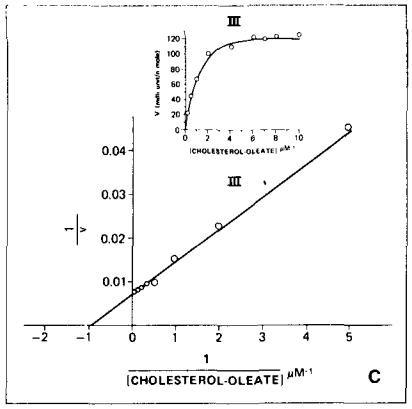


FIG. 6. Lineweaver-Burk plots of the velocity and inhibition by Na^+ (100 mM) of the sterol ester hydrolases I and II and the velocity of form III with the substrate cholesteryl oleate at pH 7.1. The apparent K_m (μM) obtained: I = 7.7×10^{-5}, II = 8.3×10^{-5} and III = 10.5×10^{-5}.

nous but contained, additionally, a number of extraneous nonactive proteins, and because the enzyme activities are destroyed by SDS, it was impractical to employ SDS analytical gel electrophoresis to examine the enzyme proteins. Our study, therefore, cannot ascertain whether the active enzyme entities observed both in Sephadex G100 chromatography and in analytical gel electrophoresis are, indeed, isoenzymes. They could represent separated and aggregated units of the enzyme or enzyme protein associated with nonenzymatic proteins. However, the additional differences in pH optima and inhibition by NaCl also suggest distinct enzyme entities. The molecular weights of 15,000, 60,000 and 75,000 obtained in this study suggest a basic unit and an aggregate, particularly because aggregates and subunits have been observed in preparations from animal sources (29,30).

The pH for optimal hydrolase activities range from 4.3 to 8.6 (1,31). Most of the animal sterol hydrolases appear to have their optimal activities between pH 6.7-pH 7 (1). Two reports (32,33) have described 3 forms of the enzyme from rat with optimal activities at pH 7.2, pH 6.0-6.6 and pH 4.2. It is difficult to compare pH optima accurately since, to a large degree, they are functions of the physical state of the respective substrates. The pH optimum for hydrolase activity obtained by Okawa and Yamaguchi (3), also using cholesterol oleate as substrate, was pH 7—similar to that obtained for the other 2 forms (I and II) in our study.

The specificity of the enzyme to hydrolyze sterol esters of various fatty acids has received much attention, primarily with the animal enzymes. We tend to agree with the argument presented by Brockerhoff and Jensen (1) that no enzyme specificity, as it is normally understood, really exists in this system, but that the differences in rates of hydrolyses observed could be due to the differences in the dispersion properties of the fatty acids and their esters.

It is of interest that the hydrolytic activity was highest with the linoleic acid and oleic acid esters, as these are the predominant fatty acids found in this organism (13) and the compounds most likely to be involved in sterol-fatty acid metabolism. Also, the higher rates of hydrolysis of the unsaturated fatty acid esters in this study possibly result from the relative differences in the physiochemical states of the esters when dispersed in the assay system (34). The pattern of hydrolysis of the various fatty acid esters obtained in this study is similar to that obtained by Okawa and Yamaguchi (3) from an extracellular hydrolase.

The inhibition of the hydrolase enzyme by the monovalent cation Na^+ is not generalized. Recently, Igarashi and Suzuki (29) demonstrated stimulation of the cholesteryl ester hydrolase from the myelin sheath of rats by sodim and potassium cations. However, Patelski et al. (35) obtained significant inhibition of rabbit aortic-wall hydrolase with Na^+, K^+ and Ca^{++} cations. The present study demonstrates the inhibition of a microbial hydrolase by NaCl. Takano (36) has shown that the inhibition of lysosomal hydrolase by $CuCl_2$ was brought about not by the chlorine but by the divalent Cu^{++} component since $CaCl_2$ at the same concentration did not inhibit the enzyme. The mechanism of inhibition by these cations has not yet been elucidated. However, a reasonable speculation, particularly for the heavy metal ions, would be that these ions effect conformational changes on the enzyme or substrate resulting the the noncompetitive type of inhibition which was obtained in this study.

The sulfhydryl reagent PCMB had no inhibitory effect on the activity of the enzyme obtained in this study. Uwajima and Terada (4) obtained similar results with the hydrolase from *Pseudomonas fluorescens*. This is in marked contrast to the inhibition obtained with this reagent in all animal sterol ester hydrolase systems which have been examined (1). The data suggest that the site involved in the catalytic mechanism is not a sulfhydryl group and that structural or mechanistic differences exist between the animal and *F. oxysporum* sterol ester hydrolases. Additional studies are required to determine the significance of these differences in the microbial and animal systems.

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The Sterols and Fatty Acids from Purified Flagella of *Chlamydomonas reinhardi*

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ABSTRACT

Purified flagella of the eukaryotic alga *Chlamydomonas reinhardi* have a sterol composition (55% ergosterol [24β-methylcholesta-5,7,22-*trans*-trien-3β-ol] and 45% 7-dehydroporiferasterol [24β-ethylcholesta-5,7,22-*trans*-trien-3β-ol]) identical to that of the whole algal cell. Fatty acids isolated from *C. reinhardi* flagella were identified as 16:0, 18:0, 18:1, 18:2 and 18:3. Whole cell fatty acids included 14:0, 16:2 and 16:3 in addition to those found in the flagella. Triunsaturates comprised 22.9% of the flagellar fatty acids and 76.4% of those from the whole cell.

INTRODUCTION

Flagella are organelles which are especially amenable to purification because they lie, except for their connecting base, external to the cell body where they function as appendages specialized for cellular motility (1-4). The fact that the membrane, which encloses the flagellar structure, appears to be contiguous with the plasma membrane (5), makes the elucidation of their lipid composition additionally interesting because uncontaminated plasma membrane is extremely difficult to purify. The functional specialization of the flagellar membrane necessary to maintain the required flexibility and fluidity suggests that the lipid composition may, indeed, be different from that of the internal cell membranes.

We report here the results of a comparison of the sterols and fatty acids in the flagella and whole cells of the eukaryotic alga *Chlamydomonas reinhardi*.

MATERIALS AND METHODS

C. reinhardi, strain 137c (mt$^+$), was grown at 26 C in a (reciprocal) shaking water bath. Illumination was on a 12-hr light:12-hr dark regime to synchronize the cells in their cell cycle. The medium used was the modified Sager and Granick medium 2 (6) containing 0.3% sodium acetate as previously described (7). Vegetative cells were used for all experiments.

Purification of Flagella

Flagella were purified by a modification of the method of Witman et al. (1). Cells were concentrated ca. 30-fold in growth medium and then placed into a beaker. The pH was then lowered to 4.6 with acetic acid for 2 min while the cells were vigorously stirred, then the pH was returned to 6.8 with KOH. This "pH shock" resulted in the deflagellation of all cells (ascertained microscopically). These deflagellated cells were then placed into a 50-ml polypropylene tube and underlayered with either a 15 or 20% sucrose solution. The suspension was then centrifuged at 870 × g for 10 min, causing the cells to pellet through the sucrose while leaving the flagella in the upper (growth medium) layer. This upper layer was then removed, underlayered again with sucrose, and centrifuged at 870 × g for 10 min. The upper layer was removed and placed into a 50-ml polycarbonate tube. This suspension appeared turbid because of the high titer of flagella it contained. The flagella were pelleted at 30,000 × g for 60 min at 4 C and then stored at -16 C until use. Flagellar purity was ascertained microscopically as well as by determining particle size with a Coulter Counter TA$_{II}$.

Extraction of Lipids

Whole cells were extracted overnight in the dark with acetone in a Soxhlet apparatus to avoid light-induced degradation of ergosterol and 7-dehydroporiferasterol. The extracted material was saponified in 10% (w/v) KOH in methanol or in 70% methanol/water; the neutral lipids, after saponification (NLAS), were extracted with ether and were further fractionated by alumina chromatography using a hexane/ether gradient (in 10% increments) as eluent.

The flagella were saponified directly with 5% (w/v) KOH in 90% methanol/water. The mixture was refluxed for 15 min in the dark and then allowed to sit overnight in the dark at room temperature. The neutral lipids were extracted with ether and desmethyl sterols purified by thin layer chromatography (TLC) on Silica Gel G using a hexane/ether (9:1, v/v) developing solution (R_f = 0.15 for both the flagellar desmethyl sterols and the desmethyl sterol standards).

Fatty acids were recovered by acidifying the

basic water mixture with HCl to pH 2, saturating with NaCl, and extracting the water layer (4 times) with ether. This material was backwashed to neutrality and then dried. The fatty acids were converted into their methyl esters by reaction with 10% (w/v) BCl_3 in methanol. The esters were then purified by TLC on Silica Gel G (R_f = 0.60 with hexane/ether [9:1, v/v]) and further separated by argentation TLC on Silica Gel G impregnated with 10% $AgNO_3$ (R_f of the methyl esters: saturates, 0.48; monoenes, 0.19; dienes, 0.14; trienes, 0.08, 0.00 with benzene/hexane [4:5, v/v]).

Analysis of Lipids

Ultraviolet spectrometry was performed using a Perkin-Elmer Model 202 UV-Visible spectrophotometer. Gas liquid chromatographic (GLC) examination of the sterols was on 1% XE-60 on Chromosorb Q (100-120 mesh) at 235 C on a Perkin-Elmer Model 881 or a Hewlett-Packard Model 5840A gas chromatograph. The fatty acid methyl esters were examined on either 12% DEGS (diethylene glycol succinate) or 1% XE-60 at 175 C on either an F&M Model 400 (with thermal conductivity detector) or an F&M Model 700 (flame ionization detector) gas chromatograph. Mass spectral data for the whole cells was obtained for us by Morgan-Schaffer (Montreal, Quebec, Canada). Mass spectral analysis of the flagellar sterols and fatty acids as well as the whole cell fatty acids was performed on a Finnigan Series 4021 gas liquid chromatography-mass spectral (GLC-MS) instrument with a Series 6000 data system. The sterols were examined both by direct probe and following GLC on a 1% XE-60 column. Mass spectra of the fatty acid methyl esters (following argentation TLC) were always obtained following separation by GLC on 1% XE-60. Proton magnetic resonance (PMR) spectra were obtained at 360 MHz at ambient temperature on a Bruker instrument, Model WH360, in $CDCl_3$ with $Si(CH_3)_4$ as internal standard at the NIH sponsored, NMR regional facility at the University of Pennsylvania.

RESULTS

Whole Cell Sterols

C. reinhardi grown mixotrophically to a titer of ca. 1×10^7 cells/ml was collected by centrifugation. In a typical lipid extraction and work-up, 67.2 mg NLAS was obtained from 5.3 g (wet wt) of cells. Preliminary inspection of the NLAS by GLC indicated that it contained ergosterol and 7-dehydroporiferasterol with retention times relative to cholesterol of 1.32 and 1.56, respectively. The presence of the $\Delta^{5,7}$-conjugated bond system was confirmed by ultraviolet spectroscopy. Following alumina chromatography, the sterol material was again examined by UV spectroscopy and absorbance maxima at 270, 282 and 294 nm were noted. Using ϵ_{282} = 12,000, we calculated that 2.0 mg of sterol was recovered (0.04% of original cell's wet wt). Area determinations from the GLC profile indicated that 55% of the sterol mixture was ergosterol and 45% was 7-dehydroporiferasterol. To confirm our identification of the 2 sterols, an aliquot was subjected to electron-impact mass spectrometry (MS). Fragments which were indicative of ergosterol included M^+, 396 (100%); M^+ -CH_3, 381 (6%); M^+ -HOH, 378 (28%); M^+ -CH_3 -HOH, 363 (70%); M^+ -C_3H_5 -HOH, 337 (48%). Fragments indicative of 7-dehydroporiferasterol included M^+, 410 (74%); M^+ -CH_3, 395 (7%); M^+ -HOH, 392 (5%); M^+ -CH_3 -HOH, 377 (69%) and M^+ -C_3H_5 -HOH, 351 (28%). Fragments which were common to both sterols included: M^+ -side chain (SC), 271 (49%); M^+ -HOH -SC, 253 (91%) and M^+ -HOH -SC-C_3H_6, 211 (59%). Because these 2 sterols differ only in the substitution at C-24, their fragmentation patterns upon electron impact are very similar. This allowed the total relative intensities of all the dissimilar peaks from the MS to serve as a quantitative measure of relative abundance. According to this analysis of MS peaks, the mixed sterol sample contained 58% ergosterol and 42% 7-dehydroporiferasterol, in complete agreement with the GLC analysis just given.

Further confirmation of our assignment of the sterol components came from analysis of the PMR spectrum. Peaks common to both ergosterol and 7-dehydroporiferasterol were: C-18 (s), 0.63 ppm and C-19 (s), 0.95 ppm. Peaks solely from ergosterol were: C-21 (d), 1.04 ppm (J = 6 Hz); C-26,27 (d), 0.83 and 0.84 ppm (J = 6,7 Hz); and C-28 (d), 0.92 ppm (J = 6 Hz). Peaks solely from 7-dehydroporiferasterol were: C-21 (d), 1.05 ppm (J = 6 Hz); C-26,27 (d), 0.80 and 0.87 ppm (J = 6,7 Hz); and C-29 (t), 0.85 ppm (J = 6 Hz). The doublet for carbon-28 of ergosterol is characteristic of a β-methyl addition at carbon-24 of the sterol side chain (8) and the triplet for C-29 indicates 7-dehydroporiferasterol. Resonances for C-21, C-26 and 27 are also characteristic of their respective sterol.

Flagellar Purification

Flagella were removed from the cells by a "pH shock" and were purified as outlined in

Methods. Phase-contrast microscopic examination of the purified flagella showed no whole cell contamination. Purity of the flagellar preparation was additionally examined by analysis of the particle size distribution using a Coulter Counter. The purified flagella (Fig. 1, A and B) appeared to have an average diameter of 0.9 μm. This determination, however, does not actually reflect the true diameter of a flagellum because of the tendency for an excised flagellum to "roll up" until it appears almost spherical (when viewed by phase contrast microscopy). Figure 1C shows that the mean diameter of the whole cells was 6 μm. The amount of material in the flagella preparation, which appears to be 6 μm in diameter, was less than 0.1% of the total number of particles.

Sterols of Purified Flagella

The flagella were removed from 1.1×10^{11} cells. Our estimated yield of flagella (based on particle counting) was ca. 60% of the theoretical maximum (which would be 2.2×10^{11} because there are 2 flagella/cell). The wet wt of the flagella was 177 mg. Two sterols exhibiting relative retention times on GLC of 1.34 and 1.56 were identified as ergosterol (55%) and 7-dehydroporiferasterol (45%), respectively. Based on A_{282}, there were 2.0×10^{-6} mol of sterol recovered which corresponds to 805 μg or 0.45% of the initial flagellar wet wt.

The sterol mixture was purified by TLC and analyzed by MS. The presence of ergosterol was indicated by M^+ at 396 (53%); M^+ -HOH, 378 (20%); M^+ -CH_3-HOH, 363 (53%) and M^+ -C_3H_5-HOH, 337 (29%). The 7-dehydroporiferasterol was indicated by M^+ of 410 (31%); M^+ -HOH, 392 (10%); M^+ -CH_3-HOH, 377 (35%) and M^+ -C_3H_5-HOH, 351 (14%). Fragments common to both sterols include: M^+ -SC, 271 (22%); M^+ -SC-HOH, 253 (100%) and M^+ -SC-C_3H_6, 211 (66%). Quantitation of the relative amounts of the sterols using MS peaks indicated 63% of the material was ergosterol and 37% was 7-dehydroporiferasterol.

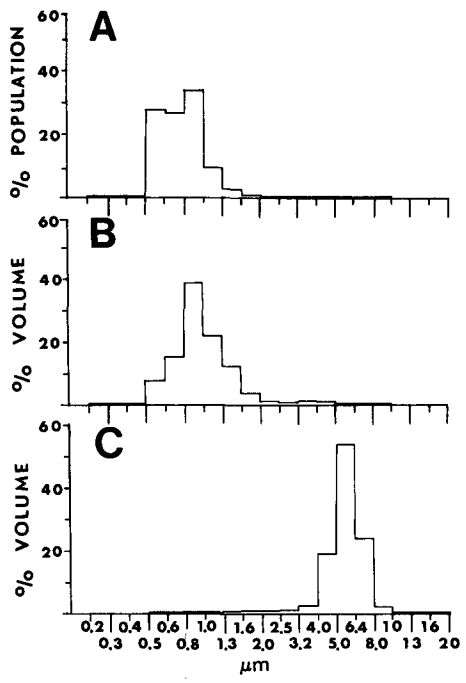

FIG. 1. Size distribution of *C. reinhardi* flagella and whole cells as measured on a Coulter Counter. The percentage of population is the number of particles with a given diameter divided by the total number of particles. The percentage of volume is the volume of particles of a given diameter divided by the total volume of all particles. (A and B) Flagella; (C) whole cells.

Fatty Acids of Whole Cells and Flagella

Although there were no differences between the sterols of the whole cells and those of flagella, there were significant differences in the fatty acid compositions. Fatty acids were identified as their methyl esters by their separation characteristics on argentation TLC and by their retention on GLC relative to known standards. The basic structure and, in many cases, the molecular weight of the fatty acid was confirmed by GLC-MS (Table I).

DISCUSSION

Purified flagella of *C. reinhardi* contain the same sterols, in similar quantities, as the whole cell, yet differ in their fatty acid content. The flagellar sterols as determined by UV, GLC and MS, are ergosterol (55%) and 7-dehydroporiferasterol (45%). A difference was observed in the amount of sterol recovered, 0.04% of the wet wt for the whole cell and 0.45% for the flagella, but this probably is more a reflection of the relatively low water content of the flagellum than of the amount of sterol in the membrane. In another alga, *Ochromonas danica*, all cellular sterols were apparently found in the flagella at approximately cellular concentrations, although the precise quantities for each of the sterols were not published (9). Our use of *C. reinhardi* showed the essentially identical composition of the flagellar and cellular sterols.

This report also confirms the identification of whole cell sterols by Patterson (10) and Eichenberger (11). Bard et al. (12) have reported the finding of considerable amounts of 24-methylcholesta-5,7-dien-3β-ol in addition to

TABLE I

Fatty Acids of *Chlamydomonas reinhardi* Flagella and Whole Cells

Fatty acid	Composition[a]		Mass spectra data after argentation chromatography[b]	
	Flagella	Whole cells	M^+	Base peak
14:0	–[c]	0.7	–[d]	43
16:0	12.8	7.0	270	74
16:1	20.0	1.5	268	55
16:2	–	2.0	266	41
16:3	–	33.0	264	41
18:0	13.8	2.0	298	74
18:1	13.3	5.8	296	55
18:2	17.1	1.5	294	41[e]
18:3	22.9	46.4	292	41

[a]Determined by GLC.

[b]All data is given as the mass-to-charge ratio (m/e). For saturated fatty acids, the base peak should be ca. 74 (14). Under our MS conditions, the monounsaturates yield a base peak of 55 and polyunsaturates, a base peak of 41. The spectra for 14:0 and 18:2 show aberrations primarily due to their weakness.

[c]None detected.

[d]Weak spectrum.

[e]Base peak for whole cells was 67 with 41 @ 94%. Base peak for flagella was 41.

the sterols we described, but we found no evidence for this compound. This difference may be the result of growth conditions, because the strains used appear to be identical.

Although no distinctiveness exists with respect to flagellar sterols, that is not true with the flagellar fatty acids. *Chlamydomonas* flagella lack some fatty acids (14:0, 16:2 and 16:3) found in the remainder of the cell. In the whole cells, 19% of the 16-carbon fatty acids were saturated and only 4% of the 18-carbon fatty acids, whereas in the flagellum, 64% of the 16-carbon and 26% of the 18-carbon material is saturated. There apparently is some regulation of the amount of unsaturated material which the flagella may possess. Because the flagellar membrane is an extension of the plasma membrane, our results suggest that the unsaturated fatty acids are located primarily in other intracellular organelles, e.g., the chloroplast, mitochondrion, or nucleus. Increases in the polyunsaturated fatty acids of a *C. reinhardi* mutant were observed under conditions where the chloroplast thylakoid membrane increased, suggesting that this organelle may have a high requirement for unsaturated fatty acids (13). It is apparent that the flagellar lipid composition is differentially regulated for the sterols and the fatty acids. The mechanism for this regulation, which occurs either during lipid synthesis or during membrane assembly, is currently under investigation.

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Subcellular Distribution of the Cytochrome P-450 Complex and Glutathione Peroxidase Activity in Vitamin E and Essential Fatty Acid Deficiency

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ABSTRACT

Glutathione peroxidase (EC 1.11.1.9.) (GSHPx) and P-450 activity were measured in hepatic mitochondrial and microsomal fractions from rats deficient in vitamin E and/or essential fatty acids (EFA). The data were compared to corresponding normal values. GSHPx was significantly decreased in the mitochondrial matrix from animals in all 3 deficiency states. In vitamin E deficiency, a non-significant decreased GSHPx activity was found in mitochondrial membranes. Opposite to these findings, GSHPx was significantly increased in mitochondrial membranes of EFA-deficient animals. In combined EFA and vitamin E deficiency, the mitochondrial membrane GSHPx activity was only insignificantly increased. The P-450 complex activity was not detectable in the mitochondrial matrix. In mitochondrial membranes and microsomes, the P-450 complex activity changed parallel to the GSHPx activity.

INTRODUCTION

Hrycay and O'Brien (1) proposed that the P-450 complex was responsible for most of the microsomal peroxidase activity. This peroxidase activity is relatively low in hepatic cytosole compared to the activity in mitochondria. Furthermore, in rats deficient in essential fatty acids (EFA) or vitamin E, microsomal mixed-function oxygenase activity and soluble GSHPx activity are decreased (2-4). The aim of this work was to study in further detail the relationship between the P-450 activity and the GSHPx activity in rats given a diet deficient in vitamin E and/or EFA. We included a comparison of the enzyme activities in mitochondria (membranes and matrix) to those of the microsomal fraction.

EXPERIMENTAL PROCEDURES

Animal Experiment

Four different diets were prepared: (A) basal diet (4), (B) diet A without addition of vitamin E, (C) diet B but without purified sunflower oil, and (D) diet A with no addition of purified sunflower oil. Each diet contained 129.0 ± 1.8 ppb Se. One g of the special food was used in the fluorescence assay for selenium described earlier (5). Young (24-day-old) Wistar specific pathogenic-free (SPF) male rats, obtained from Veterinary Doctor Møllegaards Breeding Lab., Ltd., Ejby, Denmark, were placed in cages, to a maximum of 4 animals/cage, and the animals were fed the 4 diets mentioned. Food and tap water were given ad libitum. Six months after initiation of the diets, the animals were anesthetized with ether and 10 ml blood was drawn from the inferior caval vein into 1 ml 0.1 M EDTA. The liver was perfused with 20 ml 1.14 M NaCl to remove residual blood prior to homogenization.

In rats given a vitamin-E-deficient diet, the body and liver weights were decreased 25%. Expressed as g liver/100 g whole body, the liver weights were normal. Spontaneous hemolysis was 80% (4) (normal value 10%). In rats given an EFA-deficient diet, the body weights were 35% less than normal, and the liver weights 30% less than normal. Expressed as g liver/100 g whole body, the liver weights were insignificantly increased. A significantly increased 20:3ω9/20:4ω6-ratio 2.5 was found (normal ratio, 0.01). Spontaneous hemolysis was normal (4). In rats given a combined vitamin E-EFA-deficient diet, the weights of body and whole liver were significantly decreased compared to normals. The value of g liver/100 g whole body was normal. The spontaneous hemolysis was normal (10%). The 20:3ω9/20:4ω6 ratio was 2.6 (4).

Fractionation of Tissue

After homogenization of 3 g wet liver in 21 ml 0.25 M aqueous sucrose solution, cytosol, mitochondria and microsomes were isolated as described earlier (4). Mitochondria suspended in 1 ml 0.25 M aqueous sucrose solution were sonicated for 1 min at 50 W. After adding 11 ml 0.25 M sucrose, centrifugation at 105,000 x g-av was performed at 4 C for 1 hr. The supernatant (matrix) was used for GSHPx assay. The isolated membrane was resuspended in 0.25 M

sucrose and recentrifuged. The precipitated membranes were suspended in 1 ml 0.25 M sucrose and homogenized for 2 min at 350 rpm in a Potter Elvehjem disintegrator to resuspend the precipitant before GSHPx assay. For P-450 complex activity (*p*-nitro-anisole-O-demethylation), the microsomes and whole mitochondria were washed in 0.145 M KCl-16.7 mM Tris HCl buffer, pH 7.5, recentrifuged and finally resuspended in buffered KCl solution (as just described). Before assay, the mitochondria were sonicated 1 min at 50 W.

Assay of hemolysis of erythrocytes and the estimation of the triene:tetraene ratio of the liver mitochondria was used to reveal vitamin E and EFA deficiencies (4).

Enzyme Assays

GSHPx activity was assayed by the method described previously (4) using 73 μmol/ℓ H_2O_2 as peroxide donor. Assay of P-450, i.e., the mixed function oxygenase activity, was performed by a modified method of Konat and Clausen (6). The assay system contained 400 μl Tris-HCl buffer, pH 7.5, (0.125 M) + 50 μl 0.1 M $MgCl_2$ + 50 μl 15 mM NADPH + 250 μl 3 mM *p*-nitroanisole + 100 μl source of enzyme. After incubation for 10 min, 1.2 ml 10% TCA was added and the sample was centrifuged at 11,000 x g-av (4 C) for 10 min; 1,900 μl supernatant were added to 200 μl 8.2 M KOH and the centrifugation was repeated. The supernatant was measured at 420 nm and the activity was expressed as pkat/mg protein (1 kat = 1 mol/sec of *p*-nitrophenolate produced).

The means and standard deviations were computed. Wilcoxon's test was used to determine the significance of the differences among groups of data. Spearman rank test was used for correlation (7). The level of significance was set at 5%. When lines of the best fit were drawn, they were estimated by regression analysis (8).

RESULTS

From the deficient animals, the specific GSHPx activity was significantly decreased ($p \leq 0.01$) in the hepatic mitochondrial matrix (Table I). Thus, in the group deficient in EFA, in vitamin E and in the combined deficiency, GSHPx was decreased 75, 45 and 40%, respectively. The specific GSHPx activity was increased in hepatic mitochondria membranes in EFA deficiency as well as in the combined deficiency group. The increase was 50% ($p \leq 0.05$) and 16% (NS), respectively. Deficiency in vitamin E decreased the GSHPx activity 16% (NS). An inverse relationship was found between the specific GSHPx in mitochondrial matrix and in mitochondrial membranes. Membrane-linked GSHPx accounted for 20% of the total GSHPx in EFA-deficient mitochondria. In normal and vitamin-E-deficient mitochondria, membrane-linked GSHPx was only 3% of the total GSHPx.

Although the major part of GSHPx is present in the cytosol fraction (4), we studied the minor GSHPx activity of the microsomal fraction in order to compare it to the P-450 activity. The specific microsomal GSHPx

TABLE I

Glutathione Peroxidase Activity in Mitochondria Matrix, Mitochondria Membranes and Microsomes from Rats Fed and Raised Either on the Normal Diet or on Diets Deficient in EFA and/or Vitamin E[a]

Diet (group) ($\frac{n}{\text{mean} \pm SD}$)	-Vitamin E (B)	-EFA (D)	-EFA-Vitamin E (C)	Normal (A)
Mitochondria matrix	5 10.8 ± 1.8	6 4.96 ± 1.51	4 12.0 ± 3.5	6 20.4 ± 5.1
Mitochondria membranes	4 0.72 ± 0.23	5 1.33 ± 0.41	4 1.02 ± 0.22	6 0.87 ± 0.42
Membrane activity of total mitochondrial GSHPx activity (%)	(3)	(19)	(7)	(3)
Microsomes	3 0.10 ± 0.03	7 0.04 ± 0.01	5 0.07 ± 0.02	7 0.14 ± 0.05

[a]Unit: nkat/mg protein.

TABLE II

p-Nitroanisole-O-demethylation in Liver Mitochondria and Microsomes from Perfused Rat Livers of Animals Fed and Raised Either on the Normal Diet or on Diets Deficient in EFA and/or Vitamin E[a]

Diet (group) (n / mean ± SD)	-Vitamin E (B)	-EFA (D)	-EFA-Vitamin E (C)	Normal (A)
Mitochondria	8	12	9	15
	3.2 ± 1.4	8.6 ± 2.5	5.6 ± 3.0	4.8 ± 2.0
Microsomes	8	14	9	15
	21.5 ± 8.4	15.0 ± 3.7	16.1 ± 6.8	29.0 ± 10.0

[a]Unit: pkat/mg protein.

activity in vitamin E deficiency was decreased ca. 30% (NS). The activity in the EFA and in the combined deficiency was decreased 40 and 54%, respectively ($p \leq 0.01$). In the group fed the double deficient diet, the strongest decrease was found in the microsomal fraction.

Table II shows P-450, mixed-function oxygenase activity in the mitochondrial and microsomal fractions. The activity in the mitochondrial matrix was not detectable. In the EFA-deficient mitochondrial fraction, *p*-nitroanisole-demethylation was increased 80% ($p \leq 0.05$) compared to normal activity. In animals deficient in both EFA and vitamin E, the increase was 17% (NS). In mitochondria and microsomes from vitamin-E-deficient animals, *p*-nitroanisole demethylation was decreased by 35% (NS). In microsomes from EFA-deficient animals, the activity was decreased 50% ($p \leq 0.01$). In combined deficiency, the decrease was 40% ($p \leq 0.01$). An inverse relationship between *p*-nitroanisole-O-demethylation in mitochondria and in microsomes was found. It is obvious that the demethylation activities of EFA-deficient animals differed significantly from the other groups. EFA deficiency seems to be associated with the lowest microsomal enzyme activities (Figs. 1 and 2).

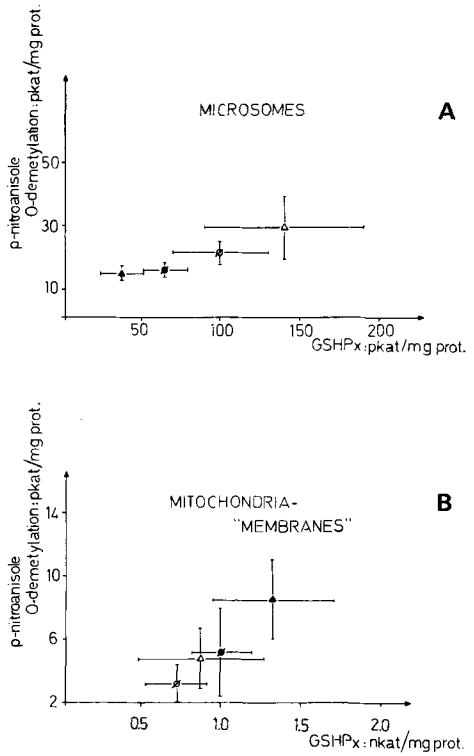

FIG. 1. The relationship between glutathione peroxidase activity and *p*-nitroanisole-O-demethylation in (A) microsomes and (B) mitochondrial membranes from perfused rat livers of normal controls and from rats deficient in EFA and/or vitamin E. Ordinate (Y): *p*-nitroanisole-O-demethylation (pkat/mg protein of rat liver microsomes or mitochondria); abscissa (X): glutathione peroxidase activity (pkat/mg protein of rat liver microsomes and nkat/mg protein of rat liver mitochondria membranes, respectively). △ = Mean ± SD, normal rats; ▲ - mean ± SD, essential vitamin-E-deficient rats; ∮ = mean ± SD, combined deficiency of EFA and vitamin E in rats.

DISCUSSION

Vitamin E is an antioxidant (9) and, therefore, in vitamin E deficiency the amounts of free radicals and hydroperoxides increase. These changes may be counteracted by GSHPx; e.g., during ozone exposure for 5-7 hr, the lipid peroxidation as well as the GSHPx activity increase (10). Thus, the enzyme level may adapt to the hydroperoxide level. However, vitamin E deficiency in rats for 26 days was

associated with normal liver cytosol GSHPx activity (11). For longer deficiency periods, i.e., the 4-6 months used here, a decrease in erythrocyte and hepatic cytosol GSHPx was traced (4,12). Furthermore, in vitamin E deficiency GSHPx activity decreased in the mitochondrial matrix ($p \leq 0.05$) and membranes (NS) and in the microsomal fraction. P-450 activity was also decreased in these fractions. Naito et al. (13) and Hochstein and Ernster (14) found inactivation of enzymes in the endoplasmatic reticulum and in mitochondria as a result of in vitro lipid peroxidation. The decrease in P-450 and GSHPx activity found in the long-term experiments such as those used here may be explained by a destruction of the proteins due to formation of toxic free radicals and hydroperoxides (15) or by a changed selenium metabolism (16). Marker-enzyme studies (4) and recovery experiments have shown that, in EFA-deficient animals, the P-450 complex activity in the mitochondria fraction cannot be accounted for by microsomal contamination. It is a possibility that the adaptation of GSHPx to the peroxide level may explain the low GSHPx activity present in microsomes and in the soluble fractions of mitochondria from the EFA-deficient animals. Multiple forms of GSHPx activity exist. Thus, besides the soluble fraction, membrane-linked GSHPx exists. This study demonstrated both enzyme activities. Furthermore, Lawrence and Burk (17) were able to separate the soluble liver GSHPx into 2 fractions, of which only one contained selenium. Recent, unpublished studies have shown that EFA and vitamin E deficiency are associated with a significant decrease in the selenium-rich GSHPx. The presence of membrane-bound as well as soluble peroxidase activity may belong to different pools with different affinities for peroxides. Thus, Hrycay and O'Brien (1) indicated that the decomposition of hydrogen peroxides results from cytochromes present in mitochondria and microsomes. Different affinities for substrates in different pools are further supported by the finding that the peroxidase assayed by means of artifical substrates, derived from *p*-phenylene diamine, is primarily membrane-linked (relative peroxidase activity in cytosol to that of microsomes is 1:8 [1]). On the other hand, GSHPx uses the substrate combination $GSH+H_2O_2$ and is primarily associated with the cytosol (4).

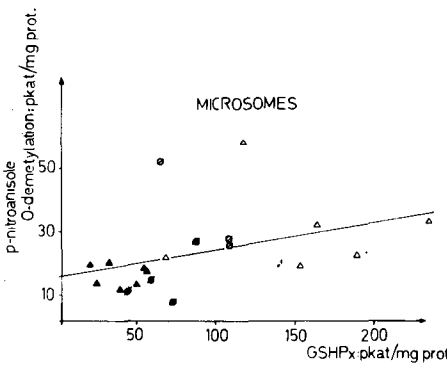

FIG. 2. The relationship between glutathione peroxidase activity and the *p*-nitroanisole-O-demethylation of the microsomal fraction of perfused rat livers of normal controls and from rats deficient in EFA and/or vitamin E. Ordinate (Y): *p*-nitroanisole-O-demethylation (pkat/mg protein of rat liver microsomes); abscissa (X): glutathione peroxidase activity (pkat/mg protein of rat liver microsomes). △ = normal rats; ▲ = essential fatty acids (EFA)-deficient rats; ϕ = vitamin-E-deficient rats; ϕ = combined deficiency of EFA and vitamin E in rats.

P-450 complex may act in vivo both as lipid hydroperoxide-producing complex and as a peroxidase using the produced lipid hydroperoxide as substrate (18). In 1975, O'Brien and Rahimtula isolated the P-450 complex from the membrane-bound enzyme system and from other membrane components and found that the initial rate of hydroperoxide decomposition was similar to the rate of lipid peroxidation (18). Thus, the ability of P-450 to act as a lipoxygenase seems to correlate with its ability to act as a peroxidase. Similar to the GSHPx, the P-450 complex peroxidase may adapt to higher hydroperoxide decomposition. Marshall and Naughton (19) suggested that it may be the peroxides that induce the hepatic microsomal cytochrome P-450 activity. In 1974, Paine and McLean (20), working with a liver cell culture system, suggested that the superoxide ion (O_2^-) may be a common intermediate in the induction of benzo(a)-pyren hydroxylase activity (20). Thus, superoxide dismutase connecting O_2^- to H_2O_2 (21) also may participate in the induction of the P-450 complex activity.

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COMMUNICATIONS

Differential Metabolism of Cholesterol and Sitosterol by *Phytophthora cactorum*

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ABSTRACT

When cultures of *Phytophthora cactorum* grown on sterol-free medium were supplemented with a mixture of cholesterol and sitosterol, the sitosterol was initially taken up more rapidly than the cholesterol. Cholesterol was preferentially incorporated into the free sterol fraction and sitosterol into esters. More polar material was formed from sitosterol. Sitosterol is more active than cholesterol in promoting reproduction; this is attributed to the greater amount of polar metabolites formed from it.

INTRODUCTION

Pythium and *Phytophthora* spp. do not synthesize sterols (1-3). Although these fungi will grow on a defined sterol-free medium, such cultures remain vegetative, and addition of sterol to the medium is necessary to bring about sexual reproduction (1).

Sterols differ quantitatively in their ability to promote sexual reproduction. Thus, more oospores are produced with sitosterol than with cholesterol. Activity depends on the size and orientation of the alkyl substituent at C-24 (4,5). In this paper, we report on attempts to see whether this difference in activity of cholesterol and sitosterol is associated with any differences in their metabolism.

METHODS

Experimental details were as given previously (6). Briefly, cultures of *Phytophthora cactorum* growing in liquid sterol-free medium were supplemented with radioactively labeled sterols. Mycelium was subsequently harvested, freeze-dried and extracted in a Soxhlet apparatus, first with acetone and then with chloroform/methanol (2:1). The extracts were evaporated and analyzed by reverse-phase chromatography on hydroxyalkoxypropyl-Sephadex LH-20/1114 using acetone/heptane/water (220:23:5) as solvent; radioactivity in the collected fractions was determined.

RESULTS

In an experiment in which *P. cactorum* was grown on sterol-free medium and then supplemented at 5 days with either cholesterol or sitosterol, it was found that the initial uptake of both sterols was rapid, and the amounts of free sterol and of sterol ester/unit wt of mycelium were maximal at 12 hr. Thereafter, with growth of the fungus, their amounts (per unit wt) declined, but the amount of ester declined much more rapidly than the free sterol. This agrees with our previous results for cholesterol (7). The results for the 2 sterols were very similar, and it was evident that an experiment of very large scale with many replicates would be required to show a significant difference between them. We therefore looked for *selective* metabolism in mycelium fed a mixture of cholesterol and sitosterol.

Table I gives results of an experiment in which cultures 5 days old were supplemented with a mixture of [^{14}C]cholesterol and [^{3}H]-sitosterol; 5 harvests were made after the time intervals indicated. Table II gives results of another experiment in which the cultures were supplemented with a mixture either of [^{3}H]-cholesterol and [^{14}C]sitosterol or of [^{14}C]-cholesterol and [^{3}H]sitosterol; mycelium was harvested after 24 hr. Table II shows the same result with the 2 methods of labeling.

Table I shows that, over the first 8 hr, sitosterol was taken up from the medium more rapidly than cholesterol; there was relatively less sitosterol in the culture filtrate and more in the acetone and chloroform/methanol extracts. Eventually, virtually all of both sterols was taken up.

Chromatography of extracts of mycelium on reverse-phase lipophilic Sephadex resolved the labeled material into 5 zones. Zone I comprised material more polar than free sterol. Zone II was free sterol alcohol and zones III-V

TABLE I

Distribution of Radioactivity in Culture Filtrate and Mycelial Extracts of *P. cactorum* Supplemented with a Mixture of [^{14}C]Cholesterol and [^{3}H]Sitosterol

Time after addition of sterol (hr)		% Material supplied recovered in:			Total recovered	Distribution of material in acetone extract zones					
		Culture filtrate	Acetone extract	Chloroform/methanol extract		I	II	III	IV	V	III+IV+V
1.5	^{14}C	70.0	8.1	2.7	80.0	0.9	81.4	2.5	12.3	3.0	17.8
	^{3}H	64.0	12.1	4.8	81.3	2.0	75.0	3.7	15.7	3.6	23.0
4	^{14}C	54.8	15.8	5.9	76.5	0.8	70.0	3.4	18.2	7.5	29.1
	^{3}H	48.9	21.0	8.7	78.6	1.6	65.3	5.0	24.6	8.5	38.1
8	^{14}C	39.4	27.3	5.5	72.2	1.0	70.4	3.0	16.8	8.7	28.5
	^{3}H	33.3	35.7	9.1	78.1	1.3	65.8	4.5	21.6	6.8	32.9
24	^{14}C	15.2	40.4	8.7	64.3	1.1	62.6	2.3	14.0	20.0	36.3
	^{3}H	14.7	52.1	12.4	79.2	4.6	55.0	2.9	18.1	19.3	40.3
48	^{14}C	0.7	42.7	16.9	60.3	1.6	74.5	2.1	12.3	9.5	23.9
	^{3}H	1.2	49.6	23.7	74.5	3.1	67.7	2.6	15.5	11.2	29.3

Each culture (flask with 10 ml medium) received ca. 50 μg cholesterol (122,000 cpm ^{14}C) and 50 μg sitosterol (496,000 cpm ^{3}H) in 1 ml 1% aqueous Tween 80 5 days after inoculation. Five harvests made at indicated times after addition of sterols: 1.5 hr, 27 flasks; 4 hr, 16 flasks; 8 hr, 9 flasks; 24 hr, 5 flasks; 48 hr, 4 flasks. Results given per flask.

Acetone extracts analyzed by reverse-phase chromatography; zone I, polar material; zone II, free sterol; zone III, steryl linolenate; zone IV, linoleate; zone V, oleate. The distribution of material between zones is shown as % material in acetone extract.

were sterol esters. Zone III was predominantly steryl linolenate, zone IV linoleate and zone V oleate (6). The chloroform/methanol extract had less than half the amount of radioactive material present in the acetone extract, but contained relatively more polar material (zone I) and ester (zones III-V) (Table II).

Sitosterol gave rise to more polar material (zone I) than cholesterol. A higher proportion of the cholesterol than of the sitosterol in the extract was the free sterol alcohol (zone II), and a higher proportion of the sitosterol was esterified (Tables I and II).

Changes with time in the distribution of steryl esters among zones III, IV and V were as described previously (6); that is, at the early harvests (Table I: harvests up to 8 hr), zone IV (linoleate) predominated, but at 24 hr zone V (oleate) was predominant. At 8 and 24 hr, the distribution of esters between zones IV and V was different for cholesterol and sitosterol; relatively more of the cholesterol esters were in zone V (Tables I and II).

The differences between cholesterol and sitosterol at each harvest in uptake and between chromatographic zones are based on a substantial number of counts and are statistically highly significant; moreover, differences of the same kind were found in all experiments (Tables I and II and data not shown).

DISCUSSION

Hendrix (8) found that the metabolism of cholesterol and sitosterol added separately to *Phytophthora* and *Pythium* spp. was similar. However, our results establish that, when *P. cactorum* is fed a mixture of cholesterol and sitosterol, the sitosterol is initially taken up more rapidly and is selectively incorporated into the ester and polar fractions; relatively more of the cholesterol appears in the free sterol fraction. Such differential uptake and metabolism of mixtures of sterols may well also occur in nature. For example, *P. infestans*, in its natural habitat of potato leaves, is faced with a mixture of sterols in which sitosterol predominates (9).

We suggest two explanations for the preferential appearance of cholesterol in the free sterol fraction. Cholesterol may pack more readily into phospholipid membranes than sitosterol, and thus, more sitosterol is available for metabolic conversion. Alternatively, sitosterol may be the preferred substrate for sterol esterases. The first hypothesis is in line with the observations of Edwards and Green (10), who found that sitosterol was not so readily incorporated into erythrocyte membranes as choles-

TABLE II

Distribution of Radioactivity in Extracts of Mycelium of *P. cactorum* Supplemented with a Mixture of (A) [^{3}H]Cholesterol and [^{14}C]Sitosterol or (B) [^{14}C]Cholesterol and [^{3}H]Sitosterol

		Relative amounts of radioactivity in zones[a]					
		I	II	III	IV	V	III+IV+V
A: Acetone							
[^{3}H]Cholesterol	b	1.58	75.90	2.21	9.32	10.99	22.52
[^{14}C]Sitosterol	b	2.86	71.60	2.67	14.10	9.77	26.54
Chloroform/methanol							
[^{3}H]Cholesterol	b	3.29	61.58	2.93	16.17	16.04	35.14
[^{14}C]Sitosterol	b	4.74	52.80	4.38	21.86	16.23	42.47
B: Acetone							
[^{14}C]Cholesterol	b	1.34	85.55	1.20	4.89	7.02	13.11
	c	1.57	100.00	1.40	5.72	8.20	
[^{3}H]Sitosterol	c	3.91	100.00	2.62	11.48	–	
Chloroform/methanol							
[^{14}C]Cholesterol	b	2.41	75.35	2.26	9.68	10.30	22.24
[^{3}H]Sitosterol	b	4.40	69.70	2.81	13.87	9.23	25.91

Each culture flask A received 50 μg cholesterol (280,000 cpm) + 50 μg sitosterol (38,000 ^{14}C); each flask B received 50 μg cholesterol (82,000 cpm ^{14}C) + 50 μg sitosterol (334,000 cpm ^{3}H).

Mycelium harvested 24 hr after addition of sterol to cultures; dried mycelium extracted first with acetone, then with chloroform/methanol (2:1).

[a]Zone I, polar material; zone II, free sterol; zone III, steryl linolenate; zone IV, steryl linoleate; zone V, steryl oleate.

[b,c]In chromatographing the acetone extract B, the last few fractions from the column were lost. It was possible to estimate the amount of cholesterol in zone V, but not of sitosterol which migrates more slowly. The results are therefore given in two forms. Rows b give the amounts of material in the five zones relative to the total recovered; rows c give them relative to the amount of free sterol (zone II).

terol. Suckling et al. (11) found that sterols with longer side chains than cholesterol are less well incorporated into liposomes than either cholesterol itself or sterols with shorter side chains. The degree of order conferred on liposome bilayers, determined by spin labeling, is greater with cholesterol than with sterols with longer or shorter side chains (11,12). Sitosterol has a side chain branched at C-24 but not longer than that of cholesterol. Suckling et al. (11,12) used sterols which had unbranched side chains or which had only a terminal isopropyl group. Other work with spin labeling (13,14) indicated that the degree of order resulting from cholesterol and sitosterol was similar. Nes et al. (15) observed that cholesterol and sitosterol have similar effects on growth rate in *P. cactorum*. They assumed that this was due to similar effects on membrane properties of these sterols.

Differences in side chain structure are known to affect the enzymatic transformation of sterols. For example, 26-norcholesterol is converted to its 7α-hydroxy-derivative by rat liver microsomal cholesterol 7α-hydroxylase less rapidly than is cholesterol, and sitosterol is not detectably affected (16). Whether this has any bearing on the preferential esterification of sitosterol, the fact that sitosterol gives rise to a greater quantity of polar metabolites than does cholesterol (Tables I and II) could well result from sitosterol being the preferred substrate for the enzymes affecting their biosynthesis from free sterol.

It has been argued that reproduction in *Phytophthora* is controlled by steroid hormones (5,17,18) and direct evidence for hormones has been provided by Ko (19,20). It is possible that the polar metabolites (zone I) include precursors of such steroid hormones. At least 10 different compounds derived from sitosterol or cholesterol have been recognized by thin layer chromatography of zone I (Elliott and Knights, unpublished). If the polar metabolites do, indeed, include precursors of hormones, the greater amount of them formed from sitosterol would provide a reason why sitosterol is more active in promoting reproduction.

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Chaulmoogric Acid: Assimilation into the Complex Lipids of Mycobacteria

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ABSTRACT

Lipid analysis of *Mycobacterium vaccae*, grown in the presence of chaulmoogric acid, demonstrates that this cyclopentenyl fatty acid is taken up by the organism and incorporated into cellular phospholipids and triacylglycerols. As cell growth is retarded by the addition of chaulmoogric acid to the growth medium, it is possible that the antimicrobial properties of this compound result from a perturbation of membrane processes.

For centuries, chaulmoogra oil, isolated from the seeds of tropical Flacourtiaceae, has been used widely in an attempt to treat leprosy; however, the efficiency of this treatment has not been precisely demonstrated (1). The major constituents of the plant oil are long-chain cyclopentenyl fatty acids which are esterified to glycerol. Hydnocarpic, chaulmoogric and gorlic acids are the predominant fatty acid species (2). These fatty acids contain an unusual monounsaturated 5-membered ring distal to the carboxylic acid function (Fig. 1). Studies with *Mycobacterium leprae*, the organism which causes human leprosy, have shown that growth of this bacterium is inhibited by either crude chaulmoogra oil or the purified cyclic fatty acids (3). We have confirmed these findings using *Mycobacterium vaccae*. Although the biosynthetic pathway (4-6) and the antimicrobial properties (3,7) of the cyclopentenyl fatty acids have been demonstrated, no evidence exists, at the biochemical level, that offers insight into the mechanism of action of these unique compounds.

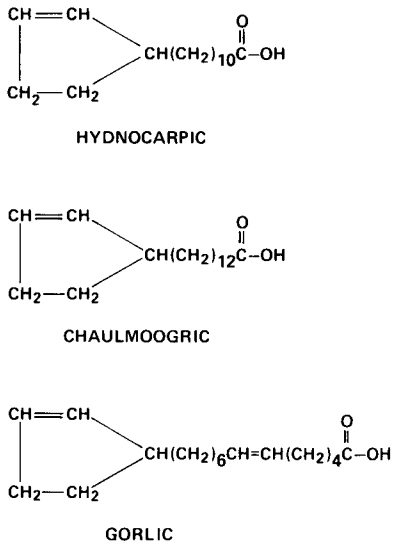

FIG. 1. The chemical structure of the most predominant fatty acids in chaulmoogra oil.

A stimulus to the investigation of the mechanism of action of these acids has been recent research in the area of membrane modifications via membrane lipid alteration. Fatty acids (8-10) and chemically modified amino alcohols (11-13) analogous to the common phospholipid polar base groups, choline and ethanolamine, when supplied to culture media, are incorporated into the complex lipid components of biomembranes. Membrane modification also has been accomplished in other organisms including protazoans, yeast and bacteria (14-18). By altering the native membrane lipid composition such that the matrix phospholipids no longer contain the "natural" complement of esterified fatty acids and polar head groups, changes in the physiochemical properties of cellular membranes (transport, membrane-bound enzyme activity, protein-lipid interactions) can occur.

The first report that described the uptake and subsequent biosynthetic incorporation of cyclopentenyl fatty acids into membrane phospholipids was done with L-M cell monolayer cultures (8). Cyclic fatty acid incorporation initiated a marked inhibition of L-M cell growth similar to that described for numerous strains of mycobacteria (7). In our study, analysis of chaulmoogric acid incorporation was made using the organism *M. vaccae*. This bacterium is antigenically similar to *M. leprae* and its growth rate was inhibited by chaulmoogric acid.

M. vaccae (TMC #1526, Lot 9A-691) were maintained on slants of Lowenstein-Jensen medium. Broth starter cultures prepared from these slants were grown for 5-7 days and used

as inocula for the growth of *M. vaccae*. The organisms were grown in 200 ml of Dubos albumin broth (Difco Laboratories) in a 500-ml Erlenmeyer flask at 30 C. To study the incorporation of chaulmoogric acid, sedimented cells from stock 200-ml cultures were suspended in growth medium, and the appropriate amount of chaulmoogric acid dissolved in absolute ethanol (3.0 mg/ml) was then added to experimental flasks. Ethanol alone was added to control cultures. The cultures were shaken gently at 30 C. Cells were harvested by centrifugation, washed 3 times in potassium phosphate buffer, 0.01 M, pH 7.2, and the total lipids were extracted by the method of Bligh and Dyer (19), modified with the methanol containing 2% glacial acetic acid. Resolution of total cellular lipids into phospholipids, diacylglycerols, free fatty acids and triacylgylcerols by thin-layer chromatography (TLC) and the gas chromatographic (GC) analyses of fatty acid methyl esters were done as previously described (8). In the event of nonspecific binding of chaulmoogric acid to washed cells, TLC of total lipids ensures that the phospholipid and triacylglycerol fatty acids identified by GC were formerly intact acyl moieties of these glycerolipids.

GC analysis of the methyl esters derived from the lipids of *M. vaccae*, grown with chaulmoogric acid, revealed the presence of a peak that was not demonstrable in the bacterial lipids from cells grown in chaulmoogric-acid-free media (Fig. 2). Identification of this unknown peak was accomplished by comparing the retention time to that of methyl chaulmoograte, prepared by methylation of chaulmoogric acid (8). The retention times of the unknown methyl ester, termed 18:1 cyclic (Fig. 2), and methyl chaulmoograte were identical. Furthermore, when a known quantity of cyclic methyl ester standard was cochromatographed with a mixture of bacterial lipids from experimental cultures, the 18:1 cyclic peak area increased in an additive manner. As seen in the chromatograms of Figure 2, chaulmoogric acid was incorporated into both the triacylglycerols (A, lower) and phospholipids (B, lower) of the bacteria and accounted for 15 and 7% of the total fatty acids in these lipid classes, respectively.

Similar experiments were done using cultures containing variable amounts of chaulmoogric acid ranging between 3 and 30 μg/ml culture medium. Inhibition of bacterial growth by chaulmoogric acid supplementation is documented by the data shown in Table I. Growth was inhibited by all amounts of chaulmoogric acid tested, an inhibition that became more pronounced with increased incubation time. Analysis of cellular triacylglycerols and phospholipids revealed an increase in the amount of esterified chaulmoogric acid relative to the amount of exogenous fatty acid supplemented. In addition, when biotin (30 μg/ml) is added to cultures together with chaulmoogric acid (30 μg/ml) and incubated for 20 hr, an enhanced esterification of chaulmoogric acid occurs. This effect was most predominant in the cellular triacylglycerols, which contained 22% of the total acyl groups as chaulmoograte.

"Chaulmoogra oil" has been used for the treatment of leprosy (1). The incorporation of cyclopentenyl fatty acid into the complex lipid

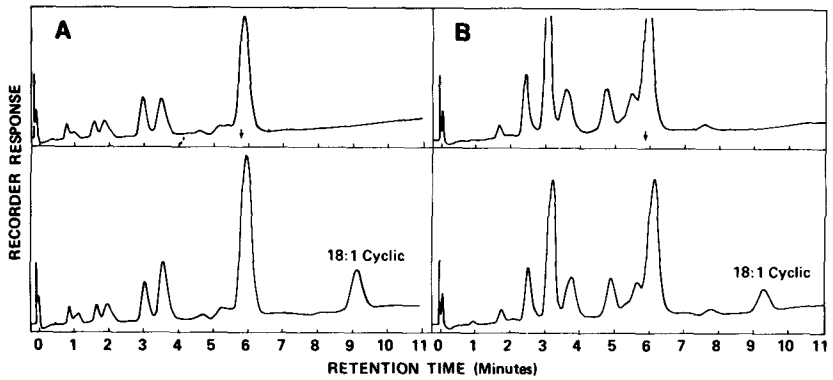

FIG. 2. Gas chromatograms of fatty acid methyl esters prepared from triacylglycerols (A) and phospholipids (B) from *M. vaccae*. Cells (4.13 mg protein) were incubated at 30 C in 100 ml of Dubos-albumin medium for 24 hr in the absence of chaulmoogric acid (upper) or cells (5.84 mg protein) were incubated in the presence of chaulmoogric acid (30 μg/ml, lower). The organisms were frozen for ca. 1 week before lipid extraction. The position of the arrow indicates the retention time of methyl oleate (18:1).

TABLE I

The Effect of Chaulmoogric Acid on the Growth of *M. vaccae*

Incubation time (days)	Chaulmoogric acid (μg/ml medium)				
	0	3	7.5	15	30
2	.08	.08	.09	.05	.05
3	.22	.21	.11	.12	.10
6	.39	.35	.22	.20	.12
12	.68	.54	.41	.37	.32

Data based on absorbance at 600 nm.

molecules of *M. vaccae*, as demonstrated here, may be one means by which these compounds inhibit bacterial growth. Interesting in this respect is that oleic acid, a straight chain monoene having the same carbon number as chaulmoogric acid, has been shown to stimulate the growth of Mycobacteria at levels as high as 100 μg/ml (20). An alteration of membrane properties brought about by the integration of cyclopentenyl fatty acids is a reasonable hypothesis that could explain the antimicrobial action of these natural plant constituents. Although the catabolism of cyclopentenyl fatty acids, either by prokaryotic or eukaryotic cells, has not been investigated, the end product of chaulmoogric acid oxidation, assuming the ring is not further oxidized, would be (2-cyclopentenyl) carboxylic acid, commonly termed aleprolic acid. These short chain cyclic acids have been synthesized recently (21) and could also be effective antimetabolites. Cyclopentenyl fatty acids and their metabolic products should be investigated further as metabolic inhibitors useful as antibacterial drugs.

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A Lysosomal Enzyme Involved in Diphosphatidylglycerol Degradation

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ABSTRACT

A soluble lysosomal phosphodiesterase in rat liver that hydrolyzes monoacylglycerophosphorylglycerophosphorylglycerol (AGPGPGase) was shown to be distinct from a lysosomal acid phosphodiesterase IV (PDase IV) which catalyzes the hydrolysis of bis(*p*-nitrophenyl) phosphate. The criteria used to distinguish lysosomal AGPGPGase from PDase IV were: separation on ion exchange celluloses, dissimilar inhibition patterns and different rates of inactivation on concentration. The lysosomal PDase IV activity was competitively inhibited by inorganic phosphate with a K_i value of 0.33 mM phosphate and was inhibited by a number of organophosphoryl compounds including AGPGPG, phosphatidylcholine, phosphatidylinositol, ATP and 4-methylumbelliferylpyrophosphate.

ABBREVIATIONS

AGPGPGase, monoacylglycerophosphorylglycerophosphorylglycerol phosphodiesterase; PDase IV, phosphodiesterase IV; K_i, dissociation constant of enzyme-inhibitor complex; DLPG, di(lysophosphatidyl)glycerol; GPGPG, glycerophosphorylglycerophosphorylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; LPG, lysophosphatidylglycerol; GPC, glycerophosphorylcholine; PG, phosphatidylglycerol.

INTRODUCTION

Among the major intermediates in the catabolism of diphosphatidylglycerol (cardiolipin) by rat liver lysosomes are the acylglycerophosphorylglycerophosphorylglycerols (AGPGPG) (1). These compounds are cleaved by a phosphodiesterase (AGPGPGase) which hydrolyzes AGPGPG to yield lysophosphatidylglycerol (LPG) and α-glycerophosphate. Genetic defects in cardiolipin catabolism may contribute to lysosomal storage diseases, including some types of Niemann-Pick disease. One form of this syndrome is a severe congenital lipidosis that is associated with a lack of the enzyme sphingomyelinase (2-4). Variant forms of the disease exhibit only partial or isoenzymic deficiencies of this enzyme and the major metabolic lesions are less well defined (5,6). It has been proposed that the lysosomal synthesis of bis(monoacylglycero)phosphate (or lysobisphosphatidic acid) in mammalian tissues may use cardiolipin degradation products such as LPG (7,8). The accumulation of bis(monoacylglycero)phosphate that is seen in patients of both severe and variant types of Niemann-Pick disease could therefore be related to increased cardiolipin catabolism (9,10). The synthetic substrate bis(*p*-nitrophenyl)phosphate has been used to monitor phosphodiesterase IV activity (PDase IV releasing *p*-nitrophenol and *p*-nitrophenylphosphate) (11) and this activity is also deficient in both severe and variant forms of Niemann-Pick disease (12).

This study was undertaken to determine if PDase IV and AGPGPGase activities are catalyzed by the same enzyme. The aim was to determine if the simpler PDase IV assay could be used to measure AGPGPGase activity, for which current specific methods are laborious and expensive. The results indicate that the 2 enzyme activities are separate and that PDase IV assays should not be used to monitor AGPGPGase activity.

MATERIALS AND METHODS

Bovine heart cardiolipin and bis(*p*-nitrophenyl)phosphate were purchased from Sigma Chemical Co., St. Louis, MO. [^{32}P] Orthophosphate was purchased from Amersham, Oakville, Ontario and from New England Nuclear, Boston, MA.

[^{32}P] Cardiolipin was prepared by injecting 200-500 g Wistar rats intraperitoneally with 20-50 mCi of [^{32}P] orthophosphate (carrier-free, pH 7). After 72-96 hr, the cardiolipin from liver, heart, lungs and kidneys was extracted as described by Courtade and McKibbin (13). Cardiolipin was separated from the other phospholipids by column chromatography through silica (mesh 40-140), eluted with a 1-ℓ linear gradient of 2-50% (v/v) methanol in benzene.

[^{32}P] Di(lysophosphatidyl)glycerol (DLPG) was prepared from [^{32}P] cardiolipin by incubation with snake venom phospholipase A_2 of

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Crotalus adamanteus and of *Naja naja* as described by Okuyama and Nojima (14). [^{32}P]-AGPGPG was prepared by repeated mild alkaline methanolysis of this [^{32}P] DLPG with 0.02 M sodium hydroxide in methanol. The neutralized [^{32}P] AGPGPG substrate was purified by preparative TLC on Silica Gel G eluted with $CHCl_3$/MeOH/2M NH_4OH (50:40:10). AGPGPG as well as its water-soluble degradation products were identified with thin layer chromatography (TLC) by comparison of R_f values and by cochromatography in at least 2 different eluting solvents with standards prepared by complete or partial mild alkaline methanolysis or by snake venom phospholipase A_2 digestion of PG and cardiolipin. The 1-5 μmol yields of [^{32}P] AGPGPG (460 μCi/mmol)/rat were assumed to be acylated at the C-1 position of glycerol on either end of the GPGPG residue because the snake venoms were specific for C-2 acyl esters of glycerol (15) and because the 1-acyl products are stable (16).

Rat liver lysosomes, containing Triton WR-1339, were prepared by Trouet's method (17) as modified by Leighton et al. (18). The lysosomal pellets, in 5 ml distilled water, were freeze-thawed 10 times and then were prepared for ion-exchange chromatography by dialysis against buffer followed by centrifugation for 1 hr at 150,000 × g (5 C) to remove membranes. Tris buffer (10 mM pH 7.2) was used for DEAE-cellulose whereas acetate buffer (10 mM pH 5.2) was used for CM-cellulose preparations. About 18-35 mg of lysosomal protein was adsorbed onto 50 g of ion-exchange gel and eluted with a 600 ml linear gradient of 0-0.35 M NaCl in buffer. The amount of protein present was determined by the method of Lowry et al. (19).

Phosphodiesterase IV activity was calculated from the difference between bis(*p*-nitrophenyl)-phosphate hydrolysis (PDase IV) and *p*-nitrophenylphosphate hydrolysis (phosphatases) activities as described by Brightwell and Tappel (11). Incubations of 1-3 hr at 37 C (linear up to 3 hr) were done with 0.23 ml vol containing 150 mM acetate buffer (pH 5.2), 4.5 mM substrate and 0.1 ml of ion-exchange fraction or 5.2 μg of enzyme protein. Phosphate liberated from *p*-nitrophenylphosphate was measured as described by Atkinson et al. (20).

AGPGPGase activity was determined by following the release of [^{32}P] label into the following aqueous methanol-soluble degradation products: AGPGPG, GPG, α-glycerophosphate and inorganic phosphate. The enzyme activity was calculated from the amount of labeled α-glycerophosphate and its degradation product, inorganic phosphate, as a percentage of total label introduced. Ten to 40 μl of ion-exchange fractions or 5.2 μg of enzyme protein were shaken for 2-22 hr at 37 C in 50 μl total vol containing 0.15 mM cold AGPGPG (from beef heart cardiolipin with 1,000-5,000 cpm of [^{32}P] AGPGPG in 50 mM 2(N-morpholino)-ethane sulfonic acid (MES) buffer (pH 6.0) or acetate buffer (pH 5.2).

After stopping the reaction by Bligh and Dyer extraction of the lipid (21), aliquots of the upper aqueous layer were analyzed by TLC on Silica Gel G plates eluted with isopropanol/water/2 M ammonium hydroxide (7:2:1). Radioactive spots were visualized by scanning plates with a Panax RTLS-1A thin-layer scanner and appropriate spots were scraped into vials containing 0.5 ml of water. Radioactivity was determined in 5 ml of Anderson's solution (22) using a Beckman LS-7000 liquid scintillation counter. Disappearance of label from the AGPGPG spot correlated well with the combined appearance of labeled inorganic phosphate and α-glycerophosphate. Overall recoveries of radioactivity ranged from 78 to 98%.

In all experiments, the extent of nonenzymic hydrolysis of AGPGPG was monitored and was subtracted from enzymic hydrolysis. It was always in the range 0.5-5% hydrolysis. No significant production of GPGPG was observed under any conditions, even at long incubation times. This confirms previous findings that the deacylation of AGPGPG to GPGPG is not significant (1). It also excludes the possibility that AGPGPG is hydrolyzed by a lysophospholipase to GPGPG and free fatty acid followed by phosphodiesterase hydrolysis of GPGPG to give GPG and α-glycerophosphate. Maximal AGPGPG hydrolysis observed was 30%, and AGPGPGase activity showed constant initial rates for up to 3 hr of hydrolysis under these conditions.

RESULTS AND DISCUSSION

Ion-exchange chromatography, on DEAE- and CM-cellulose, of the soluble enzymes of rat liver lysosomes revealed one major and 2 minor PDase IV activities, as well as one AGPGPGase activity (Fig. 1). In the CM-cellulose column, there was considerable activity of both enzymes in the void volume fractions, probably a result of overloading the column. The AGPGPGase activity had a pH optimum of 5.0-5.2 whereas the major PDase IV peak (DEAE-purified) had 3 distinct pH optima: 5.2, 6.0 and 7.2 with specific activities of 30, 35 and 34 nmol substrate hydrolyzed min^{-1} (mg protein)$^{-1}$, respectively. Dialysis against water increased PDase IV specific activities at each pH

optimum by close to 3-fold. Although ion-exchange chromatography did not completely separate the 2 lysosomal phosphodiesterases from each other or from a phosphomonoesterase, acid phosphatase (not shown), there appeared to be sufficient difference in elution patterns to indicate different enzymes for AGPGPGase and PDase IV.

Inhibition studies of DEAE-purified AGPGPGase and PDase IV were performed (Table I). Tartrate, an inhibitor of many acid phosphatases, inhibits PDase IV but stimulates AGPGPGase activity. An oxidizing agent, ferric chloride, inhibited the AGPGPGase while having little effect on PDase IV. The stimulatory effect of EDTA on AGPGPGase may result from the removal of oxidizing cations, because dithiothreitol and cystine also have some positive effect. Cystine also inhibited AGPGPGase and this suggests that disulfide bridges may be involved in the protein structure. Phosphate was inhibitory for both activities as seen for other phosphatases. The overall pattern of activation or inhibition points to a different protein for each phosphodiesterase.

When Tris buffer was removed by dialysis from the appropriate DEAE-cellulose fractions and these were then concentrated either 7-fold by dialysis against polyethylene glycol (MW 20,000) or 20-fold by lyophilization, a specific loss of AGPGPGase activity was recorded. Control values before concentration of these dialyzed fractions were 160 μg protein ml^{-1}, 567 nmol hr^{-1} ml^{-1} for PDase IV and 12.9 nmol hr^{-1} ml^{-1} for AGPGPGase. The average recoveries of protein, PDase IV and AGPGPGase were 104, 73 and 1%, respectively, after concentration by dialysis and 89, 94 and 5%, respectively, after concentration by lyophilization. The simultaneous loss of AGPGPGase activity and retention of PDase IV activity again supports the proposal of a separate enzyme for the 2 activities.

Kinetic analysis of DEAE-purified PDase IV showed a Km of 0.2 mM bis(*p*-nitrophenyl)-phosphate for the major activity. A previous determination for rat liver lysosomal PDase IV gave a Km of 0.1 mM (11). No Km was obtained for the AGPGPGase activity because not enough substrate was available. The inhibition of PDase IV activity by inorganic phosphate was competitive as shown using the Cornish-Bowden kinetic analysis (23), with a Ki of 0.33 mM phosphate.

The nature of the in vivo substrates for the enzyme responsible for PDase IV activity in rat liver lysosomes remains unclear. Competitive inhibition of this activity by various organophosphoryl compounds was used to test

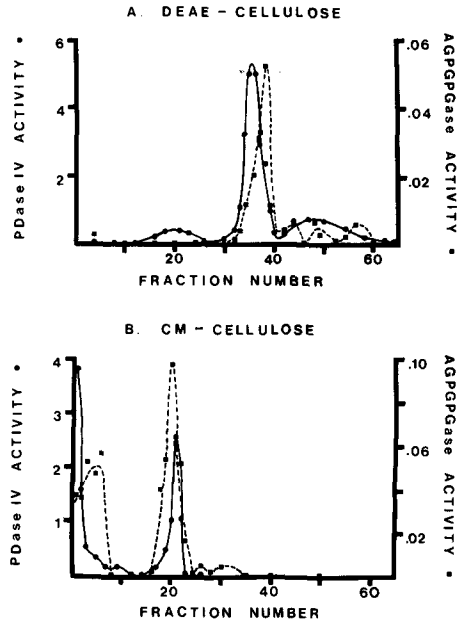

FIG. 1. Chromatography of soluble lysosomal enzymes on DEAE-cellulose pH 7.2 (24.2 mg protein) and CM-cellulose pH 5.25 (10.2 mg protein). Profile A involves PDase IV samples (•) incubated for 2 hr and AGPGPGase samples (■) of 0.03 ml enzyme solution incubated for 13 hr at pH 4.5. The chromatography was carried out 3 times and a typical profile is shown. Profile B involves PDase IV samples (•) incubated for 1 hr and AGPGPGase samples (■) of 0.04 ml enzyme solution incubated for 22.5 hr at pH 4.5. Other incubation conditions were as given in Materials and Methods. All enzyme activities were assayed at least twice and are given as nmol substrate hydrolyzed min^{-1} (ml enzyme solution)$^{-1}$.

the relative affinities of potential substrates for the enzyme active site (Table II). Substances which inhibited more than inorganic phosphate included ATP, dioctanoyl-PC, PI, AGPGPG and 4-methylumbelliferylpyrophosphate. It would seem that, due to binding with a number of organophosphates, lysosomal PDase IV is relatively nonspecific.

Inhibition of PDase IV by PC derivatives seems to require that these be in true solution or at least free of any bulky hydrophobic groups such as fatty acids. For example, micellar PC and LPC stimulate PDase IV activity whereas soluble GPC inhibits it. In a series of synthetic PC with saturated fatty acids of increasing chain length, PDase IV activity is inhibited at short chain lengths and activated by longer chain acyl compounds.

This study indicates that the rapid but nonspecific PDase IV assay cannot be used to deter-

TABLE I

Modifiers of Phosphodiesterase Activities in DEAE-Purified Soluble Lysosomal Enzymes of Rat Liver

Modifier	AGPGPGase[a] (% control activity)	PDase IV[b] (% control activity)
Control	100 ± 16	100 ± 3
Tartrate (2 mM)	128	82 ± 13
Dithiothreitol (2 mM)	162	123 ± 19
EDTA (2 mM)	159	123 ± 2
$CaCl_2$ (5 mM)	145	103 ± 1
$MgCl_2$ (5 mM)	140	110 ± 9
Phosphate (5 mM)	39	36 ± 1
Phosphate (50 mM)	39	7 ± 0
Cystine (5 mM)	43	94 ± 2
L-Cysteine (20 mM)	116	119 ± 8
$FeCl_3$ (6.5 mM)	38	108 ± 25

[a]AGPGPGase was determined at pH 5.2 with samples of 23 μg protein incubated for 2 hr with 0.30 mM [^{32}P] AGPGPG (5,000 cpm). Control activity was 0.61 nmol min^{-1} (mg protein)$^{-1}$.

[b]PDase IV was determined at pH 5.2 with samples of 48 μg of protein incubated for 2 hr with 3.70 mM substrate and was stopped with 1 ml of 0.1 M NaOH. Control activity was 37.5 nmol min^{-1} (mg protein)$^{-1}$.

TABLE II

Inhibition of PDase IV Activity in DEAE-Purified Soluble Lysosomal Enzymes of Rat Liver

Modifier[a]	Control (%)
Control[b]	100 ± 0
Phosphate	52 ± 0
AGPGPG (0.5 mM)	44
GPGPG (0.3 mM)	123
DLPG (0.5 mM)	169
Cardiolipin	170 ± 15
β-Glycerophosphate	73 ± 4
Glycerol	97 ± 4
PG	167 ± 2
PI	14 ± 0
PS	58 ± 4
Egg yolk PC	103 ± 12
Egg yolk LPC	122 ± 35
GPC	93 ± 2
PC dioctanoyl	49 ± 2
PC dihexanoyl	79 ± 1
PC dimyristoyl	104 ± 2
PC dilauroyl	105 ± 7
PC didecanoyl	116 ± 19
PC dipalmitoyl	130 ± 21
PC distearoyl	164 ± 1
p-Nitrophenylphosphate	79 ± 3
4-Methylumbelliferylpyrophosphate	6 ± 1
ATP	39 ± 6

[a]All modifiers were 1.7 mM unless given otherwise and were sonicated in 0.2 M acetate buffer (pH 5.2) before addition to reaction.

[b]Control activity was 24.9 nmol substrate hydrolyzed min^{-1} (mg protein)$^{-1}$ as determined by duplicate incubations, as described previously, for 3 hr with 2 mM bis(p-nitrophenyl)phosphate.

mine AGPGPGase levels in tissue fractions. Further work using the complex lipid substrate will be necessary to determine whether alterations in AGPGPGase levels contribute to the accumulation of bis(monoacylglycero)phosphate in Niemann-Pick disease.

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Influence of Stage of Lactation on the Triacylglycerol Composition of Buffalo Milk Fat[1]

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ABSTRACT

Milk fats obtained from colostrum and early, middle and late lactation samples of buffalo milk were analyzed for their triacylglycerol (TG) compositions. Each milk fat was first separated by thin layer chromatography (TLC) into high, medium and low molecular weight TG. The TG fractions thus obtained were further segregated by argentation TLC, according to their degree of unsaturation into saturated, *trans*-monoene, *cis*-monoene, diene and polyene species. With progressive lactation, the major changes from colostral fat were an increase in lower fatty acids and decline in oleic acid. This caused, in turn, marked variations in saturated TG and diene TG and, to a smaller extent, in polyene TG. Monoene TG, both *cis* and *trans*, remained practically constant throughout. These trends were largely reversed toward the end of lactation.

INTRODUCTION

Many factors influence the composition of milk fat. Any change in the fatty acid composition as a result of season (1) or special feeding procedures (2) has been shown to influence triacylglycerol (TG) composition of the milk fat considerably. Recent studies (3,4) have demonstrated that altered triacylglycerol composition of the fat affects, in turn, its overall physical characteristics.

Fatty acid composition is known to change with the stage of lactation of the cow (5-9). However, no information is available for any species regarding the effect of lactation on triacylglycerol composition of milk fat. The buffalo is the major milch animal in India and certain other Afro-Asian countries. This study was conducted to obtain information on the effect of the stage of lactation on the triacylglycerol composition of milk fat in the buffalo.

MATERIALS AND METHODS

Milk Samples

Milk samples were collected from the Murrah buffalo herd maintained at the National Dairy Institute, India. The animals were grouped into 4 lactation stages: colostrum, first milking after parturition; early lactation, 30-60 days after parturition; middle lactation, 120-150 days after parturition; late lactation, 270-300 days after parturition.

The animals selected were under identical conditions of feeding, age, climate and management. Milk samples were collected from at least 10 animals in each group and pooled separately.

Isolation of Triacylglycerols

The isolation of TG from milk was done as described by Breckenridge and Kuksis (10). The milk was separated by centrifugation and the lipids were extracted with chloroform/methanol (2:1, v/v). Triacylglycerols were purified on a preparative scale by thin layer chromatography (TLC) on Silica Gel G (E. Merck, Darmstadt, W. Germany).

Fractionation of Triacylglycerols According to Molecular Weight

The total TG were first resolved by TLC on the basis of molecular weight (MW) to give TG fractions of simpler composition. About 10-15 mg of TG were applied as a thin band on a thin layer (0.5 mm) of Silica Gel H (E. Merck, Darmstadt, W. Germany). The plates were developed in heptane/isopropyl ether/acetic acid (60:40:4, v/v/v). The TG bands were located by spraying the plate with a 0.05% solution of 2,7-dichlorofluorescein in 50% methanol and viewing under ultraviolet light. The fastest moving band was designated high-MW triacylglycerols (HMT) and the slowest moving band low-MW triacylglycerols (LMT). The faint band between HMT and LMT was designated medium-MW triacylglycerols (MMT). These TG bands were scraped off the plates separately, extracted 3 times with 5% methanol in diethyl ether, and the combined extracts reduced to dryness in a rotary vacuum evaporator. The TG were then dissolved in hexane and washed with water to remove the dye. The relative proportions of these fractions were determined by gas liquid chromatography (GLC) as their constituent methyl esters using methyl heptadecanoate (Applied Science Laboratories, State College,

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PA) as an internal standard.

Fractionation of Triacylglycerols on the Basis of Their Degree of Unsaturation

Each TG fraction obtained as described in the preceding section was resolved into TG classes of differing levels of unsaturation by argentation TLC. About 10 mg of the TG fraction was applied to a thin layer of Silica Gel G (0.5 mm) impregnated with 20% (w/v) silver nitrate. Chloroform/methanol (100:1, v/v) was used as the developing solvent and the TG bands were located using dichlorofluorescein. The TG bands were identified by reference to standard TG and by fatty acid analysis. The HMT, MMT and LMT fractions resolved themselves into 9 TG bands. The fastest moving one was saturated TG, followed by one *trans*-monoene TG, one *cis*-monoene TG, 3 diene TG and 3 polyene TG in order of decreasing mobility. The 3 diene and 3 polyene bands were combined for further analysis, and each TG fraction (HMT, MMT and LMT) thus yielded 5 TG classes (saturated, *trans*-monoene, *cis*-monoene, diene and polyene TG). The TG bands were removed from the plates and extracted 3 times with 5% methanol in diethyl ether. The combined extract of each class of TG was evaporated to dryness under vacuum, redissolved in hexane and washed 4 times with water to remove traces of dye and silver nitrate. The relative proportions of the TG classes were determined by GLC as their constituent methyl esters in the presence of methyl heptadecanoate as an internal standard.

Analysis of Fatty Acids

Fatty acids of TG were analyzed by GLC. The TG were transesterified by deMan's method (11) using absolute methanol containing sodium methoxide in a sealed tube. The chromatographic separations were done on a stainless steel column (5 ft. × ½ in.) packed with 100-120 Gas Chrom-P coated with 10% EGSS-X (Applied Science Laboratories, State College, PA). The sample was injected at an initial temperature of 90 C. After the emergence of 4:0, the oven temperature was programmed to 180 C at the rate of 4 C/min. The temperature of the injection port and detector were 280 and 250 C, respectively. Nitrogen (90 ml/min) was used as the carrier gas. Weight response factors (Fw) for the methyl esters were determined as described by Smith (12) by chromatography of a standard mixture of methyl esters of fatty acids (Applied Science Laboratories). Peak areas were measured by triangulation.

RESULTS AND DISCUSSION

Fatty Acid Compositions of Milk Fats

Fats obtained from buffalo colostrum and from early, middle and late lactation milk samples varied greatly in fatty acid (FA) composition (Table I). Colostrum fat had low proportions of 4:0-10:0 and 16:0 and was exceptionally rich in 18:1. As lactation progressed, 4:0-10:0 and 16:0 increased and 18:1, 18:2 and 18:3 decreased considerably. Early and middle lactation milk fats were almost identical in FA composition. Toward the end of lactation, levels of 4:0-12:0 and 16:0 declined, and those of 18:1, 18:2 and 18:3 increased substantially. Fatty acids that were almost uniform throughout lactation were 14:0 and 18:0. Changes that occurred during late lactation seemed to be mostly a reversal of those which characterized the beginning of lactation.

The increase in 4:0-10:0 and decrease in 18:1 observed in this study as lactation progressed in the buffalo are similar to the trend reported for the cow (5-9). The higher level of 12:0 and lower amount of 16:0 in buffalo colostrum fat are contrary to the findings for cow colostrum fat, but are comparable to the values reported for the buffalo by Anantakrishnan et al. (13).

Fatty acids of milk fat come from 2 sources. The fatty acids comprising C_4 to C_{16} are synthesized from acetate within the mammary

TABLE I

Fatty Acid Compositions of Buffalo Milk Fats of Different Stages of Lactation

Fatty acid	Fatty acid composition (% fatty acids)			
	Stages of lactation			
	Colostrum	Early	Middle	Late
4:0	2.9	5.1	5.4	3.7
6:0	1.8	2.8	3.0	2.2
8:0	1.1	2.2	1.9	1.7
10:0	1.5	3.3	3.2	2.1
10:1	0.1	0.1	0.1	0.2
12:0	3.8	3.2	3.1	2.4
14:0	14.6	13.5	13.7	13.2
14:1	0.8	1.2	0.6	1.5
15:0	0.3	0.5	1.0	1.7
16:0	31.1	35.3	35.4	32.8
16:1	1.2	1.6	1.8	2.3
18:0	9.0	10.4	11.0	11.3
18:1	29.1	18.8	18.0	22.5
18:2	1.5	1.2	1.0	1.3
18:3	1.2	0.8	0.8	1.1
Total saturated fatty acids	66.1	76.3	77.7	71.1
Total unsaturated fatty acids	33.9	23.7	22.3	28.9

gland. Part of the C_{16} and all of the C_{18} acids are taken up by the mammary gland from the circulating lipids of the blood. The high proportion of 18:1 acid and the low levels of short chain acids at the beginning of lactation are attributed to the animal's drawing heavily on body reserves for milk secretion. This is reflected by the decrease in body weight and increase in plasma concentration and mammary uptake of nonesterified fatty acids (14). The reason for the gradual increase during early lactation in the yields of those acids which are synthesized within the mammary gland is unclear. However, Kinsella (15) observed that, between the 18th day before parturition and the 20th day after parturition, there was a 30-fold increase in the rate of fatty acid synthesis by the homogenate of cow mammary tissue. This suggests that some metabolic regulatory factors are involved in the mammary synthesis of fatty acids that are characteristically observed in ruminant milk fat.

The precise mechanism of the chain length termination process is incompletely understood, but is presumably responsible for the presence of short and medium chain fatty acids in ruminant milk fat and their variations under different physiological conditions of the animal. A tissue-specific acyl thioesterase, thioesterase II, has been found to be responsible for the synthesis of medium chain acids in rat mammary glands (16,17). It is unknown whether the mammary tissue of the lactating buffalo contains specific acyl thioesterases and whether their rate of synthesis could account for the characteristic pattern of fatty acids in buffalo milk fat and their variations resulting from the stage of lactation.

Proportions of Triacylglycerol Fractions of Differing Molecular Weight

All 4 buffalo milk fats were separated by TLC into high-MW TG (HMT), medium-MW TG (MMT) and low-MW TG (LMT), and their relative proportions are recorded in Table II. Colostral and late lactation milk fats were characterized by a markedly higher level of HMT, and correspondingly lower proportions of MMT and LMT. Early and middle lactation samples, which were almost identical in FA composition, also carried almost identical proportions of HMT, MMT and LMT. In these 2 milk fats, the proportions of HMT were the lowest; MMT and LMT proportions were the highest among the 4 samples.

The subfractions in Table II can be matched with the FA compositions in Table I. The proportions both of the HMT fraction and 18:1 were highest in colostrum, declined during early and middle lactation, and rose again late in lactation. Because 18:1 is the only higher fatty acid to vary markedly with lactation, the HMT content of milk fat would mostly depend on the proportion of 18:1. In the MMT and LMT fractions, a different trend was seen, and this was connected with the 4:0-10:0 acids. Consistently, the proportions of short chain acids were reflected in the levels of MMT and LMT. In colostrum fat, all were drastically reduced. An increased level of these acids during early and middle lactation elevated the amounts of MMT and LMT, but during late lactation, their proportions declined to parallel the declining level of short chain acids. The association of 4:0 exclusively with the LMT fraction suggests that, of all the short chain acids, it plays a major role in regulating the proportion of this fraction. Glass et al. (18), after examining the milk fats of several species, concluded that only ruminant milk fats could be clearly segregated by TLC into faster moving, long chain TG and slower moving, short chain TG, and this happened only because of the absence of 4:0 in the fast-moving TG and almost exclusive association of this acid with the slower moving TG.

Table III shows the distribution of FA among the 3 TG subfractions. Whatever the stage of lactation, association of FA in these fractions was similar. In the HMT from all 4 milk fat samples, 14:0, 16:0, 18:0 and 18:1, comprised ca. 90% of the total acids. Quantitatively important FA of MMT were 6:0, 14:0, 16:0, 18:0 and 18:1 and in LMT it was 4:0, instead of 6:0. Certain FA showed a tendency to concentrate in a particular fraction: 18:1 in HMT, 6:0, 8:0 and 10:0 in MMT and 4:0 in LMT. Association of 4:0 with LMT, and of 6:0 with MMT, and the absence of 4:0 in HMT were particularly striking. Separation of the TG into 3 subfractions of TLC could therefore be attributed to the association of some fatty acid or acids with specific TG fractions. Also

TABLE II

Proportions of High, Medium and Low Molecular Weight Triacylglycerol Fractions of Buffalo Milk Fats of Different Stages of Lactation

Triacylglycerol fraction (MW)	Stages of lactation			
	Colostrum	Early	Middle	Late
	(wt %)			
High	65.5	42.6	42.4	48.1
Medium	8.6	16.4	17.1	14.5
Low	25.9	41.0	40.5	37.4

TABLE III

Fatty Acid Compositions of High, Medium and Low Molecular Weight Triacylglycerols of Buffalo Milk Fats of Different Stages of Lactation

	Fatty acid composition (% fatty acids)											
	High MW TG				Medium MW TG				Low MW TG			
Fatty acid	Colostrum	Early	Middle	Late	Colostrum	Early	Middle	Late	Colostrum	Early	Middle	Late
4:0	–[a]	–	–	–	1.3	1.0	2.1	1.2	24.2	22.3	23.6	22.7
6:0	0.2	0.3	0.4	0.2	10.4	12.6	12.4	11.2	4.8	6.3	5.9	5.2
8:0	0.2	1.0	0.9	0.6	2.7	6.5	4.0	5.6	1.4	2.2	1.6	1.6
10:0	1.0	3.1	2.8	1.1	2.6	6.5	6.3	4.9	0.8	2.7	2.4	1.9
10:1	–	0.1	–	0.2	–	0.3	0.4	0.3	–	0.1	0.2	0.2
12:0	4.8	3.6	2.8	2.2	5.6	4.8	4.5	3.7	4.3	3.2	3.3	2.3
14:0	13.0	14.2	14.8	13.6	14.2	13.2	13.3	13.0	13.4	14.8	14.2	12.2
14:1	0.4	1.1	1.3	1.4	0.8	0.4	0.8	1.2	0.7	0.8	0.6	0.8
15:0	0.4	1.3	1.4	1.2	0.7	0.6	0.9	1.6	0.9	0.9	0.7	0.9
16:0	32.8	35.6	36.4	33.2	29.0	31.8	32.5	27.6	27.4	31.1	30.8	31.5
16:1	1.0	2.2	1.9	3.0	1.6	1.0	1.0	2.3	1.4	0.9	1.0	1.4
18:0	10.6	11.9	12.6	11.5	6.3	7.5	8.7	7.5	4.3	5.4	6.1	5.1
18:1	32.5	22.8	22.2	28.9	22.8	12.0	11.5	18.1	14.1	8.0	8.8	12.4
18:2	1.8	1.5	1.4	1.6	1.2	1.0	1.0	1.1	1.3	0.8	0.5	1.0
18:3	1.3	1.3	1.1	1.3	0.8	0.8	0.6	0.7	1.0	0.5	0.3	0.8

[a](–) = not detected.

notable is that 14:0 and 16:0 were distributed almost uniformly among all the fractions. Breckenridge and Kuksis (10,19) reported a similar FA distribution pattern in the TG fractions of cow milk fat.

With an increase in the MW of the subfraction, there was an increase in long chain acids, particularly 18:1, and a decrease in short chain acids. This reciprocal occurrence was compounded by molecular association in TG in such a way that MW could serve as the basis of TLC separation of HMT, MMT and LMT. Accordingly, when the content of lower FA was repressed, as in colostrum and late lactation milk fats, the HMT, MMT and LMT from these fats also carried more 18:1 than did the corresponding subfractions from early and middle lactation fats.

Proportions of Triacylglycerol Classes of Different Levels of Unsaturation

The relative proportions of TG classes of differing levels of unsaturation in the HMT, MMT and LMT of the 4 milk fats, as derived by argentation TLC resolution, are presented in Table IV. Tables V, VI and VII show the FA composition of the TG of varying unsaturation derived from the HMT, MMT and LMT of colostrum, middle lactation and late lactation fats. Data for the early lactation sample have been omitted because they closely resembled those for middle lactation fat.

Although saturated TG comprised only 18.2% of the HMT of colostrum fat, compared to 29.0, 30.3 and 26.0% of the HMT of early, middle and late lactation fats, respectively, all had similar FA composition (Table V). The chief FA were 14:0, 16:0 and 18:0 and these would combine to give rise to high-melting TG. Despite the large variations in the relative proportion of saturated TG in the HMT component of the 4 milk fats, their proportions in the total fats were remarkably steady at 11.9, 12.4, 12.9 and 12.5% for colostrum and early, middle and late lactation milk fats, respectively (Table IV). This suggests a role for high-melting TG in regulating the fluidity or mobility of the milk fat as a constituent of milk. In the MMT, on the other hand, while the saturated TG also had similar FA compositions at all 4 stages of lactation (Table VI), the entire range of saturated FA of milk fat were present in fair proportions, suggesting the presence of numerous TG species.

TABLE IV

Proportions of Triacylglycerol Classes of Differing Levels of Unsaturation Prepared from Milk Fats of Different Stages of Lactation

	Proportions of TG Classes							
	Colostrum		Early		Middle		Late	
Triacylglycerols	%F[a]	%M[b]	%F[a]	%M[b]	%F[a]	%M[b]	%F[a]	%M[b]
TG fraction of high MW	100.0	65.5	100.0	42.6	100.0	42.4	100.0	48.1
Saturated TG	18.2	11.9	29.0	12.4	30.3	12.9	26.0	12.5
trans-Monoene TG	7.7	5.0	8.9	3.8	8.8	3.7	5.8	2.8
cis-Monoene TG	31.2	20.4	31.6	13.4	34.4	14.6	33.2	16.0
Diene TG	28.8	18.9	21.8	9.3	19.8	8.4	25.0	12.0
Polyene TG	14.1	9.3	8.7	3.7	6.7	2.8	10.0	4.8
TG fraction of med. MW	100.0	8.6	100.0	16.4	100.0	17.1	100.0	14.5
Saturated TG	34.6	3.0	47.3	7.8	47.1	8.1	36.9	5.4
trans-Monoene TG	3.8	0.3	7.7	1.3	6.8	1.2	6.9	1.0
cis-Monoene TG	33.9	2.9	28.9	4.7	28.3	4.8	32.0	4.6
Diene TG	19.7	1.7	11.7	1.9	11.4	1.9	14.1	2.0
Polyene TG	8.0	0.7	4.4	0.7	6.4	1.1	10.1	1.5
TG fraction of low MW	100.0	25.9	100.0	41.0	100.0	40.5	100.0	37.4
Saturated TG	36.1	9.3	60.1	24.6	61.3	24.8	47.3	17.7
trans-Monoene TG	2.9	0.8	6.6	2.7	5.0	2.0	5.9	2.2
cis-Monoene TG	35.2	9.1	24.5	10.1	24.6	10.0	31.7	11.9
Diene TG	16.6	4.3	6.4	2.6	6.2	2.5	8.7	3.2
Polyene TG	9.2	2.4	2.4	1.0	2.9	1.2	6.4	2.4
Whole milk fat		100.0		100.0		100.0		100.0
Saturated TG		24.2		44.8		45.8		35.6
trans-Monoene TG		6.1		7.8		6.9		6.0
cis-Monoene TG		32.4		28.2		29.4		32.5
Diene TG		24.9		13.8		12.8		17.2
Polyene TG		12.4		5.4		5.1		8.7

[a]Weight percentage in triacylglycerol fraction.

[b]Weight percentage in whole milk fat.

TABLE V

Fatty Acid Compositions (wt % of fatty acids) of Triacylglycerol Classes of Differing Levels of Unsaturation Prepared from High Molecular Weight Fractions of Buffalo Milk Fats of Different Stages of Lactation

	Fatty acid composition (% fatty acids)														
	Saturated TG			*trans*-Monoene TG			*cis*-Monoene TG			Diene TG			Polyene TG		
Fatty acid	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late
4:0	–[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6:0	–	0.9	0.8	0.5	1.7	1.0	0.3	0.3	0.3	0.3	0.6	0.3	0.1	0.4	0.5
8:0	0.5	2.1	1.4	1.0	1.1	1.0	0.8	1.1	1.0	0.5	0.8	0.5	0.5	0.8	0.9
10:0	1.6	5.4	4.0	1.7	2.4	2.5	1.1	2.6	2.8	0.6	2.6	1.6	0.8	1.4	0.7
10:1	–	–	–	0.1	0.1	0.1	0.1	0.1	0.2	–	0.1	0.3	0.2	0.2	0.4
12:0	7.6	6.7	5.8	5.8	3.6	2.5	3.9	3.5	3.0	2.2	2.3	1.7	2.3	2.1	0.8
14:0	26.6	21.8	22.8	17.8	16.6	15.6	17.5	15.7	14.6	8.5	7.4	7.0	9.2	8.2	7.2
14:1	–	–	–	0.5	0.4	1.0	0.4	0.3	0.8	1.6	1.0	1.9	1.2	1.7	1.9
15:0	1.2	2.4	1.7	1.4	1.2	1.3	1.1	0.8	1.3	0.5	1.0	1.2	0.5	0.8	0.8
16:0	43.4	44.3	45.7	23.9	31.9	28.4	29.7	33.8	31.6	20.4	23.1	21.6	16.7	22.5	19.5
16:1	–	–	–	0.9	0.6	0.6	0.8	1.8	2.0	2.1	2.7	2.2	4.7	3.6	4.6
18:0	19.1	16.4	17.8	14.0	13.8	14.2	11.0	11.6	11.2	5.6	7.3	6.5	5.4	8.1	6.3
18:1	–	–	–	32.4	26.6	31.8	33.3	28.4	31.2	57.7	49.5	53.5	45.8	40.7	44.9
18:2	–	–	–	–	–	–	–	–	–	–	1.6	1.7	8.9	7.5	8.0
18:3	–	–	–	–	–	–	–	–	–	–	–	–	3.7	2.0	3.5

[a](–) = not detected.

TABLE VI

Fatty Acid Compositions of Triacylglycerol Classes of Differing Levels of Unsaturation Prepared from Medium Molecular Weight Fractions of Buffalo Milk Fats of Different Stages of Lactation

	Fatty acid composition (% fatty acids)														
	Saturated TG			*trans*-Monoene TG			*cis*-Monoene TG			Diene TG			Polyene TG		
Fatty acid	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late
4:0	2.3	1.9	1.4	1.3	1.6	1.1	2.7	2.0	1.7	1.3	1.6	1.4	1.9	1.2	1.0
6:0	9.6	13.0	12.8	6.9	9.7	9.1	9.5	10.8	11.2	7.4	8.3	7.7	5.8	3.9	3.2
8:0	4.4	4.5	4.8	3.2	5.3	4.6	3.1	5.0	4.5	2.8	4.9	3.8	2.8	2.2	2.0
10:0	5.2	6.3	5.3	3.2	5.0	4.7	2.8	5.5	5.1	2.5	3.8	3.5	2.0	2.8	2.5
10:1	–[a]	–	–	0.3	0.2	0.3	0.3	0.3	0.2	0.6	1.4	1.5	1.4	0.9	1.6
12:0	8.5	5.5	5.9	6.3	5.0	3.7	5.6	3.8	3.4	3.5	3.2	2.9	4.0	3.7	2.6
14:0	21.3	18.1	16.8	15.2	14.2	14.6	12.3	11.7	10.9	8.4	8.2	8.4	8.7	12.5	10.3
14:1	–	–	–	0.5	0.4	0.5	1.3	1.0	1.3	1.5	2.9	3.9	2.1	2.2	3.7
15:0	1.3	1.5	2.2	1.3	1.4	1.5	0.6	1.1	1.0	0.8	1.1	1.2	1.0	0.9	1.1
16:0	34.6	38.1	35.9	20.4	22.9	20.6	20.1	23.7	20.2	13.5	16.8	14.6	14.4	21.7	18.8
16:1	–	–	–	0.7	0.7	1.4	1.4	1.6	2.6	2.6	3.0	5.2	1.8	1.7	3.1
18:0	12.8	11.1	14.9	9.9	8.8	9.1	7.0	6.1	6.2	2.8	3.2	3.6	3.6	5.4	3.8
18:1	–	–	–	30.9	24.8	28.8	33.3	27.4	31.7	49.5	37.9	38.7	37.5	30.8	35.7
18:2	–	–	–	–	–	–	–	–	–	2.8	3.7	3.6	7.7	7.3	6.5
18:3	–	–	–	–	–	–	–	–	–	–	–	–	5.3	2.8	4.1

[a](–) = not detected.

TABLE VII

Fatty Acid Compositions (wt % of fatty acids) of Triacylglycerol Classes of Differing Levels of Unsaturation Prepared from Low Molecular Weight Fractions of Buffalo Milk Fats of Different Stages of Lactation

	Fatty acid composition (% fatty acids)														
	Saturated TG			*trans*-Monoene TG			*cis*-Monoene TG			Diene TG			Polyene TG		
Fatty acid	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late	Colostrum	Middle	Late
4:0	23.4	22.0	20.6	17.5	16.7	16.9	23.6	20.2	21.2	18.9	17.5	18.0	11.3	14.6	13.0
6:0	4.8	6.5	5.3	3.1	4.6	4.8	3.9	6.2	5.7	3.7	5.6	5.2	1.9	3.9	4.1
8:0	1.4	2.5	2.2	1.9	2.8	2.7	1.0	1.4	1.3	0.5	1.8	1.7	1.0	1.1	1.0
10:0	1.9	4.0	4.3	1.3	2.8	2.2	0.9	1.8	2.0	0.7	2.2	0.9	1.4	1.6	1.2
10:1	–[a]	–	–	–	–	–	–	0.2	0.2	0.3	0.8	0.8	0.5	0.4	0.8
12:0	4.8	3.3	4.0	3.7	3.2	2.5	2.3	2.3	1.8	2.4	2.6	1.2	2.0	3.1	1.5
14:0	21.4	17.3	18.7	13.3	10.8	11.0	11.3	10.7	9.3	3.9	6.2	4.5	4.2	9.1	5.8
14:1	–	–	–	1.6	1.0	1.3	0.5	0.3	1.0	1.1	1.9	2.0	2.4	0.8	1.6
15:0	1.0	1.2	2.3	1.2	0.7	1.3	0.3	1.0	1.4	0.5	0.8	0.5	0.7	0.7	0.5
16:0	32.8	35.4	33.8	20.8	23.8	21.9	20.1	22.7	18.1	8.6	12.7	11.1	16.2	20.6	17.9
16:1	–	–	–	1.7	1.4	1.6	1.1	1.5	2.3	2.9	2.0	3.0	1.3	1.2	2.9
18:0	8.5	7.8	8.8	6.3	6.4	6.7	3.4	3.9	4.9	2.0	2.2	2.3	3.3	6.1	2.3
18:1	–	–	–	27.6	25.8	27.1	31.6	27.8	30.8	49.8	35.7	45.1	35.4	25.4	35.4
18:2	–	–	–	–	–	–	–	–	–	4.7	8.0	3.7	14.4	8.4	7.8
18:3	–	–	–	–	–	–	–	–	–	–	–	–	4.0	3.0	4.3

[a](–) = not detected.

The LMT from middle lactation milk fat contained 61.3% of saturated TG, compared to only 36.1 and 47.3% in the LMT from colostrum and late lactation fats. From the FA composition (Table VII), these saturated TG were likely to consist of 4:0 in combination with only 2 of the 14:0, 16:0 and 18:0 acids.

The proportions of both *trans-* and *cis-*monoene TG of the HMT, MMT and LMT constituents of the 4 fats did not vary much. *Trans-* and *cis-*monoene TG of the subfractions in all 4 milk fats had almost identical FA compositions. In the HMT, the major TG species of the *trans-* and *cis-*monoene TG were made of 14:0, 16:0, 18:0 and 18:1. In the MMT, they were likely to be composed of 6:0, 14:0, 16:0 and 18:1, and in LMT, from 4:0, 14:0, 16:0 and 18:1.

Unsaturated TG, both dienes and polyenes, were profoundly influenced by the stage of lactation. The HMT of colostrum and late lactation fats had much more diene TG (28.8 and 25.0%, respectively) than did the HMT of middle lactation fat (19.8%). Similar differences in respect to the stage of lactation, although to a lesser degree, were observed in the diene contents of both MMT and LMT.

The major FA in the diene TG of HMT (Table V) were 14:0, 16:0 and 18:1, which suggests that the TG consisted largely of 2 18:1 moieties in combination with either 16:0 or 14:0. In the MMT, the diene TG were again made of 2 18:1 FA in association with a medium chain or a short chain acid. In contrast to both HMT and MMT, in the LMT, 18:2 emerged as an important constituent of diene TG. In consequence, the TG species were likely to consist either of 2 18:1 moieties with one short or medium chain acid, or of one 18:2 with one medium chain and one short chain acid. More 18:2 was present in the diene TG of the LMT of middle lactation fat than in those of either colostrum or late lactation fat.

Polyene TG formed a major TG class of the HMT fraction in all 4 samples, and of the MMT and LMT in only colostrum and late lactation samples. Polyene TG of the HMT and MMT fractions contained 14:0, 16:0, 18:1 and 18:2 as the major acids, whereas polyene TG of LMT had 4:0, 16:0, 18:1 and 18:2 as the principal acids. The major polyene TG species of HMT could thus be made up of 2 of the unsaturated acids (18:1, 18:2 and 16:1) in combination with 16:0 or 14:0. In LMT and MMT, 18:1 and 18:2, in various combinations with a medium or a short chain acid, constituted the polyene TG.

Overall Features of TG Assembly

Data in Tables IV-VII show that an increase in unsaturation corresponded to an increase in chain length of the FA and a decrease in short chain acids of the TG classes. This inverse relationship might simply reflect the fact that the unsaturated acids are synonymous with long chain acids. This was particularly apparent in the TG classes that constituted the LMT fraction of colostrum and late lactation fats, where one long chain FA was in combination with 2 short or medium chain acids in saturated TG. In TG of greater unsaturation, i.e., diene and polyene TG, 2 unsaturated long chain acids were in association with one medium or short chain acid. If this pattern was any different in the HMT, it was because this fraction carried very small proportions of short chain acids, and only 14:0, 16:0 and 18:0 were available for combination with 18:1 and 18:2.

The greatest effect of lactation observed was on the 2 TG classes, i.e., saturated and diene TG. The HMT, MMT and LMT fractions making up colostrum fat contained the highest amount of diene TG, and the lowest level of saturated TG, when compared with the corresponding fractions derived from early or middle lactation fat. Late lactation milk fat also showed a pattern similar to colostrum, although to a lesser degree (Table (IV). Increase or decrease in saturated TG, therefore, was mostly compensated by diene TG. Variations in the unsaturation of milk fat accompanying the passage of lactation were almost entirely due to 18:1 acid (Table I). Data on TG composition (Table IV) reveal that, beyond a certain level, 18:1 contributed to increasing the proportions mostly of diene and polyene TG, and contributed to monoene TG only to a limited degree.

Thus, marked change in the TG composition of buffalo milk fat mainly occurred at either end of the lactation cycle, during the period immediately after parturition and, in reverse, late in lactation.

Comparable data in regard to the specific influence of lactation on TG composition have not been reported for any other species, although Taylor and Hawke (1) and Morrison and Hawke (2) have demonstrated that TG compositions of cow milk fat could be altered by season and by special feeding procedures.

Stereospecific analysis of cow milk-fat TG suggests that short chain and 18:1 acids show a strong preference for *sn*-3 and *sn*-2 positions, respectively, of the glycerol moleculre (10,19). A similar study on buffalo milk fat TG and their fractions would reveal if there is any dif-

ference between the positional locations of the fatty acids in buffalo and cow milk-fat TG. However, pancreatic lipase hydrolysis data (20) indicate that buffalo and cow milk-fat TG are identical in their fatty acid distribution between primary and secondary hydroxyl groups of glycerol molecules. Intact TG analysis of the buffalo milk-fat TG classes from different stages of lactation also would have revealed the association of fatty acids more clearly.

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Differential Effects of Dietary Fatty Acids on the Accumulation of Arachidonic Acid and Its Metabolic Conversion through the Cyclooxygenase and Lipoxygenase in Platelets and Vascular Tissue

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ABSTRACT

Semisynthetic diets containing either corn oil (CO) or butter (B) (11 and 2.2 en % as linoleic acid, respectively) were fed to male rabbits for periods of 3 weeks and 3 months. The CO diet, in respect to the B diet, induced higher levels of linoleic acid (LA) and lower levels of arachidonic acid (AA) in platelet phospholipids, lower levels of AA in aortic phosphatidylinositol (PI) and accumulation of both LA and AA in liver lipids. The thresholds for aggregation with AA, but not with collagen, were higher in the CO group and the formation of thromboxane B_2 (TXB_2) from [^{14}C] AA, but not from endogenous substrate after collagen stimulation, was lower in the same group. Formation of PGE_2-like material by incubated aortas was higher in the B group. In the CO group, platelet cyclooxygenase appeared to be selectively depressed. The correlations among diet-induced fatty acid changes in platelet and aortic lipids, platelet aggregation and thromboxane and prostacyclin formation are discussed.

INTRODUCTION

Dietary fatty acids are known to affect platelet aggregation as shown by studies in experimental animals and in humans (1–3). Saturated fatty acids have been consistently reported to enhance platelet aggregation, whereas unsaturated fatty acids have anti-aggregatory properties (1). More limited information is available on the effects of dietary fatty acids on metabolic processes which are considered relevant in modulating platelet aggregation, such as thromboxane production in platelets and prostacyclin (PGI_2) formation in arterial walls.

Recently, the effects of dietary fatty acid on malondialdehyde production in platelets, considered an index of nonenzymatic degradation of cyclic endoperoxides and on PGI-like material in perfused aortas and aortic pieces have been described (4).

Also, limited data are available on the correlations between diet-induced changes of the fatty acid composition of platelets and arterial wall phospholipids and corresponding changes of prostaglandin and thromboxane production in the same tissues.

Previous studies carried out in our laboratory (5) have shown that, after the administration of semisynthetic diets containing either corn oil (CO) or butter (B) as the only dietary fat to rabbits for 3 weeks, arachidonic acid (AA)-induced platelet aggregation and the conversion of [^{14}C] AA to products of the cyclooxygenase pathway were significantly reduced in platelets of the animals fed the linoleic acid (LA)-rich CO diet.

Aims of this study were to investigate the comparative effects of the administration of CO vs B in the rabbit (a) on the fatty acid composition of phospholipids in platelet and aorta in comparison to those in liver, the organ where the metabolic conversion of LA to AA has been extensively studied and found to be very active (6); (b) on platelet aggregation; (c) on the possible correlations between fatty acid changes in the tissues of a, and modifications of arachidonic acid metabolism; (d) on thromboxane formation in platelets; and (e) on prostacyclin formation by incubated aortas.

MATERIALS AND METHODS

Animals

Five male rabbits of the New Zealand strain with an average weight of 3 kg were used in each experimental group.

Diets

Semisynthetic diets were prepared with the following weight percentage composition: protein 20, carbohydrates 64.4, fibers 6.8, salmon salt mix 5.4, vitamin mix 1.0. The lipid fraction accounted for 8.0% (w/w) corresponding to ca. 25 energy percent of the diet and was composed of either corn oil or butter. The fatty acid composition of the lipid fraction of the diet is reported in Table I. The diets were fed to 2 groups of 5 animals each for 3 weeks or 3 months.

TABLE I

Fatty Acid Composition of Dietary Lipids (weight percentage)

Fatty acids[a]	Dietary lipids	
	Butter	Corn oil
10:0	1.6	–
12:0	3.2	–
14:0	9.5	0.9
15:0	2.3	–
16:0	29.6	15.4
16:1	4.9	–
18:0	9.5	2.9
18:1	25.5	24.5
18:2	9.4	48.0
18:3	1.6	2.7
Saturates	55.7	19.2
Monoenes	30.4	24.5
Polyenes	11.0	50.7
P/S	0.2	2.6
18:2 as % of calories	2.2	11.0

[a]Number of C-atoms:number of double bonds.

Collection of Blood for Platelet Studies

Animals were anesthesized with Na pentothal (30 mg/kg IV) and the carotid artery exposed and cannulated with plastic tubing. Arterial blood was collected in plastic centrifuge tubes in the presence of 10% Na citrate (3.8%) for the studies on platelet aggregation and in the presence of Na EDTA 77 mM when washed platelets were prepared for studies on the conversion of labeled AA (7) and on the production of thromboxane B_2 after stimulation with collagen.

About 70–80 ml of blood was collected from each animal. Abdominal aortas also were taken immediately after collection of blood, cleaned from the tissue, frozen in liquid nitrogen and stored at −70 C.

Analysis of Tissue Phospholipids and Their Fatty Acids

Tissues were extracted with chloroform/methanol (2:1) according to Folch et al. (8) in the presence of 5 μg/ml butyl hydroxytoluene (BHT) as antioxidant.

The phospholipid composition was measured following 2-dimensional thin layer chromatography (2D-TLC) and P-determination of the spots (9). Fatty acid methyl esters were prepared by transmethylation of the single phospholipid classes, separated by 2D-TLC, then purified on small silicic acid columns, and finally analyzed by gas liquid chromatography (GLC) on polar columns (SP-2330 on Chromosorb WAW, 100–120 mesh, Supelchem) using programming temperature (140–210 C with 2.5 C/min increments).

Preparation of Platelet-Rich Plasmas (PRP) and Washed Platelets (WP)

PRP was prepared by centrifugation according to standardized procedures. Platelets were counted by contrast phase microscopy and PRP were brought to constant platelet count with PPP, obtained by further centrifugation of the blood remaining after removal of PRP.

Washed platelets were prepared according to the procedure described by Hamberg et al. (7).

Studies on the Conversion of ^{14}C-Labeled Arachidonic Acid (AA) in Platelets

Platelets were washed and resuspended in appropriate buffer to the final concentration of 400,000/μl. Suspensions of washed platelets (0.5 ml) were then incubated for 2 min with the 2×10^{-5} M [^{14}C] arachidonic acid. At the end of the incubation period, the reaction was stopped by adding 25 ml of methanol. After evaporation of the solvent, acidified buffer (pH 3.5) was added and products were extracted with peroxide-free diethyl ether. Products were methylated with diazomethane and separated by 1-dimensional thin layer chromatography. Distribution of radioactivity was scanned by radiochromatoscanning and 3 major peaks, corresponding, respectively, to the unconverted substrate, hydroxy fatty acids (hydroxy eicosatetraenoic acid, HETE and hydroxy heptadecatrienoic acid, HHT) and thromboxane B_2 (TXB_2) were separated. Identifications of radioactive products also were made by radio gas chromatography.

Radioimmunoassay (RIA) of Thromboxane B_2

Determination of TXB_2 production by PRP from endogenous AA was done by measuring with a specific radioimmunoassay (RIA) (10) the levels of this stable metabolite of AA, prior to and after stimulation of platelets with collagen. The reaction was stopped with 25 ml of methanol and the RIA followed the extraction after acidification.

Platelet Aggregation

Platelet aggregation was studied by Born's turbidimetric technique, using an Elvi Logos aggregometer. After preparation of PRP and platelet poor plasma (PPP), PRP was brought to a concentration of 300,000/μl and was then challenged with increasing concentrations of collagen (Hormon Chemie, Munchen) and arachidonic acid sodium salt (AASS) (Sigma). Thrombin and ADP were not used as aggregating agents because of the difficulty of measuring thrombin aggregation in the presence of plasma (PRP) and because of the lack of

correlation between ADP-induced platelet aggregation and the formation of AA metabolites in platelets. Threshold concentrations of aggregation were defined for the various agents as the concentrations inducing a response corresponding to 50% of the maximum.

Prostacyclin Production by Incubated Aortas

Fragments of aorta strips with an average weight of 5–10 mg were incubated in 300 µl of Tris buffer pH 8.2 at room temperature for 20 min. At the end of the incubation, tissue was removed and the incubate was placed in ice. Fragments of tissue were then dried and weighed. Parallel incubations were done with aortas obtained from rabbits of the corn-oil- or butter-fed groups. Concentrations of prostacyclin-like material was determined by bioassay based on inhibition of platelet aggregation in comparison to authentic standards of prostacyclin (11) (Courtesy of the Wellcome Research Labs.).

Statistical Analysis

Statistical evaluations were based on determinations of the Student's t-test.

RESULTS

The phospholipid compositions of platelet of the 2 experimental groups of animals after 3 weeks on the diets are shown in Table II. Differences are small between the 2 groups of samples, except for an elevation of sphingomyelin in the animals fed the diet containing butter. Although the analyses were done on pooled samples, this difference seems appreciable.

The fatty acid compositions of platelet, aorta and liver glycerophospholipids are shown in Tables III–V. Fatty acids of sphingomyelin were not analyzed because of the absence of polyunsaturated fatty acids in this phospholipid. The contribution of the fatty acids of this phospholipid class to the prostaglandin system thus appears negligible, although changes of the very long chain fatty acids typical of sphingomyelin may occur following manipulations of dietary fatty acids. A distinct difference among the fatty acid composition of the various glycerophospholipids was observed in all tissues: 20:4 followed by 18:0 was the major fatty acid in phosphatidylethanolamine (PE); 18:0 was the major fatty acid in phosphatidylserine (PS) and phosphatidylinositol (PI), whereas 16:0 was the major fatty acid (FA) in platelet and aorta phosphatidylcholine (PC). 18:2 was the major fatty acid mainly in PC, whereas levels of 20:4 and the 20:4/18:2 ratios were considerably higher in aorta than in platelet and liver phospholipids. The dietary treatments, as expected, induced significant differences in the fatty acid composition of tissue glycerophospholipids. The CO diet containing a higher level of 18:2 generally induced an elevation of the levels of this fatty acid, especially in liver and platelets, whereas the levels in aorta lipids were only slightly modified. Significant elevations of 18:2 were observed, especially in liver PC and PI, and in platelet PE, PC and PS. The modifications of the levels of 20:4 followed a different pattern in the various tissues. Levels generally were enhanced in liver phospholipids of rabbits fed the CO diet, especially in the PE, PC and PI fractions, whereas they were reduced in the same glycerophospholipid classes of platelets from the same experimental group. In aortic glycerophospholipids, levels of 20:4 were similar in the 2 groups, except for the higher level in the PI fraction of the B group. The total levels of polyunsaturated fatty acids (18:2 + 20:4) and the unsaturation indices (U.I.) (12) were generally lower, as expected, in liver lipids of the B-fed animals, than in the CO group, whereas in platelet and aorta lipids they tended to be higher in the B group, especially in the PI fraction. When the ratio 20:4/18:2 (product/precursor relationship) was compared in the various tissues, insignificant differences between the 2 experimental groups were observed in liver phospholipids of the CO group. In the aortic tissue also, the ratio was similar in all phospholipids except for PI, where it was much higher in the B group. After 3 months on the diets (Table VI), levels of 18:2 and 20:4 were not significantly modified in platelet PE, PC

TABLE II

Phospholipid Percentage Composition of Platelets from Rabbits Fed Semisynthetic Diets Containing Either Corn Oil (CO) or Butter (B) for Three Weeks

Phospholipid class	CO	B
PC	32.1 (31.3–33.0)	29.0 (28.7–28.8)
PE	23.1 (23.4–22.8)	23.4 (23.5–22.9)
PS	13.8 (14.7–12.9)	12.1 (12.8–11.2)
S	22.8 (21.1–24.6)	28.4 (29.4–26.8)
PI	5.2 (5.4– 5.1)	4.8 (4.3– 5.4)
CL	1.7 (1.5– 1.9)	2.1 (1.5– 2.6)

Platelets from 5 animals in each experimental group were pooled and duplicate analyses were made by 2D-TLC. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; S, sphingomyelin; PI, phosphatidylinositol; CL, cardiolipin.

TABLE III

Fatty Acid Composition of Platelet Glycerophospholipids of Rabbits Fed for Three Weeks Diets Containing Either Corn Oil (CO) or Butter (B)

Fatty acids	PE		PC		PS		PI	
	CO	B	CO	B	CO	B	CO	B
16:0	9.2 ± 1.3	9.6 ± 0.8	30.9 ± 1.3	29.6 ± 1.4	–	–	5.7 ± 0.3	4.2 ± 1.9
18:0	25.0 ± 0.2	22.5 ± 1.3	19.6 ± 1.6	18.9 ± 0.8	49.0 ± 0.9	50.0 ± 1.3	48.2 ± 3.3	39.7 ± 3.1
18:1 (n−9)	9.3 ± 0.9[b]	13.3 ± 1.0[b]	9.3 ± 0.5[c]	17.2 ± 0.6[c]	10.0 ± 1.2[a]	14.5 ± 0.5[a]	6.1 ± 0.6	7.6 ± 3.0
18:2 (n−6)	22.3 ± 2.5[b]	14.3 ± 1.3[b]	34.8 ± 1.4[c]	26.6 ± 0.8[c]	33.6 ± 2.6[b]	25.7 ± 0.6[b]	3.0 ± 0.2	3.1 ± 0.7
20:4 (n−6)	26.9 ± 1.3[b]	34.6 ± 2.9[b]	5.3 ± 0.3[a]	6.9 ± 0.5[a]	6.2 ± 2.0	6.2 ± 0.8	26.2 ± 1.1[a]	32.5 ± 2.1[a]
18:2+20:4	49.2	48.9	40.1	33.5	39.8	31.9	29.2	35.6
20:4/18:2	1.21	2.42	0.15	0.23	0.18	0.24	8.73	10.48
U.I.	162	180	100	98	102	91	117	144

Values are the average ± SEM. Each experimental group was composed of 5 animals, and fatty acids of each phospholipid class were analyzed in duplicate. U.I. = unsaturation index, i.e., sum of percentage of individual unsaturated fatty acids X number of double bonds (12). Statistical significance of differences: [a]$P<0.025$; [b]$P<0.025$; [c]$P<0.005$.

TABLE IV

Fatty Acid Composition of Aorta Glycerophospholipids of Rabbits Fed for Three Weeks Diets Containing Either Corn Oil (CO) or Butter (B)

Fatty acids	PE		PC		PS		PI	
	CO	B	CO	B	CO	B	CO	B
16:0	7.1	6.3	31.5	31.9	2.7	3.9	12.4	6.7
18:0	27.4	25.5	15.8	13.6	51.2	53.8	37.1	34.7
18:1 (n-9)	16.2	17.5	18.7	22.9	35.3	33.1	15.0	8.6
18:2 (n-6)	3.2	3.1	12.2	10.5	4.2	4.5	3.9	1.8
20:4 (n-6)	45.9	47.5	21.5	20.9	6.3	4.6	31.4	48.0
18:2+20:4	49.1	50.6	33.7	31.4	10.5	9.1	35.3	49.8
20:4/18:2	14.34	15.32	1.76	1.99	1.5	1.02	8.5	26.7
U.I.	206	214	129	128	69	61	148	204

U.I. = unsaturation index, sum of percentage of individual unsaturated fatty acids X number of double bonds (12). Analyses were made on pools of 5 aortas for each experimental group.

TABLE V

Fatty Acid Composition of Liver Glycerophospholipids of Rabbits Fed Diets Containing Corn Oil (CO) or Butter (B) for Three Weeks

Fatty acids	PE		PC		PS		PI	
	CO	B	CO	B	CO	B	CO	B
16:0	13.6 ± 1.2	10.1 ± 2.1	23.2 ± 1.6[b]	32.2 ± 2.6[b]	4.9 ± 0.6	7.3 ± 0.9	3.2 ± 0.3	4.6 ± 0.8
18:0	31.7 ± 2.7	28.8 ± 3.0	16.2 ± 1.7	14.5 ± 2.2	51.5 ± 3.8	43.8 ± 4.1	49.2 ± 2.9	51.4 ± 0.9
18:1 (n-9)	8.6 ± 1.2	12.3 ± 2.9	9.1 ± 1.8[c]	21.7 ± 2.7[c]	7.4 ± 0.9	9.6 ± 1.1	8.6 ± 0.9[c]	18.8 ± 2.0[c]
18:2 (n-6)	30.2 ± 2.4	25.0 ± 1.6	45.5 ± 3.5[c]	24.9 ± 4.1[c]	24.5 ± 2.8	25.3 ± 2.7	20.5 ± 1.5[c]	10.6 ± 1.9[c]
20:4 (n-6)	15.8 ± 1.2[a]	11.8 ± 1.2[a]	6.7 ± 1.0[a]	3.9 ± 0.3[a]	13.7 ± 1.7	10.4 ± 1.3	19.1 ± 1.4[b]	12.8 ± 1.4[b]
18:2+20:4	46.0	36.8	52.5	28.8	38.2	35.7	39.6	33.4
20:4/18:2	0.52	0.47	0.15	0.16	0.56	0.41	0.93	1.20
U.I.	132	109	127	87	111	102	126	91

Values represent the average ± SEM. Each experimental group was composed of 5 animals, and fatty acids of each phospholipid class were analyzed in duplicate. U.I.= unsaturation index, i.e., sum of percentage of individual unsaturated fatty acids X number of double bonds (12). Statistical significance of differences: [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$.

and PS of the CO group, in comparison to the values found at 3 weeks. Levels of 20:4 tended, instead, to further increase in the B group in the phospholipids just described. The 20:4/18:2 ratios also were not significantly modified in platelets of the CO group after 3 months on the diet in respect to the values found at 3 weeks, whereas the ratios in the B group were raised at 3 months. Levels of 20:4 were lower and those of 18:2 higher at 3 months than at 3 weeks of treatment in the PI of both experimental groups.

Several differences between the 2 experimental groups were observed that also concerned the levels of saturated and monoenoic fatty acids, especially in liver phospholipids of the B-fed animals, in which levels of 16:0 were higher in PC and those of 18:1 were higher in PC and PI. Oleic acid (18:1) generally was significantly higher in platelet phospholipids of the B-fed animals.

The threshold concentrations for aggregation with collagen and AASS at 3 weeks and 3 months of dietary treatment are shown in Table VII. A significantly reduced threshold for aggregation with AASS at 3 weeks and 3 months of dietary treatment was observed in the B-fed rabbits in respect to the CO-fed animals, whereas no significant difference between the 2 groups was observed in the threshold concentrations for collagen.

The percentage conversions of 2×10^{-5} M [^{14}C]AA to thromboxane B_2 and hydroxy fatty acids by washed platelets of the 2 groups of rabbits after 3 weeks and 3 months of treatment are shown in Table VIII. The total metabolic conversion of [^{14}C]AA was significantly lower in the rabbits that were on the diets for 3 months than in those on the short-term treatment. Also, the balance between the 2 pathways shifted more toward the cyclooxygenase in the chronically treated animals.

At both periods of dietary treatment, the conversion of labeled AA to TXB_2 was higher in the B-fed rabbits than in the CO-fed animals, whereas the conversion to products of the lipooxygenase was not significantly affected.

The production of TXB_2 in PRP from the 2 groups of rabbits after 3 weeks on the experimental diets, after stimulation with collagen at a concentration above the threshold for aggregation (5 μg/ml), is shown in Table IX. The values of TXB_2 in PRP after collagen stimulation are spread over a rather wide range, suggesting a significant variability of responses to receptor-mediated stimulation. Levels of TXB_2 are not significantly different in the 2 groups of experimental animals.

The production of PGI_2-like material in

TABLE VI

Fatty Acid Composition of Platelet Glycerophospholipids of Rabbits Fed Diets Containing Either Corn Oil (CO) or Butter (B) for Three Months

Fatty acids	PE		PC		PS		PI	
	CO	B	CO	B	CO	B	CO	B
16:0	9.3 ± 1.1	8.0 ± 0.7	27.1 ± 2.5	28.8 ± 1.6	4.2 ± 0.7	6.1 ± 0.8	12.2 ± 1.9	14.2 ± 2.2
18:0	22.6 ± 1.1[c]	16.7 ± 0.6[c]	21.4 ± 3.5	20.3 ± 0.9	49.3 ± 3.7	45.2 ± 3.9	50.0 ± 5.6	41.3 ± 1.6
18:1 (n-9)	13.0 ± 1.6	11.6 ± 0.1	13.3 ± 0.2[c]	20.9 ± 0.2[c]	11.7 ± 1.8[a]	17.5 ± 1.5[a]	9.5 ± 0.2[a]	19.8 ± 1.2[a]
18:2 (n-6)	23.7 ± 1.9[c]	12.0 ± 1.1[c]	32.9 ± 0.1[a]	21.7 ± 1.0[a]	30.5 ± 2.8	23.1 ± 2.0	7.5 ± 1.4	9.3 ± 0.5
20:4 (n-6)	31.3 ± 1.6[c]	51.6 ± 2.1[c]	5.0 ± 1.1[a]	8.3 ± 0.2[a]	4.8 ± 0.9[a]	8.2 ± 1.0[a]	14.6 ± 2.0	15.2 ± 1.3
18:2+20:4	55.0	63.6	37.9	30.0	35.3	31.3	22.1	24.5
20:4/18:2	1.32	4.30	0.15	0.38	0.16	0.36	1.95	1.63
U.I.	186	242	99	98	92	97	83	99

Values represent the average ± SEM. Each experimental group was composed of 5 animals and fatty acids of each phospholipid class were analyzed in duplicate. U.I. = unsaturation index, i.e., sum of percentage of individual unsaturated fatty acids X number of double bonds (12). Statistical significance of differences: [a]$p<0.05$; [b]$p<0.02$; [c]$p<0.001$.

incubated aortic tissue obtained from animals fed for 3 weeks with the experimental diets is shown in Table X. Prostacyclin production during the period of incubation was significantly higher in the aortas of rabbits fed the diet containing butter.

DISCUSSION

These data indicate that manipulation of the lipid fraction of the diet, and especially the levels of LA intake, significantly modifies various parameters in the cardiovascular system.

However, the interpretation of the effects is rather difficult, considering the complexity of the interactions between the dietary intake of fatty acids and the formation of biologically active products derived from polyunsaturated fatty acids.

First of all, the administration of diets rich in LA does not result in elevation of AA levels in all tissues. In fact, higher levels of AA and higher degrees of unsaturations are found in liver lipids of CO- vs B-fed animals, but lower levels of 20:4 are found in most phospholipids in platelets, and in PI in aorta of the same experimental group. It is possible that the desaturase activity is less efficient in platelets than in liver or that high levels of LA inhibit its conversion to the long chain polyunsaturated fatty acid to a different extent in various tissues. Also it is possible that fatty acids of platelet phospholipids are mainly derived through exchange processes with the plasma compartment and, since administration of LA-rich diets results in elevation of the LA/AA ratio in plasma lipids (13), this is reflected in the fatty acid composition of platelets. Although previous reports also have shown similar findings (3), these have not been fully appreciated since the prevailing concept is that high levels of LA in the diet should generally increase the levels of arachidonic acid in tissues. After 3 months of dietary treatment, platelet aggregability, levels of AA in platelet PI and rate of TXB_2 formation were all lower than after 3 weeks, and it is tempting to speculate that these observations may be correlated.

Another interesting observation is the presence of high levels of AA in aortic phospholipids, which can be a factor in sustaining the high production of prostacyclin in the aorta. Limited information is available, to our knowledge, on the modulation of AA release for PGI_2 formation from vessel walls.

Also relevant is that the inhibiting effect of dietary LA is selective for platelet aggregation induced by AA, with little effect on that induced by collagen. The specific effect of

TABLE VII

Threshold Concentrations of Aggregating Agents in Rabbit Platelets

	3 Weeks		3 Months	
	CO	B	CO	B
Collagen μg	4.0 ± 0.6	3.2 ± 0.8	6.8 ± 0.8	6.0 ± 1.0
Sodium arachidonate (μmol)	300 ± 32[a]	160 ± 25[a]	650 ± 11[a]	275 ± 15[a]

Values are the average ± SEM of threshold concentrations of aggregating agents for aggregation of platelets obtained from 5 animals in each group; [a]$P<0.01$.

TABLE VIII

Percentage Conversion of 2×10^{-5} M [^{14}C] AA by Washed Platelets (400.000/μl)

	3 Weeks		3 Months	
	CO	B	CO	B
TXB_2	25.8 ± 1.4[a]	31.2 ± 1.6[a]	14.4 ± 1.5[a]	19.00 ± 0.5[a]
Hydroxy fatty acids	21.0 ± 1.7	21.8 ± 1.5	8.1 ± 2.0	9.8 ± 2.6

Values are the average ± SEM. Determinations of arachidonic acid conversion was studied on triplicate samples of platelets from 5 animals in each group. Statistical significance of difference: [a]$P<0.005$.

TABLE IX

Levels of TXB_2 (pg/μl) in PRP of Rabbits Fed the Corn Oil (CO) or Butter (B) Diet for Three Weeks Prior and Two Minutes after Stimulation with 5 μg/ml Collagen

	TXB_2 (pg/μl) Time (min)	
	0	2
Corn oil diet	10 ± 1	108 ± 49
Butter diet	8 ± 2	79 ± 19

Values are the average ± SEM of triplicate determinations on PRP from 5 animals in each group. TXB_2 levels were determined by a specific RIA (10). Differences between values are not statistically significant.

TABLE X

PGI_2 Production by Individually Incubated Aortas from Rabbits Fed for Three Weeks Diets Containing Corn Oil (CO) or Butter (B)

	PGI_2 (pmol/mg tissue)
CO	1.85 ± 0.7
B	5.37 ± 0.94

Incubation conditions: 20 C for 20 min, buffer pH 8.2. Reaction was stopped at 0 C. PGI_2 was assayed by inhibition of platelet aggregation in comparison to an authentic standard (11). Four aortas in each experimental group were assayed.

dietary treatment on the threshold concentration for AA-induced platelet aggregation is associated with modified formation of TXB_2 from exogenous AA, whereas both aggregation of platelets and TXB_2 formation from endogenous AA by collagen stimulation are unaffected. The selective reduction of TXB_2 formation with no changes in the production of hydroxy fatty acids from labeled AA in platelets of the CO-fed animals apparently depends on a selective inhibition of the cyclooxygenase. This may result from the inhibiting action on the enzyme of LA (14) which accumulates in platelets under these dietary conditions (Tables III and VI). Previous experiments also have shown that when the concentrations of [^{14}C] AA in incubated platelets are increased, differences in the rates of TXB_2 formation between the CO- and B-fed animals disappear (5), suggesting that the inhibition of the cyclooxygenase by the excess linoleate is reversed when the substrate concentrations are increased.

In contrast, diet-induced changes in the levels of AA in platelet phospholipids do not appear to significantly modify TXB_2 formation from endogenous substrate after stimulation with collagen.

TX is formed in platelets mainly after stimulation by aggregating agents following interaction with receptors in the case of collagen, and basal production presumably is negligible. It is thus possible that the massive release of AA occurring during aggregation (15) is

largely in excess of the rate of use by the cyclooxygenase and thus the experimentally induced variations of the total level of the precursor in platelets would be of limited impact on the total TX production.

Higher production of prostacyclin is observed in aortas from rabbits fed the butter-containing diet in respect to that measured in the CO animals after 3 weeks of dietary regimen.

Although dietary manipulations appear to selectively affect the cyclooxygenase systems when the enzyme activity is measured directly, this effect does not seem to involve the biosynthetic response of TX to receptor-mediated stimulation. Thus, the relevance for the "in vivo" thromboxane-prostacyclin balance is difficult to evaluate.

Since the aortas producing more prostacyclin in our experiment have a higher level of AA in PI, which is considered to be selectively involved in the release of the PG-precursor fatty acid (16), it is tempting to speculate that these 2 observations may be correlated. The long-term effects of our experimental diets on PGI_2 production in aortic tissues, however, may be different from those observed after 3 weeks.

Enhanced formation of PGI_2 in isolated aortas also has been reported after 2 months of atherogenic diets (17) and in spontaneously hypertensive rats (18).

It should also be considered that the formation of PGI_2-like material in incubated pieces of aortic tissue is an indication of the total capacity for production and is not representative of the rate of formation in physiological conditions.

The observed modifications in the formation of products of AA cyclooxygenase apparently are not secondary to plasma cholesterol and triglyceride changes since blood cholesterol, e.g., was not appreciably modified after 3 weeks on the experimental diets (5).

In conclusion, it appears that changes in dietary fatty acids, and especially of LA intake, significantly modify prostaglandin production in the cardiovascular system.

The AA cascade appears to be selectively affected in both thromboxane and prostacyclin formation, but the exact mechanisms involved (differential modification of the PG precursor levels in the various compartment, modification of the cyclooxygenase activity) and the biological significance in light of the thromboxane-prostacyclin balance hypothesis remains elusive.

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Accumulation of Neutral Lipids by Human Skin Fibroblasts: Differential Effects of Saturated and Unsaturated Fatty Acids

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ABSTRACT

The accumulation of neutral lipids by human skin fibroblasts grown in medium supplemented with fatty acids has been investigated. GM-10 cells incorporated exogenous fatty acids into both phospholipids and neutral lipids. More [^{14}C] oleate, linoleate, or linolenate was incorporated into triacylglycerol than was [^{14}C] palmitate or stearate. Supplementation of medium containing delipidized serum wtth unsaturated fatty acids resulted in far more stimulation of [^{14}C] glycerol incorporation into triacylglycerol than did supplementation with saturated fatty acids. Palmitate- and stearate-fed cells incorporated sizable amounts of [^{14}C] fatty acids and [^{14}C] glycerol into diacylglycerol as well as triacylglycerol, especially at higher fatty acid concentrations. Increased oleate supplementation from 10–300 μM resulted in increased triacylglycerol synthesis and accumulation of discrete cytoplasmic lipid droplets; palmitate concentrations above 70 μm were toxic. Micrographs of the palmitate-fed cells showed electron translucent slits, suggesting solid depositions of saturated fat, rather than the discrete osmiophilic droplets found in oleate-fed cells. Although GM-10 cells can synthesize fully saturated triacylglycerols, these data suggest that in cells fed saturated fatty acids, solid depositions of neutral lipids may sequester diacylglycerols and thus limit triacylglycerol synthesis.

INTRODUCTION

Mammalian cells grown in culture media which contain serum commonly contain small numbers of microscopically visible cytoplasmic lipid droplets (1) and incorporate exogenous free fatty acids into cellular triacylglycerol as well as membrane phospholipids (2). The accumulation of neutral lipids is enhanced by substitution of rabbit serum for horse serum (3), addition of albumin-bound free fatty acids (1), or use of hypertriglyceridemic human serum (4). With the hypertriglyceridemic serum, human diploid fibroblast cells have been shown to be capable of internalizing intact triacylglycerol as well as its lipolysis products (5).

Studies with L-strain transformed mouse fibroblasts have demonstrated a progressive increase in neutral lipid accumulation with increased exogenous free fatty acids in the absence of serum lipids (1). Addition of albumin-bound oleic acid results in formation of discrete, noncoalescing lipid droplets containing over 90% triacylglycerol; cells containing large numbers of these essentially triolein droplets remain mitotically active (2). Sequential exposure of the cells to oleic and linolenic acids, with markedly different affinities for osmium staining, has been used to demonstrate that the triacylglycerol droplets are in rapid biochemical equilibrium (6). The triacylglycerol acyl groups have also been shown to be in dynamic equilibrium with albumin-bound fatty acids of the serum (7).

Supplementation of normal human skin fibroblasts with oleate or linoleate also results in accumulation of triacylglycerol (8, 9). In the course of studies on use of fatty acid mixtures by these cells (10), we observed that exogenous palmitate and stearate did not stimulate fatty acid incorporation into triacylglycerol to the same extent as did oleate and linoleate. This communication describes both the quantitative differences in neutral lipid synthesis in response to unsaturated and saturated fatty acids and the significantly greater incorporation of saturated fatty acids into diacylglycerol rather than triacylglycerol.

MATERIALS AND METHODS

Cells and Media

The GM-10 line of normal human diploid fibroblasts, derived from fetal skin, was obtained from the Human General Mutant Cell Repository, Institute for Medical Research, Camden, NJ. Stock cultures were propagated in Eagle's Minimum Essential Medium with Earle's salts (MEM), supplemented with 10% nonin-activated fetal bovine serum (both from GIBCO, Grand Island, NY) in 25-cm^2 flasks at 37 C in a humidified, 5% CO_2 atmosphere and split 1:4 weekly. All experiments used cells between 15 and 30 generations in culture.

Delipidized fetal bovine serum was prepared by acetone/ethanol (1:1, v/v) extraction (11); the ether-dried protein was redissolved in Earle's Basic Salt Solution (GIBCO), filter-sterilized, and stored at −4 C. Standardization of the reconstituted serum protein solutions

was done according to Lowry et al. (12). Biotin (GIBCO) was added to the medium at 1.5 mg/ml when cells were grown for more than 24 hr with delipidized serum.

Fatty Acids and Radiolabeled Compounds

All fatty acids were obtained from Sigma (St. Louis, MO) and stored in hexane under nitrogen at −20 C; concentrations were confirmed regularly by titration (13). For each experiment, aliquots of the fatty acid solutions were evaporated to dryness under N_2 and redissolved in 95% ethanol; gentle heating was required to redissolve the stearic acid. Ethanol-sterilized glass micropipettes were then used to add these solutions directly to delipidized fetal bovine serum protein in Earles' Basic Salt Solution (EBSS). The final concentration of ethanol in the culture medium was less than 0.2%. Quantitative transfer of the fatty acids to solution was verified by addition of radiolabeled fatty acids.

[1-^{14}C] Palmitate, [1-^{14}C] stearate, [1-^{14}C]-oleate and [1-^{14}C] linoleate were obtained from New England Nuclear, Boston, MA. [1-^{14}C]-Linolenate was from Amersham, Arlington Heights, IL. Each radiolabeled fatty acid gave only a single peak upon methylation and gas liquid chromatography (GLC) with $^{14}CO_2$ detection. The [^{14}C] fatty acids were stored in hexane, and redissolved in 95% ethanol as already described. The specific activities of the radiolabled fatty acids were 46–60 mCi/m mol; solutions were adjusted to use equimolar quantities of each in a given experiment. Purity of all fatty acids and [^{14}C] fatty acids was confirmed by thin layer chromatography (TLC) and by GLC after methylation with boron trichloride-methanol.

[U-^{14}C] Glycerol (152 m Ci/m mol), [1-^{14}C]-sodium acetate (58 m Ci/m mol), and [methyl-^{14}C] thymidine (48 m Ci/m mol) were obtained from New England Nuclear in sterile aqueous or ethanol/water solutions. These were diluted directly with MEM.

Experimental Cultures

Experimental cultures were initiated by seeding replicate 25-cm^2 flasks with 3 to 4 x 10^5 cells in 4.0 ml medium with fetal bovine serum. Twenty-four hr after subculture, the cells were washed with EBSS, and the medium replaced with 4.0 or 5.0 MEM supplemented with 2.2 mg/ml delipidized serum protein in EBSS, to which had been added the required fatty acids and radiolabeled substrates. GM-10 cells remain actively mitotic in medium-containing delipidized serum protein plus biotin and have been grown through several passages in our laboratory. Thus, all experiments used log phase cell cultures in medium with defined lipid composition. Incubation time with supplemented fatty acids was 24 hr.

At the end of each experiment, the culture medium was removed, and the cells washed twice with calcium- and magnesium-free EBSS. The cells were detached from the surface with 1.5 ml/flask of 0.05% trypsin in Earle's Salts (GIBCO). Each flask was rinsed with 2.5 ml of 0.025% methyl cellulose in 0.9% NaCl. The resulting 4.0 ml aqueous cell suspension was added directly to an equal vol of acetone for extraction of cellular lipids. To measure [^{14}C]-thymidine incorporation, the cell suspensions were disrupted by sonication and the DNA precipitated with an equal vol of 10% TCA and collected on glass fiber filters (Gelman, Ann Arbor, MI). Each filter was then placed in 10 ml ACS (Amersham, Arlington Heights, IL) for scintillation counting.

Extraction and Separation of Lipids

Cellular lipids were extracted in a mixture of 2:1:1 mixture of ethyl acetate/acetone/cell suspension, as described by Slayback et al. (14). The solvent mixture was then centrifuged at 2,000 rpm at 4 C for 10 min and the organic phase removed. The remaining aqueous phase was washed with 1.5 ml ethyl acetate/acetone (2:1, v/v), and the pooled lipid extracts dried, redissolved in chloroform/methanol (1:1, v/v), and stored at -20 C under N_2.

To resolve neutral lipid classes, aliquots of the lipid extracts were applied as thin streaks of Silica Gel H coated thin layer chromatographic plates (Applied Science, State College, PA). The plates were developed by ascending chromatography in petroleum ether/diethyl ether/acetic acid (82:18:1, v/v). The phospholipids at the origin were removed and redissolved by addition of 1.0 ml water followed by 10 ml ACS scintillation fluid. The neutral lipid spots were then visualized with iodine which was allowed to sublime before the spots were scraped and dissolved in 15 ml of 0.4% Omnifluor (New England Nuclear) in toluene/ethanol (2:1, v/v). A reference mixture of neutral lipids was cochromatographed on each plate; when the samples were to be analyzed further by GLC, only the standards were exposed to iodine. For phospholipid separation, the Silica Gel H plates were developed in chloroform/methanol/acetic acid/ water (50:30:8:4, v/v).

For analysis of fatty acid composition, appropriate spots were scraped from the thin layer plates and 20 μg tripentadecanoin was added to each as an internal standard. Neutral lipids were extracted in chloroform/methanol/

ethyl ether (1:1:1, v/v) (13), phospholipids in chloroform/methanol/acetic acid/water (50:39:1:10, v/v) (15). The samples were evaporated to dryness under nitrogen, redissolved in benzene, and transmethylated with methanolic sodium methoxide (Applied Science) (16); the resultant fatty acid methyl esters were extracted with ethyl ether, dried under nitrogen, and redissolved in heptane. GLC was performed on a Packard 427 GC with F.I.D. (Packard, Downers Grove, IL) using a 6′ 10% SP 2330 on 100/120 Chromasorb W-AW glass column (Supelco, Bellefonte, PA) programmed from 170–230 C at 8 C/min. Peaks were identified by comparison of retention times with those of standard fatty acid methyl esters (FAMES); a Spectra-Physics Minigrator (Santa Clara, CA) was used for electronic integration of peak areas. To identify radioactive FAMES, the gas liquid chromatograph was interfaced with a Packard 804 gas flow proportional counter using a nominal 100:1 glass splitter; separations were isothermal at 170 C.

Electron Microscopy

Cells were fixed in situ at 4 C in 1.0% glutaraldehyde in 0.13 M phosphate buffer, pH 7.35, for 1 hr. They were then washed 3 times with phosphate buffer and post-fixed 45 min at 20 C in osmium tetroxide in phosphate buffer. The embedding procedure was adapted from Brinkley et al. (17) and involved dehydration at 20 C for 5–10 min each in 30, 50, 70, 95 and 100% ethanol. The cells were then soaked for 1 hr in 2-Hydroxypropyl Methacrylate (HPMA) (Polysciences, Warington, PA), 1 hr in HPMA/Polybed 812 (Polysciences) (1:1, v/v) both with frequent agitation, and then overnight in 100% Polybed. In preparation for polymerization, there was an additional soaking with agitation in fresh resin. Resin in excess of 1 mm thickness was then drained from the flasks. Holes were burned in the top of the flasks with a heated glass rod and the flasks placed uncapped in a vacuum oven at 60 C under 10 psi for 24–36 hr.

After forcefully crushing each culture flask, the resin was pulled from the flask. Small chips were cut and mounted cell layer upright at the flattened surface of prepolymerized resin molds with 5-min epoxy. The blocks were allowed to dry 24 hr prior to thin-sectioning on an LKB Ultratome III. Sections were mounted on copper mesh grids and doubly stained in a saturated solution of uranylacetate in 50% ethanol and Reynolds lead citrate (18). The sections were viewed in a Philips EM 301 transmission electron microscope.

RESULTS

Incorporation of [^{14}C] Fatty Acids by GM-10 Cells

GM-10 fetal human fibroblasts readily incorporate both saturated and unsaturated fatty acids and esterify them into cellular lipids. The data (Table I) indicate that cells given the exogenous fatty acid in otherwise lipid-free medium incorporated substantially more [^{14}C]-oleate, linoleate and linolenate into triacylglycerol than either palmitate or stearate. Similar, but less pronounced, differences were observed in medium containing fetal bovine serum with its mixure of free fatty acids; since the serum concentrations of oleate and linoleate were respectively higher than and lower than those of the saturated fatty acids, these results are not due to differences in dilution of [^{14}C] fatty acids. In either medium, incorporation of the saturated fatty acids into phospholipids was equal to or greater than that of the unsaturates. Linolenate was not incorporated to the same extent as the other fatty acids; this may reflect less effective conversion to acyl-CoA and/or esterification. The major [^{14}C] labeled phospholipid was phosphatidylcholine in all cases (data not shown). Incorporation of stearate into phosphatidylethanolamine and phosphatidylserine plus phosphatidylinositol was higher than that of the other [^{14}C] fatty acids. Radioactivity in sphingomyelin represented 7–9% of the total phospholipid with [^{14}C] palmitate and less than 3% with the other [^{14}C] fatty acids. Since incorporation of unsaturated fatty acids into triacylglycerol was greater than that of saturated ones both in the presence and absence of serum lipids, all subsequent experiments used delipidized serum protein so as to completely define the available exogenous fatty acids.

Time Course of [^{14}C] Fatty Acid Incorporation

The initial studies used 24 hr incubation of cells with exogenous [^{14}C] fatty acids. Oleate (high triacylglycerol) and palmitate (low triacylglycerol) were then selected for examination of rates of incorporation. As seen in Figure 1A, the incorporation of [^{14}C]oleate into neutral lipids was greater than that of [^{14}C]-palmitate for all times from 1 through 24 hr. [^{14}C] Palmitate incorporation into phospholipids was greater than that of [^{14}C]oleate; total incorporation (not shown) of palmitate was also slightly higher. The accumulated neutral lipid in cells given [^{14}C]oleate was primarily triacylglycerol (Fig. 1B). By contrast, incorporation of [^{14}C]palmitate into diacyl-

glycerol was greater than that of [^{14}C] oleate, and, by 24 hr, equal to or greater than [^{14}C]-palmitate in triacylglycerol. The differences observed between [^{14}C] oleate and [^{14}C] palmitate incorporation patterns in 24 hr thus reflect differences in initial rates. The decreased rate of accumulation of both [^{14}C] fatty acids in neutral lipid suggests turnover of diacylglycerol and triacylglycerol, whereas [^{14}C] fatty acids continue to be incorporated into phospholipids. Furthermore, the increased incorporation of [^{14}C] palmitate into diacylglycerol

TABLE I

Incorporation of [^{14}C] Fatty acids into Cellular Lipids by GM-10 Cells[a]

Serum	Lipid class	Supplemented [^{14}C] fatty acid				
		Palmitate nmol (%)	Stearate nmol (%)	Oleate nmol (%)	Linoleate nmol (%)	Linolenate nmol (%)
DL[b]	Phospholipid	32.0 (93.4)	31.1 (93.1)	28.4 (76.4)	25.2 (71.9)	20.6 (77.0)
	Diacylglycerol	.6 (1.7)	0.4 (1.3)	0.6 (1.5)	0.6 (1.7)	0.4 (1.6)
	Free fatty acid	.2 (0.6)	0.2 (0.6)	0.5 (1.2)	0.3 (0.8)	0.3 (1.3)
	Triacylglycerol	1.3 (3.8)	1.5 (4.6)	7.5 (20.3)	8.8 (25.0)	5.3 (19.7)
	Cholesterol ester	.1 (0.4)	0.1 (0.4)	0.2 (0.6)	0.2 (0.6)	0.1 (0.4)
	Total incorporation	34.2	33.9	37.2	35.1	26.8
FBS[c]	Phospholipid	6.66 (88.9)	5.49 (89.3)	5.48 (82.6)	5.07 (77.3)	3.99 (66.0)
	Diacylglycerol	0.09 (1.2)	0.06 (1.0)	0.09 (1.3)	0.12 (1.9)	0.11 (2.4)
	Free fatty acid	0.04 (0.5)	0.05 (0.8)	0.07 (1.1)	0.09 (1.3)	0.10 (2.1)
	Triacylglycerol	0.66 (8.8)	0.51 (8.3)	0.91 (13.8)	1.19 (18.1)	1.20 (26.4)
	Cholesterol ester	0.04 (0.5)	0.03 (0.5)	0.07 (1.1)	0.09 (1.4)	0.14 (3.0)
	Total incorporation	7.49	6.14	6.62	6.56	4.54

[a]GM-10 fibroblasts were seeded at 2.5 X 10^5 cells/25 cm^2 flask. After 24 hr, the culture medium was replaced with experimental media, as indicated. Incubation time with radiolabeled fatty acids was 24 hr; the cells were then harvested, and their lipids extracted and analyzed as described in Materials and Methods. Values, in nmol/flask, are means of 3 flasks from each of 2 separate experiments (N=6); all SE were less than 5%.

[b]Experimental medium was 5.0 ml plus 5% delipidized fetal bovine serum protein in EBSS (final protein concentration 2.2 mg/ml). Each flask contained 0.5 μCi[^{14}C] fatty acid, plus unlabeled fatty acid to a total of 50 nmol; final fatty acid concentration was 10 μM.

[c]Experimental medium was 5.0 ml MEM plus 10% fetal bovine serum plus 10 nmol[^{14}C] fatty acid; supplemented fatty acid concentration was 2 μM. The fetal bovine serum provided the following medium free fatty acid concentrations: palmitate, 4.6 μM; stearate, 3.3 μM; oleate, 5.7 μM; linoleate, 1.4 μM; linolenate, trace only. Incorporation is expressed as nmol/flask of the supplemented fatty acid alone.

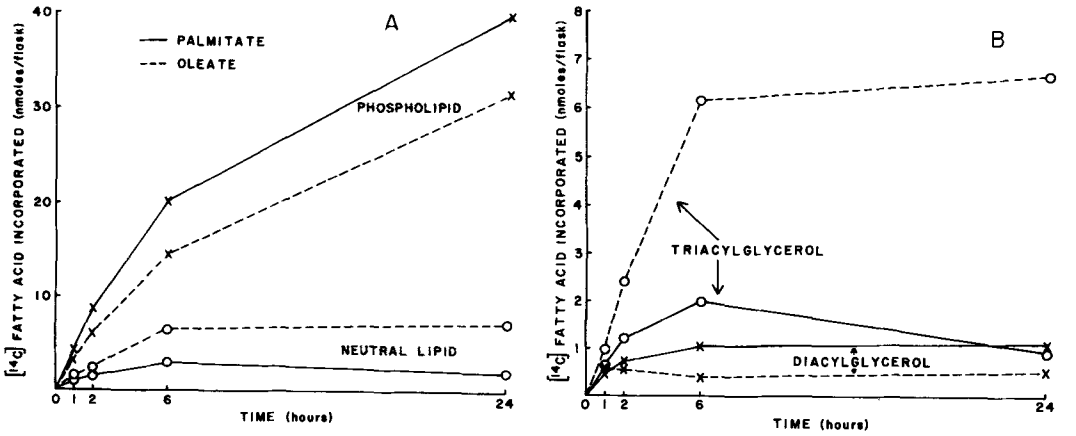

FIG. 1. Time course of incorporation of [^{14}C]-palmitate and [^{14}C] oleate into cellular lipids. GM-10 cells were incubated with [^{14}C] palmitate (solid lines) or [^{14}C] oleate (dashed lines), each diluted with the corresponding unlabeled fatty acid. Total fatty acid addition was 52 nmol, for a concentration of 13 μM in 4.0 ml MEM + 5% delipidized serum. Each point is the mean of replicate flasks.

suggests that limited [^{14}C] palmitate incorporation in triacylglycerol is not caused by lack of incorporation into diacylglycerol or immediate removal of those diacylglycerols for phospholipid synthesis.

Effects of Other Fatty Acids

In order to examine the effects of a wider spectrum of fatty acids on neutral lipid accumulation, replicate flasks were incubated with 2 μCi [^{14}C] glycerol plus 15 μM fatty acids. This concentration of palmitate, stearate, oleate or linoleate resulted in a 60–80% inhibition in endogenous fatty acid synthesis from [^{14}C]-acetate (data not shown); some of the residual [^{14}C] acetate incorporation into glycerolipids by cells given palmitate or linoleate represents elongation of these fatty acids. Thus, the repression of the fatty acid biosynthetic pathway in human fibroblasts with fatty acid supplementation of lipid-deficient serum (19) is operative under the culture conditions of these experiments. All long chain fatty acids tested (Table II) stimulated incorporation of [^{14}C]-glycerol into neutral lipids; the medium chain capric acid (10:0) did not.

Total [^{14}C] glycerol incorporation into neutral lipids was greater with unsaturated than saturated fatty acids. Whereas the major product with unsaturated fatty acids was triacylglycerol, the saturated fatty acids showed substantial [^{14}C] incorporation into diacylglycerol, as well. There was some increase in incorporation into triacylglycerol with increased chain length in both the saturated and unsaturated fatty acid series; however, palmitoleate (16:1) stimulated significantly more incorporation than did stearate (18:0). Cells fed erucate (22:1) incorporated nearly twice as much [^{14}C] glycerol into triacylglycerol as did oleate-fed cells. Heptadecanoate, an odd-chain fatty acid, had effects on neutral lipid synthesis similar to those of the even-chain fatty acids. Elaidate, (*trans*-octadecenoic acid) was similar to stearate, rather than oleate, in its effects on triacylglycerol synthesis; the lack of sizable incorporation into diacylglycerol is more characteristic, however, of an unsaturated fatty acid. These results may be due to a lower rate of activation and use of the *trans* isomer. Linolenate, which is incorporated to a lesser extent than oleate (Table I), also stimulated less [^{14}C] glycerol incorporation into triacylglycerol than did the other unsaturated fatty acids.

Use of Fatty Acid Mixtures

GM-10 fibroblasts were incubated with mixtures of palmitate and oleate; total fatty acid concentration was 18 μM. One fatty acid was ^{14}C-labeled in each set of flasts. Neither total cellular fatty acid incorporation nor total incorporation into phospholipids varied much with degree of unsaturation of the exogenous

TABLE II

The Effect of Exogenous Fatty Acids on Incorporation of [^{14}C] Glycerol into Neutral Lipids[a]

Fatty acid concentration		Incorporation (10^{-3} X dpm/flask)			
		15 μM		30 μM	
Neutral lipid class		DAG[b]	TAG[b]	DAG[b]	TAG[b]
Exogenous fatty acid					
None		1.08	1.80		
Caprate	(10:0)	1.27	1.81	–	–
Myristate	(14:0)	4.25	5.74	–	–
Palmitate	(16:0)	3.55	5.54	12.25	9.14
Heptadecanoate	(17:0)	3.71	7.96	–	–
Stearate	(18:0)	5.39	10.16	15.96	27.59
Palmitoleate	(16:1)	2.16	20.67	–	–
Oleate	(18:1)	1.26	26.04	4.12	65.86
Linoleate	(18:2)	1.35	24.54	–	–
Linolenate	(18:3)	1.46	13.21	–	–
Erucate	(22:1)	3.16	44.85	–	–
Elaidate	(18:1t)	1.92	11.73	–	–

[a]Replicate flasks of GM-10 cells were established as in Table I. They were incubated for 24 hr in 5.0 ml MEM plus 5% delipidized serum, fatty acid supplementation as indicated, plus 2 μCi/flask [^{14}C-U] glycerol (152 mCi/mmol). Values are means of 3 flasks in each of 2 separate experiments (N=6); all standard errors were less than 4%. Incorporation of [^{14}C]-glycerol into phospholipid averaged 87.9 X 10^3 dpm/flask.

[b]Abbreviations: DAG, diacylglycerol; TAG, triacylglycerol.

fatty acids at this concentration (Table III). While the percentage of exogenous [^{14}C] oleate incorporated was similar in the different mixtures, the percentage incorporation of [^{14}C] palmitate was significantly higher when most of the exogenous fatty acids were unsaturated. Thus, when palmitate was 10 and 20% of the exogenous fatty acids, it contributed 16 and 31%, respectively, of the exogenous fatty acids incorporated into phospholipids. More palmitate than oleate was also incorporated from the equimolar mixture. It would thus appear that there are mechanisms for maintaining at least somewhat more heterogeneity in phospholipid acyl groups than would be obtained by nonselective incorporation.

Total fatty acid incorporation into neutral lipids was 3.2–4.3 nmol/flask with all mixtures which were 50% or more saturated fatty acid and increased to 13.2 nmol/flask with increased unsaturated fatty acid. There were 2 opposing trends: a 5.8-fold increase in diacylglycerol with increased palmitate and 7.2-fold increase in triacylglycerol with increased oleate. The low level of diacylglycerol in cells fed unsaturated fatty acids, constituting 1–2% of the incorporated [^{14}C] oleate, is also observed at lower exogenous fatty acid concentrations, and may reflect normal metabolic intermediates. Studies on other mixtures of saturated and unsaturated fatty acids (data not shown) indicated that stearate was used similarly to palmitate and linoleate similarly to oleate; an increase in unsaturated species in the exogenous fatty acid mixture enhanced percentage uptake of the saturated fatty acid, and total incorporation of fatty acids into triacylglycerol, whereas an increase in saturated fatty acids resulted in increased incorporation into diacylglycerol and less total accumulation of neutral lipid.

Increased Exogenous Fatty Acid Concentrations

The experiments with [^{14}C] glycerol (Table II) showed that increasing the concentration of fatty acids in the medium from 15 μM to 30 μM resulted in increased synthesis of neutral lipids; the differences in incorporation between saturated and unsaturated fatty acids were maintained. While diacylglycerol remained a minor component (6%) of the neutral lipids of oleate fed cells, cells fed 30 μM palmitate incorporated more [^{14}C] glycerol into diacylglycerol than into triacylglycerol.

Incorporation of 4–75 μM [^{14}C] oleate and [^{14}C] palmitate is shown in Figure 2; the concentration of delipidized serum protein was constant throughout. There was little incorporation into neutral lipids at concentrations

TABLE III

Incorporation of Fatty Acids into Cellular Lipids from Exogenous Mixtures of Oleate and Palmitate[a]

Fatty acid mixture (nmol/flask)		Total incorporation (nmol/flask [% uptake])			Phospholipid (nmol/flask)			Diacylglycerol (nmol/flask)			Triacylglycerol (nmol/flask)		
18:1	16:0	18:1	16:0	Total	18:1	16:0	Total	18:1	16:0	Total	18:1	16:0	Total
90	–	39.2 (43.6)	–	39.2	26.1	–	26.1	0.47	–	0.47	12.71	–	12.71
81	9	33.9 (41.8)	5.4 (60.1)	39.3	24.7	4.78	29.5	0.61	0.05	0.66	8.57	0.59	9.16
72	18	25.9 (35.9)	10.1 (55.9)	36.0	19.9	9.00	28.9	0.49	0.11	0.60	5.43	0.96	6.39
45	45	14.2 (31.6)	18.8 (41.7)	33.0	12.6	16.9	29.5	0.36	0.41	0.77	1.24	1.48	2.72
18	72	6.9 (38.2)	27.2 (37.8)	34.1	6.47	24.2	30.7	0.12	0.87	0.99	0.30	2.12	2.42
9	81	3.8 (43.0)	28.3 (34.9)	32.1	3.73	25.2	28.9	0.06	1.16	1.22	0.08	1.89	1.97
–	90	–	34.6 (38.4)	34.6	–	30.3	30.3	–	2.73	2.73	–	1.69	1.61

[a]Log phase GM-10 cells were incubated for 24 hr in MEM + 5% delipidized serum, with 18 μM fatty acid mixture, molar composition as indicated. Within each mixture, 2 flasks contained 0.5 μCi [^{14}C] oleate and 2 [^{14}C] palmitate. Values are calculated from mean dpm/flask and specific activity of each fatty acid.

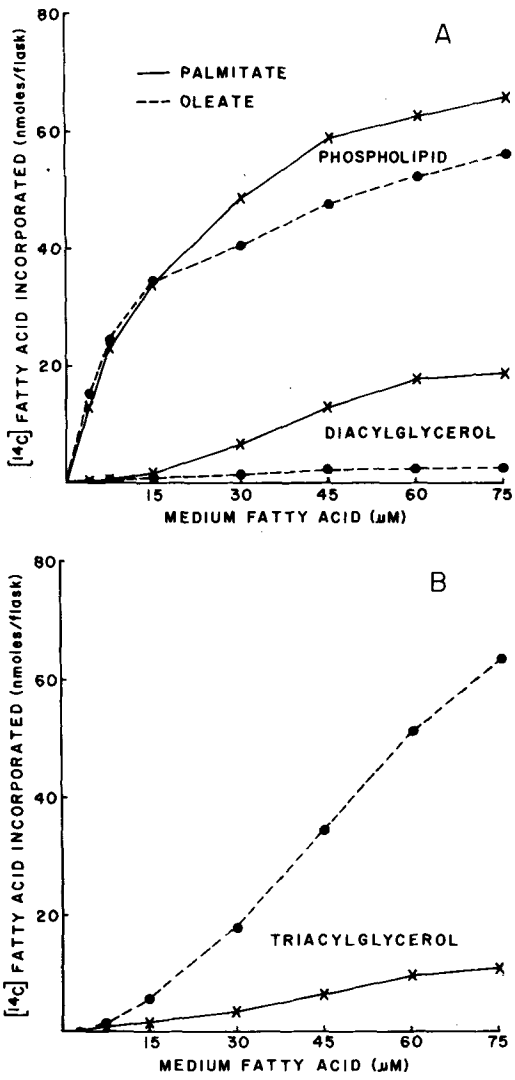

FIG. 2. The effect of increased concentrations of free fatty acids in the medium on incorporation of palmitate and oleate into cellular lipids. GM-10 cells were incubated for 24 hr with 0.4 μCi [1-^{14}C] palmitate (solid lines) or [1-^{14}C] oleate (dashed lines), diluted with unlabeled palmitate and oleate, respectively, to result in the indicated concentrations of free fatty acid in MEM + 5% delipidized serum. Each point is the mean of 3 replicate flasks.

below 15 μM; above 15 μM, synthesis of both triacylglycerol and diacylglycerol increased with increased exogenous fatty acids. At the higher concentrations of palmitate, incorporation of [^{14}C] palmitate into diacylglycerol was nearly twice that into triacylglycerol. At this cell density, medium supplementation with 100–150 μM palmitate resulted in some detachment and cell death within 24 hr. Increased oleate resulted in massive incorporation into triacylglycerol with little into diacylglycerol. Incorporation of oleate into triacylglycerol continued to increase with exogenous fatty acid concentrations up to 300 μM as described previously (10), whereas that into phospholipids leveled off. The latter presumably reflects limits of saturation of phospholipid synthesis and reacylation with oleate; Spector et al. (9) have shown that exogenous oleate does not increase total cellular phospholipid.

Electron Microscopy

As reported previously (8, 10), the increased exogenous oleate resulted in an accumulation of discrete intracellular lipid droplets. Except at the highest concentrations of oleate, the GM-10 cells maintained mitotic activity and were morphologically similar to cells grown without exogenous fatty acids. Figure 3A is an electron micrograph of cells grown with 35 μM oleate, showing numerous spherical osmiophilic droplets, similar to those described by Schneeberger et al. (6) in L cells. Cells incubated with 140 μM oleate (Fig. 3B) showed a greater accumulation of lipid droplets. The droplets remain as distinct spheres demarcated from the surrounding cytoplasm by a thin coat of osmiophilic material but not by a unit membrane (see Insert). By contrast, cells incubated with 35 μM palmitate appeared grainy under light microscopy. Electron micrographs (Fig. 3C) revealed large electron translucent slit-like spaces in the cytoplasm, but no lipid droplets. Gordon (20) described similar cytoplasmic clefts in palmitate-fed L cells, and suggested that these spaces represented sites of deposition of saturated fat lost during fixation. Cells incubated for 24 hr with 70 μM palmitate (Fig. 3D) showed an increased number and size of these spaces, as well as debris, autophagic vacuoles and mitochondrial swelling. While 70 μM palmitate clearly resulted in necrosis, 140 μM oleate (Fig. 3B) stimulated accumulation of lipid droplets in an otherwise normal cell.

Incorporation of Thymidine

Cells grown with 15–20 μM saturated fatty acid did not show any morphological evidence of toxicity and appeared mitotic. To investigate further, cells were incubated with 15 μM fatty acid plus 1.5 μCi/flask [methyl-^{14}C] thymidine. The results (data not shown) indicated that in 48 hr, twice our usual incubation time, DNA synthesis in the presence of palmitate or stearate was equal to that in the absence of any

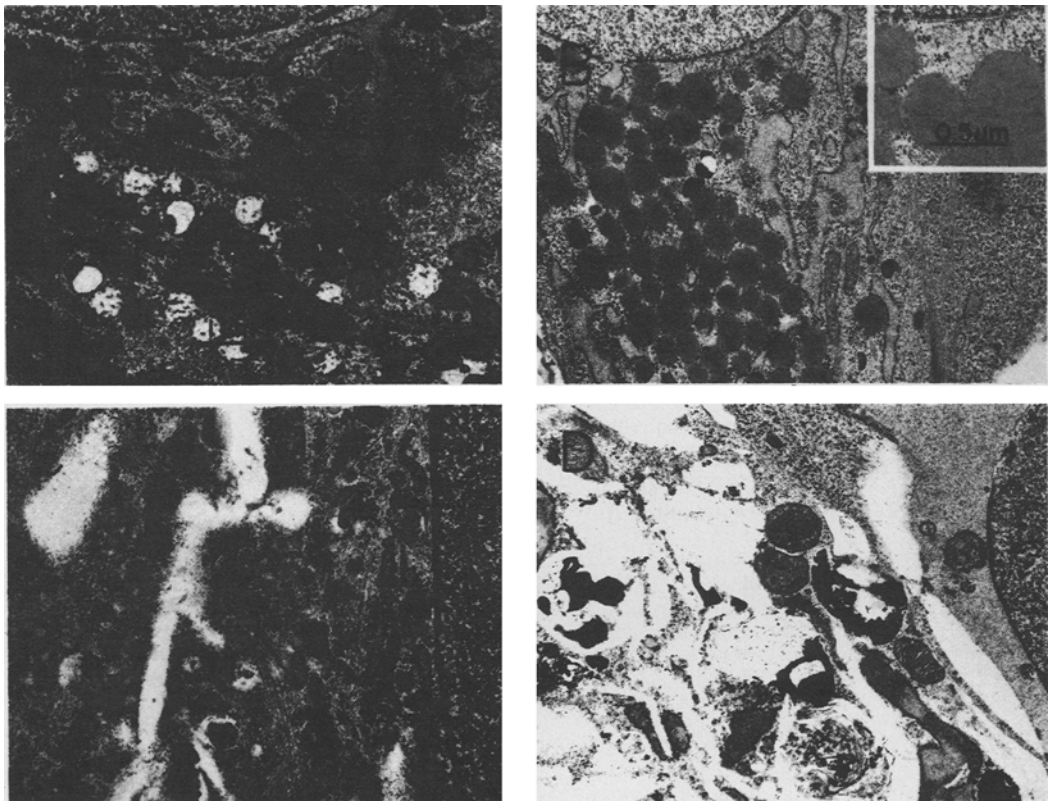

FIG. 3. Electron micrographs of cells grown for 24 hr in medium supplemented with fatty acids. (A) 35 μM oleate; (B) 140 μM oleate; (C) 35 μM palmitate; (D) 70 μM palmitate.

exogenous fatty acid, and at least 85% of that with oleate or linoleate. Thus, there is no evidence of saturated fatty acid toxicity at the concentrations used in most experiments. Furthermore, the differences in growth per se are not sufficient to explain the decreased triacylglycerol accumulation in cells incubated with 15 μM palmitate instead of oleate.

Acyl Composition of Triacylglycerols

Table IV shows the fatty acid composition of triacylglycerols synthesized by cells grown in 35 μM and 140 μM oleate and palmitate. The higher concentration of palmitate was used to enhance fatty acid enrichment although there were definite signs of saturated fatty acid toxicity. Direct determination of total triacylglycerol, using an internal standard, confirmed the far greater accumulation of triacylglycerol in oleate-fed cells compared to those fed palmitate. Control cells grown without exogenous fatty acid contained less than 0.8 μg triacylglycerol/flask. The triacylglycerols synthesized in response to exogenous oleate were primarily unsaturated, whereas those in palmitate-fed cells contained over 80% saturated fatty acids. Thus, GM-10 cells can synthesize fully saturated triacylglycerols; in cells grown with 140 μM palmitate, at least 50% of the synthesized triacylglycerol was tripalmitin. The percentage of saturation of diacylglycerols of the palmitate-fed cells were similar to those of their triacylglycerols (data not shown). Cellular phospholipids also were enriched with oleate or palmitate, respectively, but not to the extent of the triacylglycerols. At the 2 concentrations of exogenous palmitate, the phospholipids were 35 and 50% saturated, respectively, whereas exogenous oleate resulted in 19 and 16% phospholipid saturation.

DISCUSSION

This study has shown that human skin fibroblasts synthesize triacylglycerol in response to increased medium concentrations of all long chain fatty acids studied. Saturated fatty acids are, however, far less effective than unsaturated ones in stimulating triacylglycerol

TABLE IV

Composition of Triacylglycerol Synthesized by GM-10 Cells Grown with Exogenous Palmitate or Oleate[a]

Fatty acid	Palmitate		Oleate	
Concentration	35 μM	140 μM	35 μM	140 μM
	(mol %)			
16:0	71.5	85.8	7.8	3.0
16:1	tr	1.2	2.1	1.5
18:0	13.2	4.2	4.5	2.1
18:1	9.5	3.5	63.5	85.2
18:2	ND	ND	7.8	0.7
20:4	3.1	ND	4.5	1.2
Others	2.7	5.2	8.9	6.3
Percentage saturated[b]	84.7	90.0	12.5	5.1
Total triacylglycerol[c] (μg/flask)	4.9	26.5[d]	25.4	128

tr = trace; ND = none detected.

[a]GM-10 cells were incubated for 24 hr in MEM + 5% delipidized serum, fatty acid supplementation as indicated. Cellular lipids were then extracted and separated by TLC. The triacylglycerol spots were analyzed by GLC. Values are means of duplicate determinations in each of 2 separate cultures.

[b]Small amounts of 14:0, included with "others," would increase these values slightly.

[c]An internal standard of 20 μg tripentadecanoin was added to each triacylglycerol sample before extraction from the silica gel and methylation.

[d]This concentration of palmitate resulted in necrosis and in some cell death (see Fig. 3). The adherent cells were analyzed; this value is therefore 10–20% low.

accumulation. Furthermore, increased concentrations of exogenous saturated fatty acids result in increased incorporation of fatty acids and glycerol into diacylglycerol above the low levels normally observed in these cells, presumably as metabolic intermediates. Oleate-fed L cells (mouse tumor fibroblasts) have been shown to accumulate more neutral lipid or triacylglycerol than those fed palmitate (1, 20); those studies did not determine diacylglycerol concentrations.

Spector et al. (9) have shown that 10–20 μM fatty acids are nontoxic to human diploid fibroblasts and can be used to obtain extensive modification of cellular acyl composition; triacylglycerols are modified to a greater extent than are phospholipids. Our study, using delipidized serum protein to replace the serum in the culture medium, confirms those results. Furthermore, uptake of fatty acids by GM-10 cells does not passively reflect the mixture available in the medium. Particularly when scarce, saturated fatty acids are incorporated to a greater extent than are unsaturates. Ehrlich ascites tumor cells also incorporate more palmitate than oleate from equimolar mixtures (21). Since quantity of cellular triacylglycerol and diacylglycerol and acyl composition of both neutral lipids and phospholipids vary with medium fatty acid composition, changes in culture media, such as replacement of fetal bovine serum with calf serum or use of serum-free media, may significantly alter the lipid composition of cells in culture.

GM-10 cells incorporate exogenous palmitate and stearate into phospholipids to an equal or greater extent than oleate or linoleate over a wide concentration range, indicating that the saturated fatty acids readily enter the cell and are activated to fatty acyl-CoA. Oleate and linoleate inhibit fatty acid synthesis from [^{14}C]acetate to a similar extent as do the saturated fatty acids; total acyl pools are thus similar in cells fed saturated and unsaturated fatty acids. The differences in incorporation of palmitate and oleate into triacylglycerol are observed within 1 hr (Fig. 1), indicating that triacylglycerol synthesis from oleate in these cells does not require the enzyme induction observed in adipocyte conversion of 3T3 cells (22, 23). Thus, the deficit in triacylglycerol synthesis in cells fed saturated fatty acids would appear to involve the reaction of diacylglycerol with acyl-CoA.

Studies with rat liver diacylglycerol acyltransferase (EC 6.2.1.3) have shown a broad acyl-donor and diacylglycerol specificity (24, 25). Recently, however, Kobayashi and Kanoh (26) have shown a marked accumulation of diacylglycerols in rat liver slices incubated in the presence of long chain saturated fatty acids. They suggest that there may be a mechanism limiting the synthesis of fully saturated triacylglycerols. The enzymes of isolated fat cells may have a similar mechanism, because with palmitoyl-CoA as the acyl donor, there was greater activity using dioleoylglycerol or 1-stearate-2-oleoyl diacylglycerol than with dipalmitoylglycerol (27). Our results indicate that GM-10 cells can synthesize fully saturated triacylglycerols, but to a lesser extent than less saturated species (Table IV), and incorporate progressively more fatty acids into diacylglycerol as the percentage of saturated fatty acids in exogenous mixtures is increased (Table III). While these data are compatible with diacylglycerol specificity of the diacylglycerol acyltransferase, the use of intact cells makes possible an alternative explanation. Cells fed saturated fatty acids accumulate neutral lipids as solid cytoplasmic depositions; these deposi-

tions may act to sequester diacylglycerols and render them less accessible to the membrane-bound enzyme. Sequestering of diacylglycerols would result in limited triacylglycerol synthesis and an increase in intracellular diacylglycerols, which, rather than consisting solely of disaturated species, would be similar in acyl composition to the triacylglycerol.

Saturated fatty acids have been shown to be toxic at lower concentrations than are unsaturated ones in both L cells (1, 21, 28) and human diploid fibroblasts (9). In this study, 70 μM palmitate resulted in cytotoxicity in 24 hr (Fig. 3); cells grown in 140 μM oleate contained numerous lipid droplets but were otherwise apparently normal. While toxicity of oleic acid appears to occur through cell lysis due to excess accumulation of discrete triacylglycerol droplets (2), that of the saturated fatty acid presumably involves a different mechanism. Gordon (20) has suggested that the accumulation of clefts or slits of crystalline solid neutral lipid within the endoplasmic reticulum may be responsible for cell injury. Doi et al. (28) have shown that reduction of unsaturated fatty acids in membrane phospholipids to less than 50% is inhibitory of cell growth, presumably by decreasing membrane fluidity. This study suggests an additional possible mechanism: increased intracellular diacylglycerol may be toxic whereas cytoplasmic triacylglycerol is inert and relatively benign. Further investigation is needed to determine the actual mechanism(s) of cytotoxicity of excess saturated fatty acids; the protective role of additional unsaturated fatty acids is compatible with all of the hypotheses discussed here.

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Effect of Fat and Microflora on Hepatic, Small Intestinal and Colonic HMG CoA Reductase, Cytochrome P_{450} and Cytochrome B_5

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ABSTRACT

High levels of dietary fat caused a significant reduction in HMG CoA reductase activity in the liver of germ-free rats whereas significantly elevated small intestinal enzyme activity was observed. Dietary fat had no significant effect on HMG CoA reductase activity in any tissue studied in the conventional rat. No significant change in colonic HMG CoA reductase activity was observed between any of the experimental groups. Rats fed a high-fat diet tended to exhibit higher cytochrome P_{450} levels in all tissues studied, regardless of the presence of intestinal microflora.

INTRODUCTION

The role of high-fat diets in the etiology of colon cancer has been identified by numerous investigators (1-3). To date, several hypotheses have been forwarded to explain diet-colon cancer interactions. These include diet-induced alterations in cholesterol and cholesterol metabolites (4), microsomal drug-metabolizing systems (5,6) and intestinal microflora (7,8). One previously unexamined possibility is that dietary fat and intestinal microflora directly and/or indirectly affect the rate of cholesterol synthesis in the colon. Increased levels of fecal cholesterol and cholesterol metabolites have been reported in colon cancer patients (9) and individuals predisposed to cancer (9,10), as well as in populations consuming high-fat diets (11). In addition, intestinal microflora (7,11), as well as the level of fat in the diet (12), have been shown to alter fecal cholesterol levels.

Diet-induced changes in fecal cholesterol levels may, in part, be regulated by the microsomal enzyme 3-hydroxy-3-methyl glutaryl CoA reductase (HMG CoA reductase, EC 1.1.1.34), the key rate-limiting enzyme in cholesterol biosynthesis (13). However, we know of no studies which have simultaneously examined the effect of dietary fat and intestinal microflora on hepatic small intestinal and colonic cholesterol synthesis.

The intestine and liver of man and animals are primary target organs through which diet-related changes in the levels of potential procarcinogens, such as cholesterol and cholesterol metabolites, can be metabolized by the microsomal mixed-function oxidase system (6). The ability of the hepatic mixed-function oxidase system to metabolize drugs has been shown to be enhanced when the level of corn oil in the diet is increased (7, 14). However, these investigators did not examine the role of extrahepatic microsomal mixed-function oxidase systems in metabolizing lipid-soluble compounds.

The purpose of our investigation was to determine the role of dietary fat and intestinal microflora on hepatic, small intestinal and colonic cholesterogenesis, cytochrome P_{450} and cytochrome b_5 levels in the rat.

MATERIALS AND METHODS

Animals and Diets

Weanling male and female F344 germ-free (GF) and conventional (C) rats were obtained commercially (Charles River Breeding Laboratory, Wilmington, MA) and maintained in a temperature (74 ± 2 F), light (12-hr cycle, 3:00 p.m.-3:00 a.m.) and humidity (50%) controlled room. Germ-free rats were maintained in Trexler flexible plastic isolators (Standard Safety Equipment Co., Palatine, IL). Germ-free status of the animals was verified biweekly and at the termination of the experiment (15). Conventional rats were housed 3/cage in plastic cages (19 × 10.5 × 8 in.) with stainless steel tops.

At the onset of the experiment, rats were randomly fed a control low-fat diet (LF) (5% corn oil) or a high-fat (HF) (20% corn oil) diet (Table I). Food and water were available ad libitum. All animals were checked twice daily to ensure constant supply of food and water.

Biochemical Studies

Animals were killed by decapitation after an 8-month exposure to the various dietary regimens. The livers were quickly removed,

[1]To whom reprint requests should be sent.

TABLE I

Composition of Semipurified Diets

Diet composition	Low fat (g/100 g diet)	High fat (g/100 g diet)
Casein (vitamin-free)	22.0	22.0
DL-Methionine	0.3	0.3
Cornstarch	58.7	43.7
Alphacel	7.0	7.0
Corn oil	5.0	20.0
Salt mix[a]	4.5	4.5
Vitamins[b]	2.5	2.5

[a]The salt mix contained (in %) sodium chloride, 13.9325; potassium phosphate, 38.8967; magnesium sulfate, 2.292; calcium carbonate, 38.142; ferrous sulfate, 2.696; potassium iodide, 0.079; magnesium sulfate, 0.9453; zinc chloride, 0.0259; copper sulfate, 0.0475; and cobalt chloride, 0.0022.

[b]The vitamin mixture (/100 g) consisted of: vitamin A (200,000 IU/g); 50 mg vitamin D concentrate (400,000 IU/g); 11.1 mg α-tocopherol; 100 mg ascorbic acid; 11.1 mg inositol; 167 mg choline chloride; 5 mg menadione; 11.1 mg pyrioxine hydrochloride; 2.2 mg thiamin hydrochloride; 6.7 mg calcium pantothenate; 0.04 mg biotin; 0.2 mg folic acid and 0.003 mg vitamin B_{12}.

rinsed 3 times in ice-cold 0.9% NaCl, gently blotted on cheesecloth and weighed. The entire small intestine or colon was quickly removed, freed from adhering material, cut longitudinally with a pair of scissors, washed 3 times with ice-cold 0.9% NaCl and gently blotted dry on cheesecloth. The tissues were laid out on a flat, ice-cold surface and the mucosal layer scraped with the aid of a microscope slide. Mucosal scrapings from 2 rats were pooled and weighed.

Microsomal pellets were prepared by a modified method of Goldfarb and Pitot (13). Each liver was minced with a scissors and homogenized in 3.5 vol of ice-cold buffer (50 mM potassium phosphate buffer, pH 7.3, containing 0.25 M sucrose, 10 mM EDTA and 10 mM glutathione) using a Williams Polytron. Mucosal scrapings were homogenized in 3.5 vol of ice-cold buffer of similar composition to that used for liver homogenization, except trypsin inhibitor (25 mg%, type 1S, Sigma Chemical Corp., St. Louis, MO) was added to intestinal and colonic buffer to prevent enzyme inactivation by pancreatic enzymes.

The liver, small and large intestinal mucosal homogenates were centrifuged in a Sorvall RC-2B centrifuge at 10,000 × g and 4 C for 20 min. The supernatant was freed of any existing floating layer of fat and the 10,000 × g postmitochondrial supernatant was recentrifuged in a Spinco ultracentrifuge at 105,000 × g and 4 C for 1 hr. The resulting microsomal pellet was resuspended in 0.2 times the original vol of homogenization buffer. Microsomal pellet suspensions were frozen at −60 C in a Revco ultra-low freezer for no longer than 1 wk. Preliminary studies indicated that HMG CoA reductase activity, cytochrome P_{450} and cytochrome b_5 activity is stable for this amount of time. Microsomal protein content was determined by the method of Lowry et al. (16).

HMG-CoA Reductase Activity

Frozen microsomal pellet suspensions were brought to room temperature and analyzed for HMG CoA reductase activity (EC 1.1.1.34) by an adaptation of the methods of Bochenek and Rogers (17) and Goldfarb and Pitot (13). Aliquots containing 0.2–0.5 mg of microsomal protein/ml were placed in 1 ml of 0.1 M potassium phosphate buffer, pH 7.3, with a final concentration of 10 mM glucose 6-P, 50 mM glutathione, 3 mM NADP, 3 mg of soybean trypsin inhibitor type 1-S, 0.2 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Corp., St. Louis, MO), 0.1 M EDTA and were equilibrated at 37 C for 15 min in a Dubnoff Metabolic shaking incubator. About 9,000 dpm of DL-hydroxy [3-^{14}C] methylglutaryl-CoA (New England Nuclear, Boston, MA) was added to each incubation tube. After a 30-min incubation period at 37 C, 0.3 ml of 6 N HCl was added to each tube to lactonize the mevalonate, followed by addition of ca. 2.2 × 10^6 dpm of [H^3]-DL-mevalonic acid (New England Nuclear, Boston, MA). All samples were incubated an additional 30 min at 37 C, capped and refrigerated overnight.

The mevalonic acid lactone was extracted using 3 successive 3-ml aliquots of petroleum ether and, was evaporated to dryness in a water bath at 37 C under a stream of nitrogen gas. The dried samples were taken up in 0.2 ml of acetone, spotted on silica 250 GF plates and developed with acetone/benzene (1:1, v/v). The mevalonic acid lactone was isolated and scraped directly into scintillation vials containing 15 ml of Scintisol Complete and ^{3}H and ^{14}C was determined simultaneously in an Intertechnique Liquid Scintillation Counter, Model SL-36. Quenching was corrected for using the external standard method. All samples were assayed in duplicate at 2 concentrations to ensure linearity.

Cytochromes P_{450} and b_5

The level of hepatic, small and large intestinal cytochrome P_{450} was measured by the McClean and Day method (18). Cytochrome b_5 levels were measured by the Omura and Sato method (19). Liver microsomal suspensions used for analysis contained ca. 3 mg protein/ml

TABLE II

HMG CoA Reductase* Activity in Germ-Free and Conventional Rats Fed Low-Fat and High-Fat Diets

	Liver	Smlll intestine**	Colon**
Low-fat germ-free	2.18 ± 0.20[a]	10.50 ± 1.46[a]	12.80 ± 2.89[a]
High-fat germ-free	1.89 ± 0.30[b]	18.26 ± 2.70[b]	8.46 ± 1.00[a]
Low-fat conventional	2.80 ± 0.71[c,d]	3.43 ± 1.10[c]	8.72 ± 1.10[a]
High-fat conventional	3.27 ± 0.66[a,d]	3.20 ± 1.10[c]	7.95 ± 1.48[a]

[a–d]Values in the same column with different superscript letters are significantly different.

*Each value is the mean of 12 animals ± the standard error of the mean of the nmol of mevalonate formed/mg of microsomal protein. Statistical significance was determined by a Direct Difference Student's t-ratio at the 95% confidence interval.

**Mucosa from 2 rats were pooled for analysis.

whereas intestinal and colonic microsomal mucosal suspensions contained ca. 6 mg protein/ml. All measurements were made on an Aminco DW-2a recording spectrophotometer.

Statistical Significance

Statistical significance at the $p<0.05$ level was determined by a Direct Difference Student's t ratio (20). This particular statistical design was chosen because it enabled us to determine the role of diet and intestinal microflora on HMG CoA reductase activity without confounding the results with the strong diurnal rhythm in HMG CoA reductase activity (7). Germ-free and conventional rats were killed simultaneously in matched pairs. Statistical significance was established by a comparison of the difference in enzyme activity between a particular germ-free rat and its conventional matched pair, and not by comparing the mean enzyme activity for the entire germ-free and conventional group.

RESULTS

Table II shows HMG CoA reductase activity in germ-free and conventional rats fed low-fat and high-fat diets. Hepatic HMG CoA reductase activity was significantly higher in the low-fat, germ-free than in the high-fat, germ-free group. Germ-free animals had significantly lower hepatic HMG CoA reductase activity than conventional animals when exposed to both low-fat and high-fat diets. HMG CoA reductase activity was not significantly different between low-fat and high-fat conventional rats in any of the tissues studied. Small intestinal HMG CoA reductase activity was significantly higher in the high-fat, germ-free than in the low-fat, germ-free group. High levels of dietary fat did not significantly alter colonic HMG CoA reductase activity in any of the groups studied.

Hepatic cytochrome P_{450} and cytochrome b_5 levels in germ-free and conventional rats are shown in Figure 1. HF GF group had significantly elevated cytochrome P_{450} and cytochrome b_5 levels than all other experimental groups. Figure 2 shows that small-intestinal cytochrome P_{450} levels were significantly elevated in the HF GF than in the LF GF and LF C groups. In addition, HF GF rats demonstrated a significant elevation in cytochrome b_5

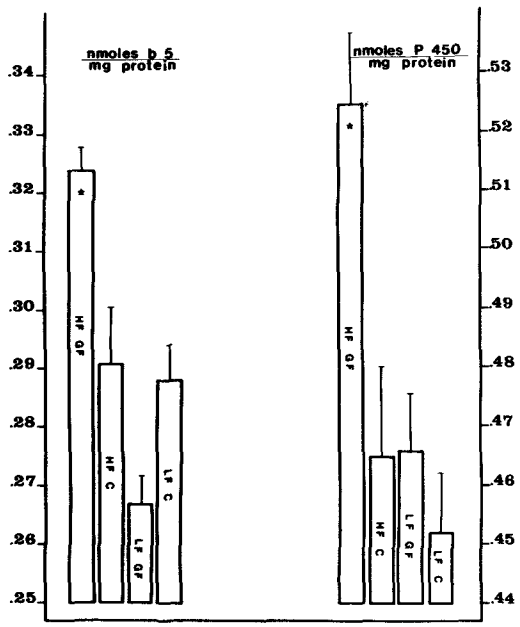

FIG. 1. The effect of high-fat diets and intestinal microflora on hepatic P_{450} and cytochrome b_5 levels. Each bar represents the mean ± the standard error of the mean of 12 livers. A single star denotes that the HF GF group had significantly elevated cytochrome P_{450} and cytochrome b_5 levels than all other experimental groups.

levels compared to the HF C group. Figure 3 shows a significant elevation in colonic cytochrome P_{450} levels in the HF C group compared to all other experimental groups.

DISCUSSION

Results from this study suggest that dietary fat differentially affects HMG CoA reductase activity in the liver and small intestine of germ-free and conventional rats. We found a 15% increase in hepatic HMG CoA reductase activity in the high-fat, conventional group when compared to the low-fat, conventional group. These results are substantiated by Goldfarb and Pitot (13), who reported that conventionally maintained rats fed a 20% corn-oil diet and killed at a similar biorhythmic interval demonstrated 20% higher hepatic HMG CoA reductase activity than control rats maintained on a 5% corn-oil diet.

We know of no previous studies that have examined the interaction of dietary fat, intestinal microflora and HMG CoA reductase activity. We found that feeding a 20% fat diet led to a significant decrease in HMG CoA reductase activity in small intestinal mucosal preparations from germ-free rats, but not conventional rats fed a high-fat diet. A reported increase in the lifespan of the villous fraction of intestinal mucosal cells in germ-free rats might, in part, be responsible for the observed increase in HMG CoA reductase activity (21). Studies with isolated mucosal cells of villous and crypt origin have shown that feeding a high-fat diet caused a significant increase in HMG CoA reductase activity in the villous fraction, but not in the crypt fraction of the small intestine (22).

The liver, as well as the small intestine, exhibits diet-responsive changes in HMG CoA reductase activity (23, 24). Hepatic HMG CoA reductase activity was higher in conventional rats than in germ-free rats, regardless of the level of fat in the diet. However, previous reports have indicated that hepatic cholesterol levels are significantly lower in conventional rats compared to values found in germ-free animals (25). This could be attributed to a

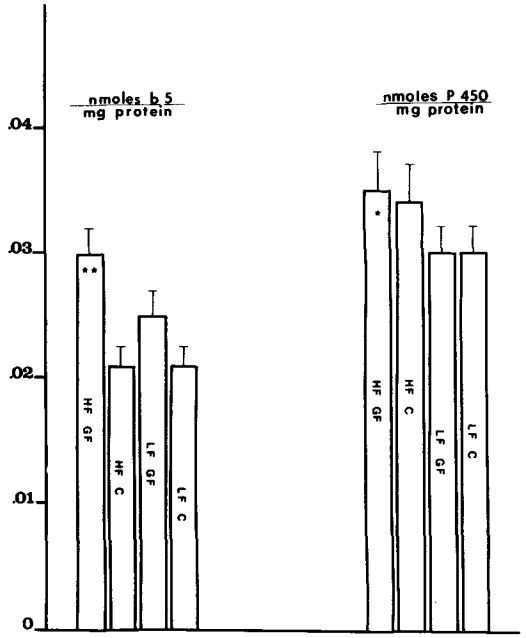

FIG. 2. The effect of high-fat diets and intestinal microflora on small intestinal cytochrome P_{450} and cytochrome b_5 levels. Each bar represents the mean ± the standard error of the mean of pooled mucosa from 12 rats. A single star denotes that the HF GF group had significantly higher cytochrome P_{450} levels than the LF GF and LF C groups. A double star denotes that the HF GF group had significantly elevated cytochrome b_5 levels compared to the HF C and LF C groups.

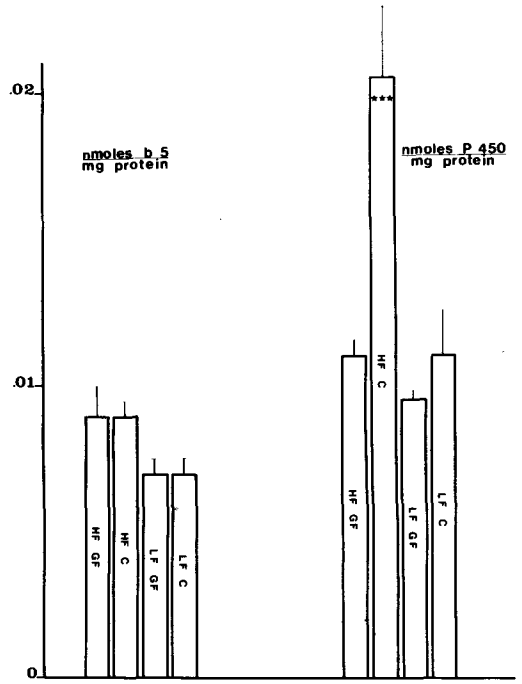

FIG. 3. The effect of high-fat diets and intestinal microflora on colonic cytochrome P_{450} and cytochrome b_5 levels. Each bar represents the mean ± the standard error of the mean of the pooled mucosa from 12 rats. A triple star denotes that the HF C group had significantly higher cytochrome P_{450} levels than all other experimental groups.

reported increased conversion of cholesterol to bile acids and neutral sterols such as coprostanol (2,13,26–28) occurring in conventional rats (29), rather than in increased rate of hepatic cholesterogenic activity. This is supported by data from Lakshmanan et al. (30) who showed that the conventional rat model diet-induced increase in HMG CoA reductase activity was accompanied by a decline in hepatic cholesterol levels.

Several investigators have shown that cytochrome P_{450} plays a role in the 7α-hydroxylation of cholesterol to bile acids (31). In addition, cytochrome P_{450} is involved in other types of hydroxylation reactions, such as drug-metabolizing reactions (6,32). Studies have shown that the ability of hepatic cytochrome P_{450} to detoxify potential carcinogens is related to the type of diet fed (14,33,34). Recent evidence suggests that the small intestine and colon of rats contain a mixed-function oxidase system capable of metabolizing carcinogens (33, 35).

Studies by Norred and Wade (14) demonstrated that the content of hepatic cytochrome P_{450} increased as the level of dietary fat was increased. However, they did not examine the role of dietary fat on extrahepatic, mixed-function oxidase systems. We found cytochrome P_{450} levels were elevated in all high-fat-fed groups in all tissues studied compared to values observed in the appropriate low-fat control group.

Reddy et al. (36) reported that the incidence of 1,2-dimethylhydrazine-induced colorectal tumors was higher in rats maintained on a 20% corn-oil diet than in rats fed a 5% corn-oil diet. Results from the present study showed a trend toward elevated cytochrome P_{450} levels in all high-fat-fed animals, regardless of the tissue studied. Wattenberg (6,32) has suggested that the mixed function oxidase system could act either as an initial defense against dietary carcinogens by detoxifying carcinogens or could play an activating role by metabolically converting procarcinogens to their proximate reactive form. Because rats fed a high-fat diet appear to be more susceptible to chemically induced colon cancer (15), it is possible that, during high-fat feeding, the mixed function oxidase system plays an activating role, rather than a detoxificating role. Recent studies by Strobel et al. (5) demonstrated that the colon contains a mixed-function oxidase system capable of activating carcinogens. However, additional studies are necessary to elucidate the mechanism by which dietary fat alters the functioning of the mixed-function oxidase system.

Results from this study suggest that a relationship exists between high levels of dietary fat, the microsomal mixed-function oxidase system, cholesterol synthesis and intestinal microflora. These factors should be considered potential risk factors associated with colon carcinogenesis.

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Sulfite-Induced Lipid Peroxidation

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ABSTRACT

Sulfite initiated the peroxidation of linoleic acid and linolenic acid emulsions via a free radical mechanism. Peroxidation of these fatty acids required oxygen and sulfite and occurred with concomitant oxidation of sulfite to sulfate. In reaction mixtures containing linoleic acid, the formation of conjugated diene equaled the formation of hydroperoxide. In reaction mixtures containing linolenic acid emulsions, thiobarbituric acid reactive materials were also formed. Peroxidation was pH-dependent; peroxidation of linoleic acid proceeded between pH 4 and 7, but linolenic acid peroxidation was significant only if pH was below pH 6. The linoleic acid hydroperoxides thus formed were reduced and methylated to methyl hydroxystearate. Analysis of methyl hydroxystearate by gas chromatography-mass spectrometry indicated that sulfite-induced peroxidation gave rise to the 9- and 13-hydroperoxy isomers. In addition to the hydroperoxides, sulfite adducts were detected. Hydroquinone, butylated hydroxytoluene and α-tocopherol effectively inhibited both sulfite oxidation and hydroperoxide formation. Conjugated diene formation also was inhibited by 4-thiouridine, suggesting that the reaction is mediated by the sulfite radical. No significant inhibition was observed with the addition of superoxide dismutase, catalase, or the hydroxyl radical scavengers, mannitol or *t*-butanol. A possible mechanism is presented to account for sulfite-induced peroxidation of linoleic acid.

INTRODUCTION

The oxygen-catalyzed addition reaction of sulfite (sulfite will be used to designate all dissolved species of SO_2, HSO_3^- and SO_3^{2-}) to unsaturated fatty oils and esters has been known for some time. It has been suggested that the addition involves a free radical reaction which is analogous to the addition of SO_3^{2-} to alkenes (1, 2). That the SO_3^{2-} addition to polyunsaturated fatty acids (PUFA) is accompanied by peroxidation is indicated by the report that the sulfitation of unsaturated oils under certain conditions leads not only to the formation of sulfonated products, but also oxidized fatty acid derivatives (3). Sulfite-induced peroxidation of PUFA is further suggested by the detection of thiobarbituric-acid-reacting material (TBARM) in corn oil emulsions treated with sulfite (4).

Sulfite can undergo aerobic oxidation to sulfate via a free radical mechanism which forms oxidizing free radicals (5–8) involved in the oxidation of a variety of biologically important compounds (8–12). This study was carried out to determine the role of sulfite in initiating the peroxidation of linoleic acid (LH) and linolenic acid (LnH) and to characterize the reactions involved.

PROCEDURES

Materials

All reagents were of the highest chemical purity available, unless specified otherwise. [U-^{14}C] Linoleic acid was obtained from New England Nuclear and sodium [^{35}S] sulfite from Searle.

Reactions were done in 125-ml Erlenmeyer flasks, which contained emulsions consisting of 0.9 mM LH or LnH, 0.1% Tween-20, 0.1 M phosphate buffer, 0.1 mM EDTA, and 0.33 mM sulfite, in a total vol of 7 ml. Reaction was initiated by the addition of sulfite to the emulsions. Unless otherwise specified, LH emulsions were buffered at pH 7 and LnH were added as ethanolic solutions, thus giving a final concentration of 0.2 M ethanol in the reaction mixtures.

Conjugated diene (CD) concentrations were estimated from the absorbance at 232 nm (13) and hydroperoxides by the ferrous thiocyanate assay (14). Linoleic acid hydroperoxide (LOOH) was prepared by reacting LH with soybean lipoxygenase (15). The course of LnH peroxidation was followed by measuring TBARM according to the Dahle et al. method (16). Sulfite concentration was determined spectrophotometrically with 5,5′-dithiobis(2-nitrobenzoic acid) reagent (17). The UV absorption spectra of diluted aliquots of the reaction mixture were obtained with a scanning spectrophotometer. Anaerobic reactions were conducted in Thunberg tubes which were alternately evacuated and flushed with nitrogen prior to mixing of reactants. After mixing, the reactants were incubated for 15 min under a continuous steam of nitrogen.

Thin layer chromatography (TLC) was carried out by spotting reaction mixtures containing either labeled or unlabeled LH on silica gel plates (Bakerflex IB or IB-F), using hexane/ether/acetic acid (9:1:1, v/v) as developing solvent. Radioactive loci on developed chromatograms were located and relatively

quantified with a radioscanner.

Inhibition studies were conducted by adding the appropriate inhibitors as ethanolic or aqueous solutions to the reaction mixture and following the absorbance at 232 nm or the radioactivity in the unreacted LH after TLC separation.

LOOH formed in the reaction mixtures was separated by TLC and eluted with 95% ethanol. The LOOH was then reduced to hydroxylinoleic acid with $NaBH_4$, followed by methylation with diazomethane. The methyl hydroxylinoleates were then hydrogenated to methylhydroxystearates at atmospheric pressure using Pd black as catalyst. Gas chromatography-mass spectrometry (CG-MS) analysis of the positional isomers of the methyl hydroxystearates was done using a Varian 2700 equipped with a 3% QF-1 column and a flame ionization detector coupled to a high resolution DuPont 492 dual beam magnetic analyzer. Identification of the isomers was based on the peaks corresponding to the following m/e ratios: 155, 158 and 187 for the 9-isomer, and 211, 214 and 243 for the 13-isomer (18).

For analysis of the hydrocarbons evolved from the mixtures, reactions were done in stoppered Erlenmeyer flasks. A 2-ml gas sample was withdrawn from the headspace with a syringe and injected into a gas chromatograph equipped with an alumina column and a flame ionization detector.

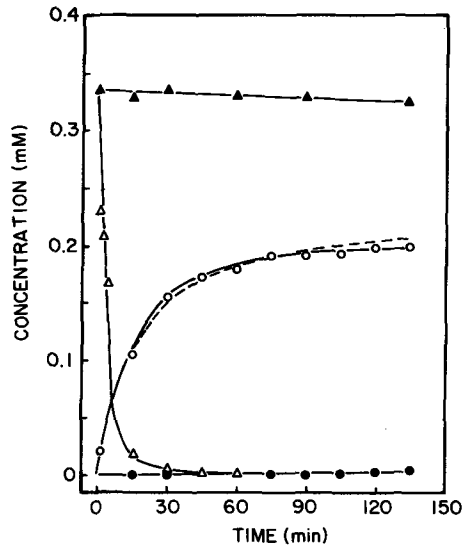

FIG. 1. Time course of CD and LOOH formation and SO_3^{2-} consumption. CD concentrations in the complete reaction mixture (○) and control (●) (no added SO_3^{2-}). SO_3^{2-} concentrations in the complete reaction mixture (△) and blank (▲) (no LH). ----- Represents LOOH concentrations as measured by the ferrous thiocyanate assay carried out on complete reaction mixtures in which more than 90% of the SO_3^{2-} had been oxidized. CD and SO_3^{2-} concentrations represent the averages obtained from 6 runs.

RESULTS

The UV spectra of aliquots withdrawn from the LH and LnH reaction mixtures exhibited an absorption band at 232 nm which has been attributed to the presence of CD (13). Controls consisting of emulsions incubated without sulfite exhibited no spectral change after 1 hr.

Figure 1 shows the progress of CD formation and the disappearance of sulfite in LH emulsions buffered at pH 7. The rate of CD formation was greatest during the first 5 min of incubation and declined considerably after 30 min when more than 95% of the sulfite initially added had been consumed. CD concentration leveled off after all the sulfite had been oxidized. Using a molar absorptivity of 26,000, CD concentration was estimated to be 0.12 mM and 0.19 mM after 15 and 45 min, respectively. The 0.19 mM represents 21% of the LH initially present in the mixture. As the initial concentration of sulfite was 0.33 mM, it is estimated that there was 0.64 mol of CD formed/mol of sulfite oxidized. That the reaction products were conjugated diene hydroperoxides was indicated by the observation that the molar concentration of CD equaled that of peroxide as determined by the ferrous thiocyanate reagent (Fig. 1). The requirements for sulfite and oxygen for the formation of CD is shown in Table I; when one of these reaction components was omitted, there was little formation of CD. Under a nitrogen atmosphere, both sulfite oxidation and CD formation was inhibited. It should be noted that in the absence of LH, sulfite oxidation did not proceed.

The optimal pH for CD formation from LH was 6 (Fig. 2), and there was a sharp drop in

TABLE I

Oxygen Requirement of the Sulfite-Induced Peroxidation of LH (incubation time 15 min)

Composition of emulsion	Increase in conjugated diene (mM)	SO_3^{2-} consumed (%)
In air		
Complete	0.125	91
SO_3^{2-} omitted	0.001	–
LH omitted	–	3
Under N_2		
Complete	0.004	8

CD formation when the pH was above 7. More than 90% of the sulfite initially added was consumed after 15 min at pH 7 or lower. At pH 8, however, sulfite consumption dropped to less than 20% and there was a corresponding decrease in CD formation. In contrast to LH peroxidation, significant LnH peroxidation occurred only below pH 6. Figure 3 shows the time course of CD formation and sulfite oxidation in LnH emulsions incubated at pH 7 or 4.5. Under the standard reaction conditions, the maximal peroxidation of LnH was only half that of LH (Figs. 1 and 3). The time course of TBARM formation from LnH emulsions in the presence or absence of sulfite is illustrated in Figure 4. In the absence of sulfite, there was very little TBARM formation. At the end of a 60 min incubation period, the amount of TBARM formed (expressed as malonaldehyde) was 66 nmol, which was equivalent to 9% of the CD formed during the same incubation period. When LH was employed, there was no significant production of TBARM.

Hydroperoxides are known to react with sulfite at low pH via a homolytic mechanism (19), and it is therefore important to determine whether the observed reaction was initiated by the contaminating hydroperoxides in the LH samples used. LH samples were treated with $NaBH_4$ to reduce any contaminating hydroperoxides. $NaBH_4$-treated LH underwent peroxidation in the presence of sulfite, although at an initial rate that was 10% less than that observed with the nontreated samples. Reaction

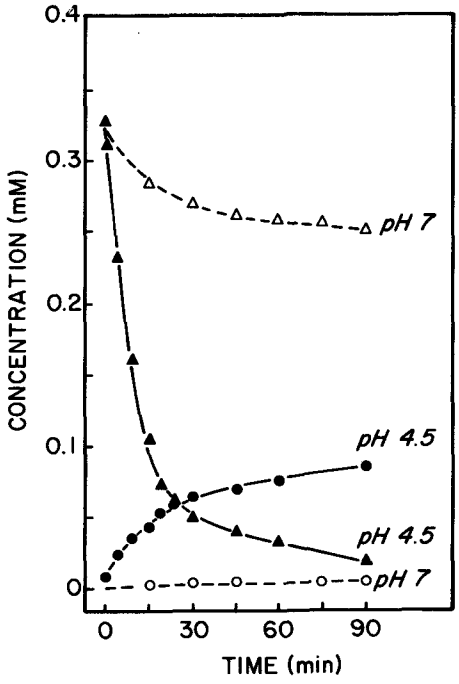

FIG. 3. Time course of CD (○, ●) formation and SO_3^{2-} (△, ▲) consumption in LnH emulsions at pH 7 or 4.5.

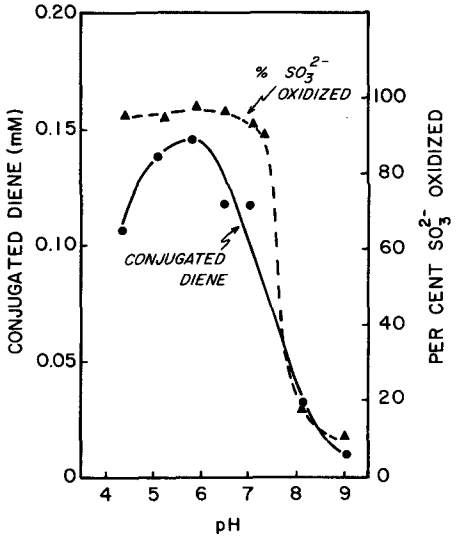

FIG. 2. Effect of pH on CD formation and SO_3^{2-} consumption in LH emulsions. Incubation time was 15 min.

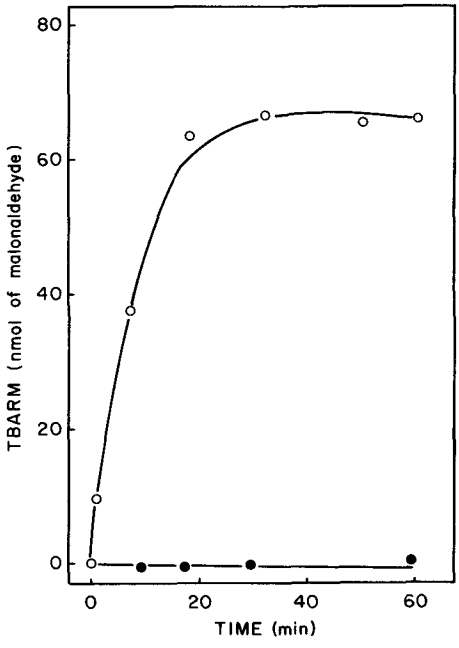

FIG. 4. Time course of sulfite-induced formation of TBARM from LnH emulsions incubated with (○) and without (●) added SO_3^{2-}. Reaction mixtures containing 0.9 mM LnH were buffered at pH 4.7.

mixtures containing 0.4 mM [U-^{14}C] LH (50 nCi) and 0.4 mM sulfite in a total vol of 0.1 ml were incubated for 45 min and analyzed by thin layer radiochromatography. Two radioactive peaks were found, in addition to the one identified as unreacted LH (R_f = 0.40): Peak I remained at the origin and Peak II cochromatographed with LOOH (R_f = 0.20). Peak I represented 17% of the total radioactivity applied. By paper electrophoresis at pH 2.1, it was resolved as 2 components: one (IA) which remained at the origin and the other (IB) which migrated toward the anode with a mobility of 0.76 relative to DNP-cysteic acid. When $Na_2{}^{35}SO_3$ was employed, IB became labeled, indicating that it was a sulfite adduct. Component II gave a positive test with ferrous thiocyanate reagent and represented 25% of the total radioactivity applied. Successive reduction, methylation and hydrogenation of II gave a mixture of methyl hydroxystearates, which were found to consist of the 9- and the 13-hydroxy isomers by GC-MS analysis.

Analysis for pentane hydrocarbons in the headspace of the reaction mixture containing LH revealed that a small quantity of pentane (5.1 pmol) was produced. When LnH was employed, approximately equal amounts (0.6 nmol) of ethane and ethylene were found.

The formation of CD from LH was dependent on sulfite concentration (Fig. 5). After a 15-min incubation period, the CD concentration increased as the initial sulfite concentration was increased up to ca. 0.33 mM. With increasing sulfite concentrations above 0.33 mM, CD production decreased. This decrease in CD formation could not be attributed to the failure of sulfite oxidation, because most of the sulfite, independent of its initial concentration, was oxidized during the incubation period (Fig. 5). Radiochromatography revealed that increasing the sulfite concentration led to increased radioactivity in Peak I and a decrease in Peak II.

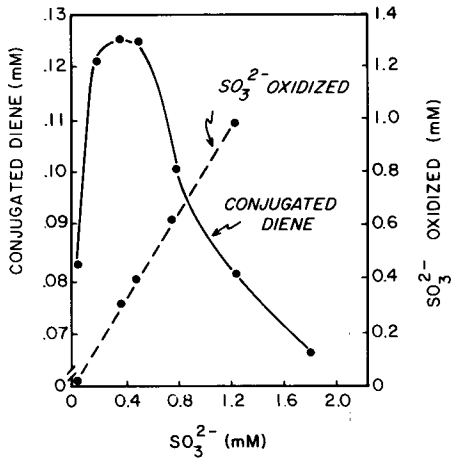

FIG. 5. Effect of the initial $SO_3{}^{2-}$ concentration on CD formation and on $SO_3{}^{2-}$ oxidation. A fixed concentration of LH (0.9 mM) was incubated 15 min with various initial concentrations of sulfite.

As trace metals are known to catalyze the autoxidation of PUFA (20), EDTA (0.1 mM) was routinely added to the reaction mixtures to eliminate possible interference by trace metals. The course of the reaction was not significantly altered, however, when EDTA was omitted from the mixture. The substitution of Tris buffer for phosphate had no effect.

To identify the reactive species which might be involved in the sulfite-induced peroxidation of LH, the effect of various inhibitors was studied. Ethanol, *t*-butanol and mannitol are known hydroxyl radical scavengers. In this study, LH was added to the reaction mixture as an ethanolic solution, providing a final ethanol concentration of 0.2 M. When ethanol was deleted from the reaction mixture, CD production after a 45-min incubation was 15% lower than that in the presence of 0.2 M ethanol, indicating that ethanol did not inhibit CD formation. Neither *t*-butanol nor mannitol inhibited CD formation. Superoxide dismutase (SOD) at concentrations of 7–33 μg/ml also was ineffective as an inhibitor, suggesting that CD formation is not mediated by the superoxide radical. No inhibition by catalase was observed when used alone or in combination with SOD. Sodium formate, which effects the conversion of OH to O_2^-, did not enhance nor inhibit CD formation when used at concentrations ranging from 0.1 mM to 0.1 M. The singlet oxygen scavenger, 2,5-dimethylfuran, exhibited no effect on LOOH formation as revealed by radiochromatograms of reaction mixtures containing [U-^{14}C] LH. No significant inhibition of sulfite oxidation was observed when any of these inhibitors were tested.

In contrast, the free radical scavengers, hydroquinone, butylated hydroxytoluene (BHT) and α-tocopherol, were effective inhibitors of both CD formation and sulfite oxidation (Fig. 6).

Sulfite is known to react with 4-thiouracil derivatives in an oxygen-mediated radical reaction. On the basis of the inhibitory effects obtained with hydroquinone, Hayatsu and Inoue (21) suggested that the reaction involves the addition of the SO_3^- radical. The inclusion of 4-thiouridine at a final concentration of 0.1 mM resulted in a 50% inhibition of CD formation, indicating involvement of SO_3^- in the sulfite-induced peroxidation of LH.

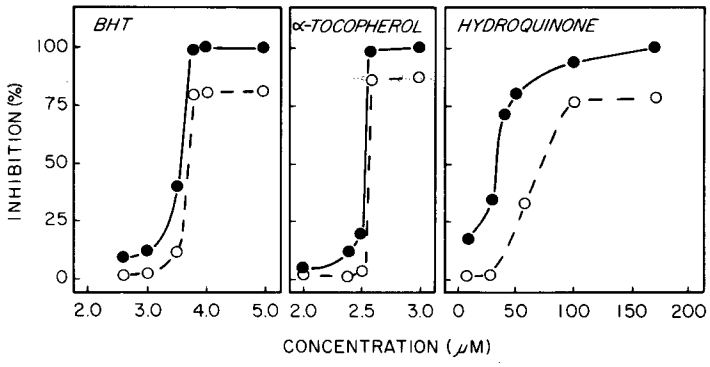

FIG. 6. Effect of radical scavengers on CD formation (○) and SO_3^{2-} oxidation (●). Complete reaction mixtures were incubated for 15 min.

DISCUSSION

The reactive free radicals O_2^-, OH and SO_3^- are generated during the aerobic oxidation of sulfite to sulfate (5, 6). One or more of these species has been implicated in the cooxidation of methionine (8), indole compounds (9, 10) reduced pyridine nucleotides (11) and pyrimidine ribonucleosides (12). Our present observations tend to preclude the suggestion of involvement of OH radicals in the sulfite-induced LH peroxidation. The failure of SOD to inhibit LH peroxidation in this system might be attributed to the fact that it is not as lipid-soluble as α-tocopherol, BHT, or hydroquinone, which were found to be effective inhibitors of the reaction. However, the absence of inhibition by SOD of sulfite oxidation is consistent with the view that sulfite is aerobically oxidized chiefly via a nonsuperoxide-mediated mechanism. From spectroscopic studies, Hayon et al. (7) proposed a mechanism of sulfite oxidation which does not involve the participation of O_2^- in the initiation or propagation step:

Initiation:	SO_3^{2-}	$\rightarrow SO_3^- + e^-$	[I]
	$SO_3^- + O_2$	$\rightarrow SO_5^-$	[II]
Propagation:	$SO_5^- + SO_3^{2-}$	$\rightarrow SO_4^- + SO_4^{2-}$	[III]
	$SO_4^- + SO_3^{2-}$	$\rightarrow SO_4^{2-} + SO_3^-$	[IV]

Although these proposals were based on results obtained from the photooxidation of SO_3^{2-} solutions in which SO_3^- formation is effected by light, Hayon et al. proposed that the process also can be achieved by thermal autoxidation by electron transfer, such as to a metal (Eq. I). Although EDTA was routinely added to all reaction mixtures, metal involvement was not precluded, because complexed metals can still participate in electron transfer processes (22). Alternatively, the formation of initiating radicals may be achieved by reaction between preexisting LOOH and sulfite as expressed by Equation VI. The involvement of Equation VI is consistent with the observation that $NaBH_4$ treatment of LH samples led to a decrease in the initial rates of both CD formation and SO_3^{2-} oxidation.

Initiation:	$HSO_3^- + M^{n+}$	$\rightarrow$	
	$SO_3^- + M^{(n-1)+} + H^+$		[V]
	$HSO_3^- + LOOH$	$\rightarrow$	
	$LO\cdot + SO_3^- + OH^-$		[VI]
Propagation:	$SO_3^- + LH$	$\rightarrow \cdot LHSO_3^-$	[VII]
	$\cdot LHSO_3^- + HSO_3^-$	$\rightarrow$	
	$LH_2SO_3^- + SO_3^-$		[VIII]
	$SO_3^- + LH$	$\rightarrow$	
	$\cdot L + HSO_3^-$		[IX]
	$\circ L + O_2$	$\rightarrow LOO\cdot$	[X]
	$LOO\circ + LH$	$\rightarrow$	
	$LOOH + \circ L$		[XI]
Termination:	$\circ L + \circ L$	$\rightarrow LL$	[XII]
	$LOO\circ + L\cdot$	$\rightarrow LOOL$	[XIII]

In emulsions containing nontreated LH, this reaction would be important during the early stages of the reaction when SO_3^{2-} concentration is high. Once the chain-initiating species (SO_3^- and SO_5^- from Eqs. I and II) are formed, chain-propagating reactions are maintained with the concomitant formation of sulfate via Equations III and IV, of $LH_2SO_3^-$ (compound I) via Equations VII and VIII, and of LOOH via Equations IX–XI. In these reactions, it is assumed that SO_3^- serves as the common carrier. Such a view is consistent with the present observations that these reactions are intimately interrelated and are inhibited by the same inhibitors. Equations VII and VIII are reactions analogous to those which have been postulated for the addition of SO_3^{2-} to alkenes (23, 24). The ability of SO_3^- to abstract a hydrogen atom from reduced nicotinamide adenine dinucleotide (NADH) during the

sulfite-mediated oxidation of NADH to NAD has been demonstrated (11). Similarly, SO_3^- abstracts a hydrogen atom from LH to form L• radical (Eq. IX), which, in turn, undergoes oxidation as depicted by Equations X and XI. We have shown that the formation of I (LH_2SO_3) is favored over the formation of LOOH as the concentration of sulfite is increased in the reaction mixture. This may be explained on the basis of relative rates: as the sulfite concentration is increased, the overall reaction rates of Equations VII and VIII are increased more than those of Equations IX–XI.

LH was readily oxidized at both pH 7 and pH 4.5, with concomitant oxidation of sulfite to sulfate. In contrast, peroxidation of LnH and the cooxidation of sulfite occurred at pH 4.5, but both processes were suppressed at pH 7. It is possible that the occurrence of sulfite oxidation was dependent on the peroxidation of LnH. The kinetics of peroxidation in aqueous systems has been shown to be dependent on the state of aggregation of fatty acids (25). The low pH requirement of sulfite-induced peroxidation of LnH may thus result from the pH dependence of the aggregation of LnH in the emulsion.

The inhibition observed upon the addition of hydroquinone, BHT, or α-tocopherol can be attributed to the ability of phenolic compounds to act as radical chain-terminating antioxidants (26). Although α-tocopherol is known to scavenge singlet oxygen, its participation can be excluded from consideration based on the observation that 2,5-dimethylfuran failed to inhibit LH peroxidation. The formation of the 9- and 13-hydroperoxyisomers detected in the reaction mixture cannot be attributed to the direct participation of singlet oxygen, because $^1O_2$ is known to give rise to 10- and 12-hydroperoxyisomers in addition to the 9- and 13-isomers when reacting with double bonds of LH (27, 28).

This investigation shows that sulfite can cause peroxidation of PUFA via a free radical mechanism. These observations suggest the possibility that SO_2/sulfite can inflict biological damage in vivo by initiating peroxidation of PUFA, particularly in membranes.

ACKNOWLEDGMENTS

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Metabolism of Sterols by Anaerobic *Saccharomyces cerevisiae*

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ABSTRACT

Anaerobically grown *Saccharomyces cerevisiae* retained the ability to transfer a C_1-group to the C-24 position of a $\Delta^{24(25)}$-sterol and to reduce the $\Delta^{24(28)}$-bond of a 24-methylenesterol. Both desmosterol and 24-methylenecholesterol yielded 24β-methylcholesterol. However, when the substituent at C-24 was enlarged to a 24-ethylidene group (fucosterol), reduction of the $\Delta^{24(28)}$-bond did not occur. In no cases was a Δ^7- or a Δ^{22}-bond introduced. Because the $\Delta^{24(28)}$-bond was reduced in the absence of the Δ^{22}-bond, the Δ^{22}-bond is not an obligatory requirement for reduction.

INTRODUCTION

In the aerobic biosynthesis of sterols by *Saccharomyces cerevisiae*, there are a number of reactions which do not have any obvious requirement for oxygen. These include C_1-transfer to C-24 of a $\Delta^{24(25)}$-intermediate (1–6) and the reduction (3,7–13) of the $\Delta^{24(28)}$-bond of the 24-methylenesterol which is produced in the C_1-transfer. However, because the C_1-transferase (14) and probably the reductase are particulate, oxygen could conceivably play a secondary role in maintenance of the appropriate structures of the subcellular particles containing the enzymatic apparatus. C_1-Transfer, for instance, is known (14) to be mitochondrial and mitochondria are altered under anaerobiosis (15). In order to determine experimentally whether the absence of oxygen would prevent these 2 reactions (C_1-transfer and $\Delta^{24(28)}$-reduction), we examined the fates of 24(25)-dehydrocholesterol (desmosterol) and of 24-methylenecholesterol in *S. cerevisiae* under anerobic conditions.

MATERIALS AND METHODS

Incubations

A wild-type diploid *S. cerevisiae* (ATCC 18790) was grown for 72 h under anaerobic conditions in 1-ℓ vessels as described previously (16). With ergosterol, stationary phase was reached at 72 hr yielding 10^8 cells/ml (17). Sterols were added in a suspension of ethanol and Tween-80. The Tween-80 also served as a source of fatty acids. Final concentration was 5 mg of sterol, 2 ml of ethanol and 15 ml of Tween-80/ℓ of medium.

Reference Sterols and Substrates

Desmosterol was purchased from Applied Science Laboratories, Inc., State College, PA. 24-Methylenecholesterol was isolated from the pollen of the Saguaro cactus (18). 24-Methyldesmosterol and its Δ^{23}-isomer (24-methylcholesta-5,23-dien-3β-ol) were prepared as the acetates by isomerization of 24-methylenecholesteryl acetate with iodine by A. Friedman and M. Naftulin in work to be published separately. The structures of the acetates were demonstrated by the presence of strong allylic cleavage at C–20 (22) for the Δ^{23}-isomer and at C–22 (23) for the $\Delta^{24(25)}$-isomer during mass spectroscopy. 24β-Methylcholesterol was synthesized from ergosterol (19). Fucosterol was isolated from *Fucus vesiculosus* (20).

Isolation of Sterols

Sterols were isolated by Soxhlet extraction (19 h, acetone) and saponification (10% KOH in ethanol) of the material so obtained followed by separation from other lipids by thin layer chromatography (TLC). The amount of sterols obtained was a fraction of one mg. Quantitation was achieved by gas liquid chromatography (GLC), and the result is expressed in terms of the wt/cell. The percentage of growth was estimated from the observed growth (cells/ml) compared to that of ergosterol.

GLC was performed analytically on 1% XE-60 at 235 C and the rate of movement (RRT) is given relative to cholesterol for sterols and relative to cholesteryl acetate for steryl acetates. Silica Gel G was used for TLC, and spots were visualized with 2′,7′-dichlorofluorescein. In the absence of $AgNO_3$, the solvent was benzene/ether (90:10, v/v). When the silica gel was impregnated with $AgNO_3$ (9% w/w), the sterols were acetylated (Ac_2O, pyridine), and the solvent was chloroform/light petroleum ether/acetone/acetic acid (75:25:1:1, v/v). Ultraviolet spectra were determined in ethanol on a Cary instrument, Model 15. Cell counting and sizing was accomplished with a Coulter Model TA$_{II}$ equipped with a 16-channel population accessory. Two distributions were obtained (16,17). In one, the particle diameter (actually a range of particle diameters) was plotted against the percentage of such particles

in the population. In the other, the diameter was plotted against the contribution (as %) that such particles made to the total vol.

RESULTS

Reduction of the $\Delta^{24\ (28)}$-Bond

24-Methylenecholesterol supported growth of the yeast to the same extent as did 24β-methylcholesterol (16). At stationary phase, the cell count was 76% (standard deviation [SD] of 4%) of that obtained with ergosterol (which we used as a standard). With 24β-methylcholesterol, we obtained 75% (SD 3%). This distribution of particle diameters had a peak of 5.0–6.4 μm, and the principal contributors to total vol were cells of 10–13 μm as also happens with 24β-methylcholesterol (16).

The sterol isolated from the cells was equivalent to 2.7×10^{-12} mg/cell and was a single component by GLC (RRT 1.26), ordinary TLC (in which its rate of movement corresponded to that of 24β-methylcholesterol), and argentation TLC as the acetate (R_f 0.53). In argentation TLC, 24β-methylcholesterol also possessed R_f 0.53 as the acetate. The UV spectrum of the metabolite was devoid of absorption bands above 210 nm; this information proves that no dehydrogenations at C-7 and C-22 had occurred to give sterols with a $\Delta^{5,7}$- or $\Delta^{22,24\ (28)}$-diene system. The possibility that the product was unchanged substrate is excluded by the rate of movement on argentation TLC. 24-Methylenecholesteryl acetate was found to move R_f 0.21, and, when this region of the TLC plate was scraped and extracted, no steryl acetate was observable by GLC. The isomeric dienyl acetates ($\Delta^{5,23}$ and $\Delta^{5,24\ (25)}$) were similarly excluded. The relative rates of movement of steryl acetates in argentation TLC follow the degree of unsaturation and extent of substitution on the double bond giving the following order for R_f values: $\Delta^5 > \Delta^{5,24\ (25)} > \Delta^{5,23} > \Delta^{5,24\ (28)}$. As all of our steryl acetate was recovered from the Δ^5-region of the plate, it must have had only the Δ^5-bond. Furthermore, the RRT of the $\Delta^{5,24\ (25)}$-compound (24-methyldesmosteryl acetate) which moves closest to the Δ^5-steryl acetate in argentation TLC is 1.47. This number is in marked contrast to the observed 1.26 for the yeast metabolite. Both the RRT and the R_f of the metabolite (or its acetate) show it to be 24β-methylcholesterol (RRT 1.28; as the acetate: R_f 0.53). Although too little sample was available for nuclear magnetic resonance (NMR) spectroscopy which would have proven the configuration at C-24, there can be little doubt that it was β. It is already established (7–13) that, in aerobic *S. cerevisiae*, the methyl group arises from the methylene group, as happened in our study, and that the configuration produced is exclusively β (21). There is no reason to believe the stereochemistry of this reduction would be reversed in the absence of oxygen. Certainly, no epimerization occurs, since we have previously incubated yeast anaerobically with ergosterol and recovered it unchanged as shown by NMR (22).

C_1-Transfer to C-24 and Reduction of the $\Delta^{24\ (28)}$-Bond

Unlike the growth response to 24-methylenecholesterol, which was consistently good, yeast grew poorly and inconsistently (as little as 18% with an average of 62% compared to ergosterol) when desmosterol was the substrate. Two cultures (31 and 64%) were examined by cell-sizing and chemical isolation. The cells which had grown to the smaller population showed a distribution of particles similar to but slightly bigger than those obtained with 24-methylenecholesterol (see earlier). The principal difference was an enhancement in the percentage of particles with a diameter of 6.4–8.0 μm, which was sufficiently great to make them the peak of the distribution of particle sizes. At the larger growth, the particles were smaller (peak of 5.0–6.4 μm) and the population and vol distributions were nearly the same as with 24β-methylcholesterol, except that particles of 10–13 μm made a lesser contribution of total vol, leading to a biphasic distribution with peaks at 5.0–6.4 and 10–13 μm. The emergence of the peak at 5.0–6.4 μm was suggestive of the distribution found when ergosterol is substrate (16). We observed by spectroscopy a small amount of $\Delta^{5,7}$-sterol in the cells, so the variation in the growth almost certainly reflected, in part, the effect of trace levels of oxygen in the medium (see Discussion in relation to the importance of the 24β-methyl group).

The sterols isolated from the cells by extraction and TLC were composed in both cases (31 and 64% growth) of one major and 2 minor components as shown by GLC. The relative amounts were so similar (6–7% with RRT 1.16, 10–15% RRT 1.51, and 79–83% RRT 1.26) that the samples from the 2 cultures were combined for further study. The sterols amounted to the equivalent of 1.74×10^{-12} mg/cell. By UV analysis, 11% of the combined mixtures was $\Delta^{5,7}$-sterol, most or all of which presumably corresponded to the component with RRT 1.51 (authentic 22-dihydroergosterol, RRT 1.51). In earlier work (22) with yeast cultures grown in the presence of trace amounts of

oxygen without added sterol, 22-dihydroergosterol was identified by MS and other means as the major component. In our investigation, the components (as the acetates) with RRT 1.51 and 1.16 were located on an argentation TLC plate in the region where 24-methylenecholesteryl acetate is found. This information is consistent with the 2 minor components' being substrate (authentic desmosterol, RRT 1.16) and 22-dihydroergosterol. The material isolated from this region also displayed (unquantitated) absorption for the $\Delta^{5,7}$-chromophore.

The major component of the sterol mixture from the cells was identified as 24β-methylcholesterol in the same manner as described for the metabolite of 24-methylenecholesterol. The region of the argentation TLC plate corresponding to 24β-methylcholesterol yielded material lacking UV absorption and with an RRT (1.28) the same as that of 24β-methylcholesteryl acetate. Except for the 2 previously described minor components, extraction of the rest of the plate in increments by distance yielded no other steryl acetate.

Incubation with Fucosterol

Fucosterol induced good growth of yeast (83% compared to ergosterol). The population of cells showed a nearly symmetrical distribution with particles of 6.4–8.0 μm at the peak, but slight fragmentation occurred as shown by a few percentages of very small particles (<2.5 μm). The principal contributors to the total vol were particles of 8.0–10.0 μm.

The sterol isolated by extraction and TLC amounted to an equivalent of 0.87×10^{-12} mg/cell, had <7% $\Delta^{5,7}$-dienol as shown by UV analysis, and exhibited a single peak in GLC at RRT 1.56 (authentic fucosterol, RRT 1.60; authentic 24(28)-dihydrofucosterol, RRT 1.56). After argentation TLC of the acetylated material, and region corresponding to fucosteryl acetate yielded material with RRT 1.58, and no steryl acetate was found by GLC in the region corresponding to the dihydro derivatives, sitosteryl acetate or clinonasteryl acetates, which are inseparable by argentation TLC.

DISCUSSION

Although we were unable to detect the presence of 24-methylenecholesterol as a metabolite of desmosterol, the known reactions (23) which occur in aerobic yeast and our demonstration that both desmosterol and 24-methylenecholesterol yield 24β-methylcholesterol can leave no doubt that the sequence of events shown in Figure 1 occurred

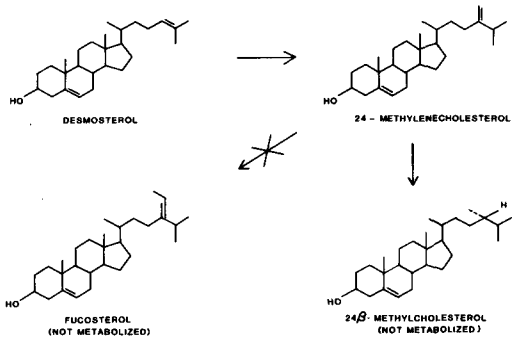

FIG. 1. Metabolism of sterols in yeast demonstrated to occur under anaerobic conditions.

anaerobically, i.e., C_1-transfer to desmosterol yielding 24-methylenecholesterol which was reduced to 24β-methylcholesterol. In the experiments with 24-methylenecholesterol as substrate, no evidence for oxygen could be obtained either in the distribution of cell sizes or in the structure of the products. Had significant amounts of ergosterol or 22-dihydroergosterol been formed by endogenous biosynthesis, we should have observed a much smaller peak (6.4–8.0 μm) in the plot of particle size against the percentage of total vol (16), and we should also have found $\Delta^{5,7}$-sterol by UV analysis (22). With desmosterol, less rigid anaerobiosis was achieved by these same parameters, but the amount of oxygen present was still very small, because only 1/10 of the sterol present possessed the $\Delta^{5,7}$-system. The amount of oxygen necessary to produce these results is in the μℓ/ℓ region (22) and is well within the limits commonly regarded as anaerobic (22).

The variable growth achieved with desmosterol finds a possible explanation, not only in the problem of varied traces of oxygen, but also in 4 earlier findings: (a) the presence of a C_1-group at C-24 is functionally important for yeast (16); (b) the principal substrate for C_1-transfer in aerobic biosynthesis is zymosterol (6, 24, 25); (c) the rate of C_1-transfer to desmosterol, which is not biosynthesized in yeast, is only half of the rate found when zymosterol is the substrate (25), and (d) the level of C_1-transferase is lowered in anaerobiosis (14) and increased by the presence of zymosterol (24). The lowest growth (18%) achieved with desmosterol probably corresponded to the most anaerobic conditions in which all of the 24-methylsterol had to arise (slowly) from desmosterol, whereas the larger growths may be explained by the presence of trace amounts of oxygen which provided zymosterol as a substrate and increased C_1-

transferase level. The resulting trace amounts of endogenously biosynthesized 24-methylsterol could then have increased the initial growth rate, but an exact definition of these complicated dynamics was beyond the scope of this investigation.

The absence of any introduction of a Δ^{22}-bond under anaerobic conditions is consistent with other evidence (26) indicating that this dehydrogenation requires oxygen. In the major biosynthetic sequence to ergosterol in aerobic *S. cerevisiae*, introduction of the Δ^5- and Δ^{22}-bonds precedes reduction of the $\Delta^{24(28)}$-bond (23), and under semianaerobic conditions in a cell-free system, the Δ^7-analog (24-methylenelathosterol, episterol) of 24-methylenecholesterol is reported to accumulate (26). If the sequence is dictated by enzymatic specificities, then our results indicate that it is the presence of the Δ^5-bond, rather than of the Δ^{22}-bond, which is the important factor in promoting reduction of the $\Delta^{24(28)}$-bond.

The addition or removal of a single carbon atom can, but does not necessarily have, a marked effect on the biological properties of sterols. Thus, if a methyl group is added to C-20 of cholesterol, there is a drastic reduction in the extent of metabolism by *Tetrahymena pyriformis* (27) and in the extent of growth support given to yeast (16). On the other hand, removal of one of the *gem*-dimethyl groups from cholesterol has little or no affect in these 2 biological properties (28, J. M. Joseph and W. R. Nes, unpublished observations). From this, it is evident that no simple rule operates which would permit a prediction as to whether the addition of a methyl group to C-28 of 24-methylenecholesterol giving fucosterol would or would not strongly change the ability of yeast to reduce the $\Delta^{24\ (28)}$-bond. Our work shows, however, that a very large influence does result, because fucosterol was not metabolized. The yeast reductase apparently has a very precise "hole" for the methylene group which is too small for the ethylidene group to fit into.

The ability of yeast to perform C_1-transfer and $\Delta^{24\ (28)}$-reduction of an appropriate substrate under anaerobic conditions shows that the integrity of the subcellular organelles which contain the appropriate enzymes for these 2 reactions is not altered by the absence of oxygen in such a way as to prevent the enzymatic apparatus from functioning.

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COMMUNICATIONS

Abnormal Lipid Composition of Fat Tissue in Human Mesenteric Panniculitis

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ABSTRACT

Mesenteric fat tissue obtained at autopsy from 6 patients with mesenteric panniculitis (MP) were found to contain significant amounts of cholesteryl esters (CE). In addition, samples from 3 of these cases were found to contain 0.5-1.3% free cholesterol, 0.9-1.9% free fatty acids (FFA), 0.6-2.5% 1-alkyl glyceryl ether diesters and small amounts of squalene. Two of these tissues also contained alk-1-enyl glyceryl ether diesters. The fatty acid compositions of the CE, FFA, triacylglycerides and glyceryl ether diesters (GEDE) were determined and oleic acid (18:1) was found to be the major fatty acid. The alkyl group composition of the GEDE consisted essentially of 16:0 and 18:0 and 18:1 carbon atoms in both types of ethers.

INTRODUCTION

Mesenteric panniculitis (MP) also known as "retractile mesenteritis," "sclerosing lipogranuloma," "xanthogranuloma" and "isolated lipodystrophy," is a form of tumor in which the fat tissue surrounding the small intestine is involved in inflammatory changes (1) and shows varied morphologic expression (2). Fresh frozen sections observed in polarized light reveals that the abnormal fat cells contain birefringent material resembling liquid crystals. These lesions have been found to be similar to bovine lipomatosis and fat necrosis (3,4). Because there are serious limitations to the histochemical methods, we have examined the tissues by direct chemical analyses. A preliminary report has been presented (5).

MATERIALS AND METHODS

Human Tissues

Twenty-nine separate specimens including mesenteric, retroperitoneal and subcutaneous fat tissue from 12 autopsy and 4 surgical cases from both sexes, 31-82-yr-olds, were received and kept frozen at -18 C and were used within 1-4 weeks for the initial screening, either by direct thin layer chromatography (TLC) of frozen sections (6) or by TLC after extraction. Autopsy samples used for further study were carefully trimmed to remove portions exposed to air and analyzed between 3 and 39 months after autopsy, except for the uninvolved retroperitoneal tissue of case A260, the analysis of which was completed after 57 months. Long-term preservation did not significantly alter the composition of the lipids (unpublished results). The tissues were classified on the basis of clinical history, gross anatomy and histologic observations.

Chemicals

Silica Gel G and Silica Gel HR were products of E. Merck AG, Darmstadt, Germany. Unisil, 100-200 mesh was obtained from Clarkson Chemical Co., Williamsport, PA. All solvents were analytical reagents from Mallinckrodt Chemical Works, St. Louis, MO. All ratios of solvent mixtures given in the text are on a volume basis.

Experimental Procedures

For the direct TLC of frozen sections, plates coated with Silica Gel G or HR in the solvent petroleum ether/diethyl ether/acetic acid (90:10:1) were used and the spots were visualized with sulfuric acid/chromic acid spray, as reported previously (6). Cholesterol and cholesteryl ester (CE) spots were further identified by spraying duplicate plates with the ferric

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chloride reagent spray as reported by Lowery (7).

For the isolation of lipids, samples of tissue (0.8-9.1 g) were homogenized with chloroform/methanol, 2:1, and filtered and the filtrates were dried in a flash evaporator. Portions of lipids (95-271 mg) were suspended in hexane and chromatographed on Unisil and 4 fractions (A-D) were collected: 7% benzene in hexane for fraction A (squalene and other hydrocarbons), 21% benzene in hexane for B (CE), 10% ether in hexane for C (triacylglycerides [TG] and glyceryl ether diesters [GEDE]) and 30% ether in hexane or chloroform for D (cholesterol and free fatty acids [FFA]). Squalene was purified by TLC on Silica Gel G (8). Squalene was also analyzed by gas liquid chromatography (GLC) and GLC-mass spectrometry (GLC-MS). Alk-1-enyl- and 1-alkyl-GEDE were isolated by TLC on Silica Gel G impregnated with Rhodamine 6G in the solvent hexane/ether/acetic acid (90:10:1). The spots were visualized by ultraviolet (UV) light and the TG, alk-1-enyl- and 1-alkyl-GEDE were extracted with chloroform and weighed. Fatty acid methyl esters were prepared from CE, TG and GEDE by alkaline transmethylation (9) and the methyl esters from CE and TG and the methyl esters and aldehydes from alk-1-enyl-GEDE were analyzed by GLC as reported before (10). Free and esterified cholesterol were quantified by colorimetry (11). The fatty acid methyl esters and the 1-alkyl glyceryl ethers (GE) derived from the methanolysis of 1-alkyl-GEDE were separated by chromatography on Unisil (12) and the methyl esters were analyzed by GLC (10). The GE were then converted to their isopropylidine derivatives (12) and analyzed by GLC on 12% DEGS. The alk-1-enyl-GEDE were dissolved in methanol and treated with aqueous HCl to yield aldehydes and diacylglycerides (13). The aldehydes were then extracted with hexane and analyzed by GLC. The free fatty acids (FFA) isolated by TLC were methylated with diazomethane and quantified (14). The fatty acid compositions of the methyl esters from FFA were also determined by GLC (10).

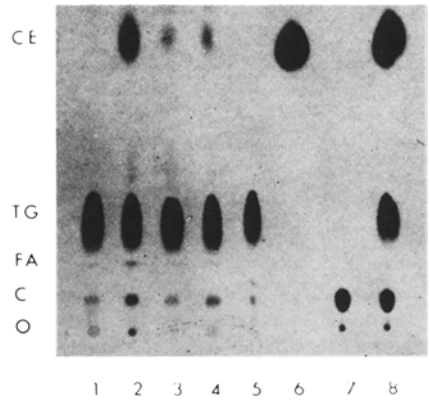

FIG. 1. Thin layer chromatography of lipids extracted from fat tissue on Silica Gel HR, in petroleum ether/ether/acetic acid, 90:10:1, (v/v/v). Lane 1, A399, normal-appearing subcutaneous fat, 150 μg; 2, A260, mesenteric panniculitis (MP), 150 μg; 3, A399, MP, 150 μg; 4, A46, MP, 125 μg; 5, triolein, 100 μg; 6, cholesteryl oleate, 100 μg, 7, cholesterol, 50 μg; 8, mixture of 5-7. CE, cholesteryl esters; TG, triacylglycerides; spot above TG, glyceryl ether diesters; FA, fatty acids; C, cholesterol; O, origin.

RESULTS AND DISCUSSION

In all samples except 2 cases of MP (A260 and A407), the total lipids accounted for 88-96% of the dry weight of tissue, whereas in A260 and A407, the percentages were lower (74-80%).

TLC of lipids from 3 cases of MP (Fig. 1) showed the presence of CE in all of them.

The total lipids of 2 controls (A260, normal retroperitoneal and A458, mesentery) and 6 specimens from cases of MP (A19, A46, A260, A293, A399, A407) were separated by column chromatography into 4 fractions, A-D. GLC of fractions A revealed that, in 3 cases of MP (A46, A260 and A399), the major peak was that of squalene and the mass spectra showed the following fragment ions characteristic of squalene: m/e 410 (M), 369 (M-41), 341 (M-69), 328 (M-82), 273 (M-137), 231, 137, 81 and 69. In the above 3 cases of MP judged as advanced cases (2), the CE were found to be 3.4-27.0% of the dry wt of tissue. The other 3 cases contained 0.9-1.3% of CE. Controls had only traces of CE. Preparative TLC of fractions C from the same advanced cases of MP yielded 0.6-2.5% of the dry wt of tissue of 1-alkyl GEDE. Fractions D from the same cases of MP showed that 0.5-1.3% of the dry wt of tissue consisted of free cholesterol. The determination of cholesterol in fraction D of uninvolved retroperitoneal tissue from A260 was calculated to be 0.2% of the dry wt of tissue. The amounts of FFA from fractions D, calculated as stearic acid (13), from the 3 samples of MP were 0.9-1.9% of the dry wt of tissue. The relative percentage composition of the fatty acids of the CE, FFA, TG, 1-alkyl- and alk-1-enyl-GEDE indicated that, in all cases of MP, the principal acid (45-65% of the total) was 18:1 (Table I). The alkyl group composition of the GEDE showed that saturated alkyl groups with 16 and 18 carbon atoms constituted the major portion

TABLE I

Relative Percentage Composition of Fatty Acids and Alkyl Groups in Various Classes of Lipids from Mesenteric Panniculitis (MP)

Fatty acid or alkyl group (chain length: no. of double bonds)	MP		Controls	MP	MP			
					1-Alkyl-GEDE[a]		Alk-1-enyl-GEDE[a]	
	Cholesteryl ester fatty acids (6)	Free fatty acids (3)	Triacylglyceride fatty acids (2)	Triacylglyceride fatty acids (5)	Fatty acids (5)	Alkyl groups (5)	Fatty acids (2)	Alkyl groups (2)
14:0	tr[a]	0.9 ± 0.3	2.3 ± 0.6	1.6 ± 1.2	2.0 ± 2.4	tr	–	–
15:0	–	–	–	–	–	–	1.6 ± 1.6	2.1 ± 1.2
16:0	13.7 ± 3.6	10.1 ± 5.4	23.9 ± 0.1	23.0 ± 1.8	26.1 ± 1.1	32.5 ± 4.0	19.8 ± 0.5	50.2 ± 1.6
16:1	4.0 ± 1.2	5.4 ± 1.2	7.4 ± 1.4	5.4 ± 1.2	7.4 ± 3.3	0.5 ± 1.1	5.8 ± 2.7	–
17:0	–	–	–	–	–	tr	–	1.7 ± 1.7
18:0	3.1 ± 0.8	2.9 ± 1.5	3.0 ± 0.4	4.4 ± 1.0	4.8 ± 2.1	30.1 ± 0.9	4.1 ± 1.0	27.2 ± 0.8
18:1	64.5 ± 3.3	64.6 ± 1.1	54.2 ± 0.4	52.2 ± 2.1	45.1 ± 4.7	30.8 ± 5.2	53.8 ± 1.0	15.5 ± 0.4
18:2 (n-6)	11.1 ± 1.0	14.4 ± 5.0	8.3 ± 2.7	12.3 ± 2.4	9.8 ± 2.7	tr	13.1 ± 1.6	–
18:3 + 20:1	2.4 ± 0.6	1.1 ± 0.2	1.0 ± 0.5	0.7 ± 0.5	0.5 ± 0.3	2.0 ± 1.7	–	–
20:0	–	–	–	tr	tr	2.6 ± 1.7	–	3.5 ± 1.0
20:4 (n-6)	0.5 ± 0.8	0.5 ± 0.4	tr	tr	1.3 ± 2.2	–	1.9 ± 1.1	–
20:5 (n-3)	–	–	–	–	0.6 ± 1.3	–	–	–
22:0	–	–	–	–	–	1.1 ± 1.2	–	–
22:4 (n-6)	–	–	–	–	0.7 ± 1.0	–	–	–
22:5 (n-3)	–	–	–	–	1.0 ± 1.6	–	–	–
22:6 (n-3)	–	–	–	–	0.7 ± 1.3	–	–	–

Fatty acids and alkyl groups were isolated and analyzed by GLC as described in Experimental Procedures. Values are mean plus or minus SD or deviation from mean; minor peaks with less than 14 carbon atoms are not included. The figures in parentheses indicate the number of samples.

[a]GEDE, glyceryl ether diesters; tr, trace (less than 0.5%).

of the alkyl groups (63-77% of total). Our data are in general agreement with those reported by Schmid et al. for GEDE from subcutaneous fat tissue (15).

The identification of substantial amounts (0.9-27.0% of the dry wt of tissue or 1.1-36.5% of the total lipids) of CE in fat tissue from cases of MP is a new finding. Because the amounts of CE isolated were large in spite of wide variations between individual cases, it excludes the possibility that the CE were derived from traces of blood, although precautions were taken to remove all blood. The total cholesterol content of rat adipose tissue has been reported to be in the range of 0.06-0.16% of fresh wt of tissue (16). Similar levels were reported in the fat tissue from humans (17). The free cholesterol content of the mesenteric fat tissue in MP reported in this study varied between 0.5 and 1.3% of the dry wt. Although we did not quantify the amounts of squalene isolated, our TLC and GLC studies indicated increased proportions of squalene in MP compared to controls and this may indicate an increase in the precursor pool, because squalene is known to be an intermediate in the biosynthesis of cholesterol. The concentrations of GEDE in MP in at least 3 cases out of 5 examined (0.8-2.8% of the lipid) was higher than the values reported by Schmid et al. (0.3% of the lipid) for subcutaneous tissue (15). Snyder and Wood (18) found higher levels of GE probably derived from 1-alkyl GEDE in several types of human neoplastic tissues. The detection of higher-than-normal levels of 1-alkyl GEDE in the mesenteric fat in at least some cases of MP indicates that their increased concentration in human tumor tissues is not limited to neoplasms.

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Quantitative Estimation of C-24 Epimeric Sterol Mixtures by 220 MHz Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

Four pairs of 24-alkyl epimeric sterols: 24α- and 24β-methylcholesterol; 24α- and 24β-ethylcholesterol; 24α- and 24β-methyl-5, 22-E-cholestadienol; and 24α- and 24β-ethyl-5, 22-E-cholestadienol, were mixed in known proportions (0, 20, 40, 50, 60, 80 and 100% of β-epimer to α-epimer). The 220 MHz nuclear magnetic resonance spectra of each of the mixtures were obtained and compared. Areas of these spectra useful in quantitative determinations were identified and discussed.

INTRODUCTION

The sterols of plants and primitive animals frequently contain a methyl or an ethyl group at C-24 which may be oriented in the α or the β position (1). These isomers have always been difficult to identify and no system has been demonstrated to separate the epimeric mixture. Most phytosterols were identified as 24α or 24β as a result of slightly different melting points or optical rotations until it was demonstrated that nuclear magnetic resonance (NMR) could distinguish between sterols isomeric at C-24 (2). Prior to the use of NMR, each sterol from an organism was considered to be a single epimeric form. In general, algae (3) and fungi (4) were believed to produce sterols with the 24β-configuration, whereas most of the higher plants were believed to contain the 24α-configuration (5). Because some of the sterol epimers are indistinguishable even by 60 MHz and 100 MHz spectrometer (2) unless a lanthanide shift reagent is added (6), more and more researchers are using 220 MHz or higher resolution NMR spectrometers to distinguish 24α and 24β epimers (7–15). Recently, by using these high resolution NMR spectrometers, a number of plants and marine invertebrates were found to contain sterols which were epimeric mixtures at C-24 (8,11,13,15). However, the relative proportions of the epimers were estimated at 2:1 or 1:1 or else no specific estimates were made. Although spectral information from numerous pure sterols is available in the literature (2,8, 10,11,13,15), published spectra of quantitative epimeric mixtures are unavailable. Since many C-28 and C-29 sterols isolated from plants and marine invertebrates appear to be C-24 epimeric mixtures, NMR spectra of a series of 24α-methylcholesterol- 24β-methylcholesterol mixtures, e.g., could serve as quantitative standards with which to compare an unknown mixture of epimeric 24-methylcholesterols. For this reason, we have prepared appropriate mixtures of four C-24 epimeric sterols in known proportions and obtained their 220 MHz NMR spectra.

MATERIALS AND METHODS

Eight sterols comprising 4 epimeric pairs were used for the study. The 24α-methylcholesterol (campesterol) was a gift from Dr. Henry Kircher; pure 24β-methylcholesterol (22-dihydrobrassicasterol) and 24β-ethyl-5,22-choles-

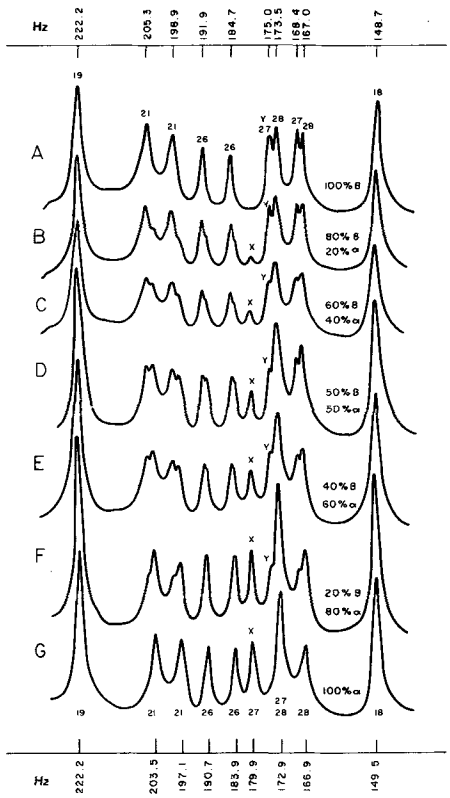

FIG. 1. Comparison of NMR spectra of pure and mixed epimers of 24-methylcholesterol.

tadienol (poriferasterol) were isolated from *Chlorella ellipsoidea* Indiana culture collection #247; 24α-ethylcholestadienol (sitosterol) and 24β-methyl-5,22-cholestadienol (brassicasterol) were isolated from rapeseed; 24 β-ethylcholesterol (clionasterol) was isolated from oysters in estimated 84% stereochemical purity; 24α-methyl-5,22-cholestadienol (diatomsterol) was isolated from the diatom, *Phaeodactylum tricornutum*; and 24α-ethyl-5,22-cholestadienol (stigmasterol) was purchased from Nutritional Biochemicals Co.

The NMR spectra of sterols dissolved in $CDCl_3$ were recorded at 220 MHz with a Varian HR-220 NMR spectrometer under the following conditions: temperature 20–21 C, sweep speed 250 sec, sweep width 250 Hz, sweep offset 100 Hz. Spectra were obtained from pure sterols and from 20, 40, 50, 60 and 80% mixtures of the components of each epimeric pair.

RESULTS AND DISCUSSION

24β-Methylcholesterol and 24α-Methylcholesterol

The difference between 24α-methylcholesterol and 24β-methylcholesterol is most distinctive at the chemical shifts of C-27 protons as described by Rubinstein et al. (10). The 24β-methylcholesterol has a unique 2-doublets pattern contributed by the C-28 and C-27 protons (Fig. 1A). The purity of the 24β-methylcholesterol can be determined by an examination of the baseline within the 175 and 185 Hz region. If a small amount of 24α-methylcholesterol is present together with 24β-methylcholesterol as indicated in the spectra of B and C in Figure 1, there will be a small peak at about 180 Hz due to the C-27 methyl of the C-24α epimer (peak x, Fig. 1). The height of this peak is in proportion to the amount of 24α-methylcholesterol present. Pure 24α-methylcholesterol produces a single absorption peak at 172.9 Hz (Fig. 1G), but if a small amount of 24β-methylcholesterol is present together with 24α-methylcholesterol, a shoulder at 175 Hz will be observed as in E and F in Figure 1 (peak y, Fig. 1).

In addition, the chemical shift of C-21 protons also gives a very prominent quantitative clue to the purity of the epimeric mixtures (8,10,13,15). The C-21 proton absorption peak of 24α-methylcholesterol compared to that of 24β-methylcholesterol is different by about 2 Hz. A mixture of equal amounts of the 24α- and 24β-epimers shows 4 peaks for C-21 (Fig. 1D) in contrast to the clear doublets seen for the pure individual epimers (Fig. 1,A and G).

24β-Ethylcholesterol and 24α-Ethylcholesterol

In pure 24β-ethylcholesterol, according to Rubinstein et al. (10), one peak of the C-26 doublet will overlap one of the C-29 triplet peaks due to the downfield shifting of the C-26 protons and upfield shifting of the C-29 protons. 24α-Ethylcholesterol has a characteristic peak at 192.9 Hz which is one of the resonance peaks from the triplet of C-29 protons (peak x, Fig. 2). Therefore, the 24-ethylcholesterol from oysters cannot be pure clionasterol because of the presence of a shoulder at 193 Hz which is a resonance peak from the C-29 proton triplet of 24α-ethylcholesterol. When the oyster 24-ethylcholesterol was mixed with pure 24α-ethylcholesterol in known proportions, the relative heights of the 2 peaks between 200.3 and 187.5 Hz became a good tool for quantitative measurement (Fig. 2, B-F). An equal mixing of pure α- and β-epimers should give 2 peaks in the region 200.3–187.5 Hz with the same area (or height). Therefore, the propor-

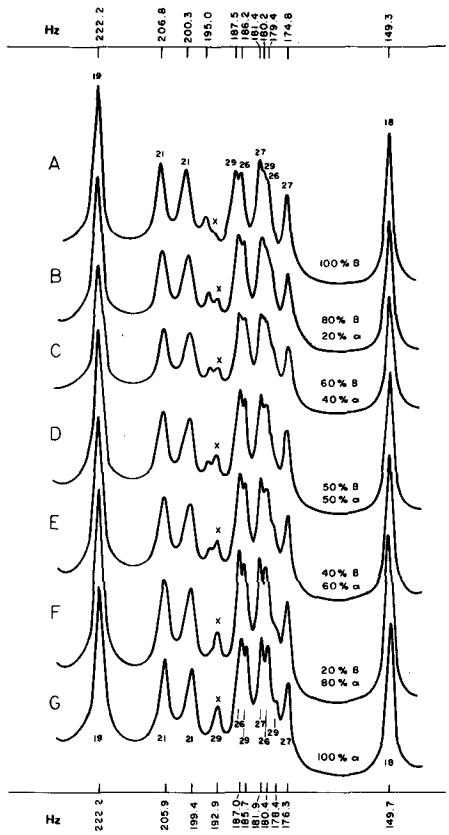

FIG. 2. Comparison of NMR spectra of pure 24α-ethylcholesterol, 84% stereochemically pure 24β-ethylcholesterol, and mixtures of the two.

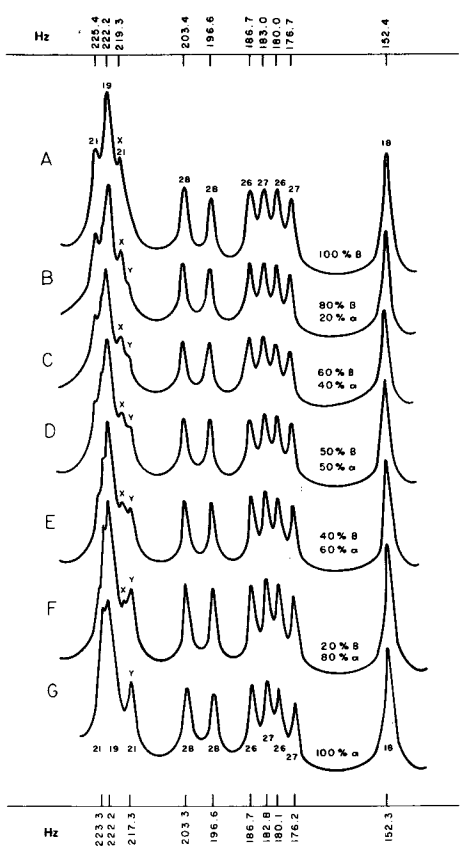

FIG. 3. Comparison of NMR spectra from pure and mixed epimers of 24-methyl-5,22-cholestadienol.

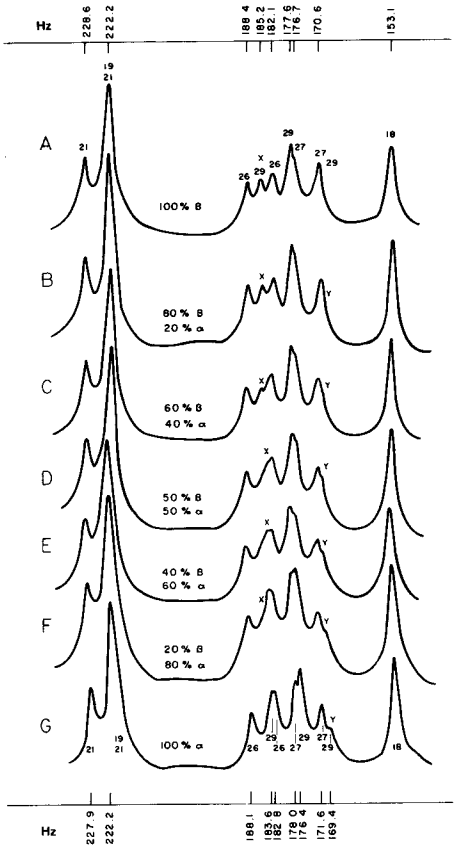

FIG. 4. Comparison of NMR spectra from pure and mixed epimers of 24-ethyl-5,22-cholestadienol.

tion of α- and β-epimers in Figure 2C is shown to be 50:50 rather than 60:40. From this information, the 24-ethylcholesterol in oyster (Fig. 2A) is estimated to contain ca. 83–84% of 24β-ethylcholesterol and 16–17% 24α-ethylcholesterol. The actual proportion of β- and α-epimers is extrapolated to be 66% β to 34% α in Figure 2B; 42% β and 58% α in Figure 2D; 34% β to 66% α in Figure 2E; 17% β to 83% α in Figure 2F.

24β-Methyl-5,22-cholestadienol and 24α-Methyl-5,22-cholestadienol

The NMR spectra of 24β-methyl-5,22-cholestadienol and 24α-5,22-cholestadienol are so similar that the only significant differences are the absorption peaks of the C-21 protons. In the β-epimer, the doublet of C-21 is at 225.4 and 219.3 Hz, whereas in the α-epimer, it is slightly shifted upfield to 223.3 and 217.3 Hz. When both epimers are mixed in different, known proportions, one can trace the increase and decrease of the corresponding peaks of the C-21 proton doublets (Fig. 3, A-G, peaks x and y).

24β-Ethyl-5,22-cholestadienol and 24α-Ethyl-5,22-cholestadienol

The spectra of pure 24β-ethyl-5,22-cholestradienol and 24α-ethyl-5,22-cholestadienol are very different (Fig. 4, A and G). The difference is mainly caused by the downfield shifting of C-29 protons in the 24β-ethyl-5,22-cholestadienol. Pure 24β-ethyl-5,22-cholestadienol is characterized by the presence of 3 clearly separated peaks at 188.4, 185.2 and 182.1 Hz. The peak in the middle (185.2 Hz, peak x, Fig. 4) is one from the C-29 triplet, whereas the other 2 are the C-26 doublet. When mixing increasing amounts of 24α-epimer with 24β-ethyl-5,22-cholestadienol (Fig. 4, B and C), one notices the fusing together of the 2 peaks at 185.2 and 182.1 Hz and that they become less distinctive because of the upfield shifting of C-29 protons. Pure 24α-ethyl-5,22-cholesta-

dienol is characterized by the presence of the shoulder at 169.4 Hz which is one absorption peak of the C-29 triplet (peak y, Fig. 4). When increasing amounts of 24β-epimer are mixed in known proportions (Fig. 4), this peak gradually disappears (Fig. 4, D-F). It is relatively more difficult here, than in the case of 24-methylcholesterol, 24-ethylcholesterol and 24-methyl-5,22-cholestadienol, to use one particular characteristic peak in the spectrum for a quantitative estimate. However, the mixing study in Figure 4 serves as a good reference for quantitation.

ACKNOWLEDGMENTS

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Suppression of Hepatocyte Fatty Acid Synthesis by Albumin-Bound Linoleate Involves Depolymerization of Acetyl-CoA Carboxylase Filaments

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ABSTRACT

Digitonin treatment of chick liver cells in monolayer culture results in plasma membrane perforations due to digitonin removal of membrane cholesterol. The amount and rate of acetyl-CoA carboxylase activity that escapes from the hepatocyte during digitonin treatment is positively related to the amount of protomeric carboxylase in the cells. Incubation of chick liver cells in culture with albumin-bound linoleate (60 min) caused a 3-fold increase in the amount of carboxylase activity released during exposure of cells to digitonin. Concomitant with the enhanced release of carboxylase activity was an 85% reduction in fatty acid synthesis induced by linoleate. Apparently, acute suppression of hepatocyte fatty acid synthesis by media free fatty acids resulted, in part, from a change in carboxylase conformation from the active polymeric state to the inactive protomeric form.

INTRODUCTION

The suppression of acetyl-CoA carboxylase (EC 6.4.1.2) function has often been the proposed explanation for the acute inhibition of fatty acid synthesis by free fatty acids in isolated hepatocytes (1,2), skin fibroblasts (3) and Ehrlich cells (4). Purified acetyl-CoA carboxylase is inhibited by free fatty acids and their acyl-CoA esters in a manner competitive with citrate activation (4-6). Further, fatty acyl-CoA esters may promote the depolymerization of carboxylase from its high molecular weight, catalytically active, filamentous state to its inactive protomeric state (7). The physiological occurrence of this carboxylase transition has little supportive data for the intact cell. Meredith and Lane (8) have found that a 90% decrease in cellular citrate concentration induced by cyclic AMP (cAMP) was accompanied by marked depolymerization of carboxylase in chick liver cell monolayers. However, such evidence with intact cells for the depolymerization of carboxylase by fatty acids is unavailable.

In this communication, a technique was used in which digitonin treatment of hepatocytes in culture removes membrane cholesterol, resulting in perforations of the membrane (9). Under these conditions, the amount and rate of carboxylase activity released from the cells after digitonin perforation is inversely related to the degree of carboxylase polymerization (8). The data indicate that the acute suppression of hepatocyte fatty acid synthesis caused by albumin-bound free fatty acids was partially due to a lesser amount of active, polymeric acetyl-CoA carboxylase.

EXPERIMENTAL PROCEDURES

Chick hepatocytes were isolated from male leghorn chicks (15- to 18-days-old) according to the methods of Tarlow et al. (10). Each hepatocyte preparation consisted of four livers to minimize individual bird variations. After hepatocyte isolation, the cells were plated in 60 mm-Falcon cell culture dishes in a basal Eagles medium (4 ml) containing glucose (25 mm), amino acids, rooster serum (3%) and insulin (5 μg/ml). The cells were maintained in an atmosphere of 90% air-10% CO_2 (10). After 6 hr, the cells had firmly attached to the plates and the medium was changed to one which contained no serum. Acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthesis studies were conducted with the hepatocytes after 40-48 hr of culture. Media changes occurred at 6 and 24 hr of incubation. Insulin was generously provided by Dr. Walter N. Shaw, Eli Lilly (Indianapolis, IN). The plating density was 10×10^6 cells/60 mm dish and, after 48 hr in culture, were 90-95% viable.

One hr prior to determining the amount of acetyl-CoA carboxylase activity released during digitonin treatment and the rate of fatty acid synthesis, the culture medium was replaced with fresh medium which included 5 mM sodium acetate plus the addition of (a) 0.5 mM albumin-bound linoleate, (b) 0.1 mM N^6,O^2-dibutyryl cAMP, or (c) no additional constituents. After a 1-hr incubation, the medium was removed; the plates were washed twice with 10 ml cold phosphate-buffered saline; and a buffered digitonin solution (1.5 ml) containing: mannitol (250 mM), potassium morpholinopropane sulfonate (17 mM, pH 7.0), EDTA (2.5

mM), glucose (18.5 mM), dithiothreitol (1 mM), benzene-1,2,3-tricarboxylic acid (4.2 mM, pH 7.0), hexokinase (35 U/ml), and digitonin (0.8 mg/ml), was added to each plate (11). At the times reported in Results, the digitonin solution was gently removed and immediately adjusted to contain potassium phosphate (50 mM, pH 7.0), citrate (5 mM) and dithiothreitol (1 mM). The residual cell monolayer was scraped from the plate in 2.0 ml potassium phosphate (5 mM), citrate (5 mM), EDTA (0.5 mM) and dithiothreitol (1 mM). After homogenizing in a glass Dounce homogenizer, the buffer was adjusted to 40 mM phosphate.

Acetyl-CoA carboxylase activity released into the buffered digitonin solution, and that residual activity associated with the disrupted cells, was determined by the procedure of Gregolin et al. (12). The period allowed for $H^{14}CO_3$ fixation was only 1 min and the concentration of $KHCO_3$ was 10 mM. One milliunit of carboxylase activity was that which catalyzed the fixation of 1 nmol of $H^{14}CO_3$ into acid-stable product/min at 37 C.

The acetyl-CoA carboxylase activity released from the hepatocytes after digitonin treatment has been assumed to represent the protomeric form of carboxylase. Similarly, the activity associated with the cells after scraping was termed residual activity and has been considered to represent the aggregated or polymeric carboxylase form (8).

Total cellular citrate concentrations were quantitated as described by Clarke et al. (13) using a spectrophotometric procedure (14). Long-chain acyl-CoA concentrations were determined as described by Goodridge (2) using the CoA assay of Allred and Guy (15). Citrate and long-chain acyl-CoA concentrates were determined in hepatocyte suspensions rather than cultures, because concentration of these metabolites in cultured cells was too low for detection. Fatty acid synthesis in monolayer cultures and in hepatocyte suspensions was quantitated using [1-^{14}C]acetate as described by Watkins et al. (11). Albumin was washed with charcoal to remove citrate (16) and used to bind linoleic acid by the technique of Spector and Hoak (17).

RESULTS AND DISCUSSION

Digitonin binds and removes cholesterol from the plasma membrane of hepatocytes, resulting in a perforated membrane (8,9). The cytosolic contents of the cells can subsequently exit. The rate of release of the cytosolic constituents depends on molecular size (8). Hence, the protomeric carboxylase subunit would be released from cell after digitonin treatment but the high molecular weight filamentous acetyl-CoA carboxylase would be retained in the cell (Fig. 1). Supporting this concept was the large amount of carboxylase activity released when the potent depolymerizing agent, malonyl-CoA, was included in the digitonin disruption buffer (Table I; ref. 8). In contrast, the inclusion of citrate (a strong polymerizing agent of carboxylase [7]) in the digitonin solution caused most of the carboxylase activity to be retained in the cell (Table I). Furthermore, citrate entirely reversed the depolyermizing action of linoleate, cAMP and malonyl-CoA (Table I).

Preincubation of cultured chick hepatocytes with albumin-bound linoleate greatly increased the amount and rate of carboxylase activity released upon treatment of the cells with digitonin (Fig. 1). Linoleate and cAMP were not present in the digitonin-disruption buffer, but had been removed with the culture medium and further washed out by saline prior to the addition of digitonin solution. Therefore, the

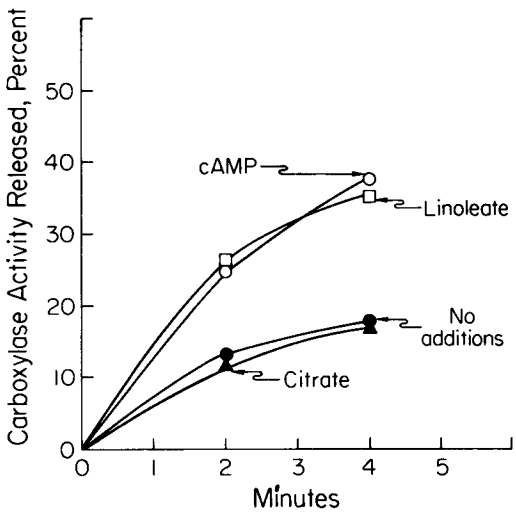

FIG. 1. Rate of release of acetyl-CoA carboxylase from chick hepatocytes in monolayer following digitonin treatment. Chick hepatocytes in monolayer culture were incubated for 60 min with 0.5 mM albumin-bound linoleic acid or 0.1 mM dibutyryl cyclic AMP. The molar ratio of linoleic acid to albumin was 8.0. After removing the incubation medium, a buffered solution of digitonin was added to each plate; at the designated times (room temperature), the solution was removed and assayed for acetyl-CoA carboxylase activity as described in Experimental Procedures. Citrate (10 mM) was added to the digitonin solution of untreated cells. Each point represents the average of three plates.

TABLE I

Effect of Linoleate and Dibutyryl Cyclic AMP on the Degree of Acetyl-CoA Carboxylase Polymerization in Chick Liver Cell Monolayers

Treatment	Distribution of acetyl-CoA carboxylase activity (%)	
	Released	Residual
Minus citrate:		
None	14	86
+ linoleate (0.5 mM)	41	59
+ cyclic AMP (0.1 mM)	40	60
+ malonyl-CoA (0.1 mM)	51	49
Plus citrate:		
None	18	82
+ linoleate (0.5 mM)	25	75
+ cyclic AMP (0.1 mM)	21	79
+ malonyl-CoA (0.1 mM)	22	78

Chick liver cells were cultured and incubated in presence of linoleic acid or dibutyryl cyclic AMP as described in Experimental Procedures. Malonyl-CoA was included in the buffered digitonin solution. Plus and minus citrate indicates the addition or deletion of 10 mM citrate from the digitonin solution. "Released" or "residual" activities are considered to represent protomeric and polymeric carboxylase forms, respectively. The molar ratio of linoleic acid to albumin was 8.0. Data are expressed as the average of two experiments with variation between experiments less than 10%. The average total carboxylase activity per plate was 17.7 ± 2 milliunits (n=16 plates).

disaggregation of carboxylase by linoleate or cAMP occurred during the incubation period of the cells and was not due to depolymerization by these agents upon cell membrane disruption.

Linoleate and cAMP both inhibited fatty acid synthesis (Table II) and increased the amount of carboxylase activity that could be released (Fig. 1, Table I). Unlike cAMP, the linoleate-induced depolymerization of carboxylase was independent of citrate as well as long chain fatty-acyl-CoA concentrations (Table II; refs. 2 and 8). Thus, although citrate is an important determinant of the proportion of active filamentous acetyl-CoA carboxylase (2,8), it is not the only metabolite which can alter the degree of carboxylase polymerization.

A failure of media fatty acids to lower citrate or raise acyl-CoA levels has been found with hepatocytes as well as fibroblasts and Ehrlich cells (2-4). The intracellular compartmentation of these metabolites was not determined and could differ between treatments, i.e., albumin-bound linoleate may lower cytosolic citrate while raising cytosolic acyl-CoA concentrations, thereby facilitating carboxylase depolymerization. Alternatively, although the total concentration of acyl-CoA remained unchanged with linoleate, the composition of the fatty acid esterified to CoA may have changed in favor of a more inhibitory variety (4).

In conclusion, these data strongly suggest that the suppression of fatty acid synthesis by albumin-bound free fatty acids was partly due to a decrease in the amount of catalytically active, filamentous acetyl-CoA carboxylase. Furthermore, the data provide the first evidence with the intact cell that the degree of polymerization can be altered by fatty acids in the medium and that the rate of fatty acid synthesis is negatively correlated to the amount of filamentous acetyl-CoA carboxylase.

ACKNOWLEDGMENT

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TABLE II

Effect of Linoleate and Dibutyryl Cyclic AMP on Lipogenesis in Chick Liver Cells

Cell treatment	Cultures	Suspensions		
	Fatty acid synthesis (nmol/min/plate)	Fatty acid synthesis (nmol/min/mg dry cells)	Citrate (nmol/mg dry cells)	Long chain acyl-CoA (pmol/mg dry cells)
None	16.7 ± 0.1	1.63 ± 0.44	1.60 ± 0.2	148 ± 31
+ 0.5 mM linoleate	2.5 ± 0.01	0.40 ± 0.12	1.50 ± 0.2	146 ± 29
+ 0.1 mM $B+_2$ cAMP	1.1 ± 0.01	–	0.30 ± 0.1	–

Chick liver cells were cultured and incubated in presence of linoleic acid or dibutyryl cyclic AMP as described in Experimental Procedures. The molar ratio of linoleate to albumin was 8.0. One mg dry cells was roughly equivalent to 4 million cells. Fatty acid synthesis was determined using [1-^{14}C]acetate incorporation into saponifiable lipid (13). Data are expressed as means ± SEM (n=3).

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Alteration of Liver Microsomal Proteins from Rainbow Trout *(Salmo gairdneri)* Fed Cyclopropenoid Fatty Acids

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ABSTRACT

Rainbow trout were fed diets containing cyclopropenoid fatty acids (CPFA) at 50 and 300 ppm with appropriate controls fed CPFA-free diets. Treatment with CPFA altered the overall microsomal protein composition in a manner suggesting a reduction of high molecular weight components. One protein found in low concentration in controls appeared dominant in experimental animals, with the effect more pronounced as dietary levels of CPFA increased. The estimated molecular weight of this component was 41,500 daltons. Membrane fractions from CPFA-fed fish separated on a Bio-Gel P-150 column revealed a significant number of small molecular weight components that suggest degradation of microsomal proteins. These data suggest an alteration by CPFA of membrane protein composition.

INTRODUCTION

It is well established that cyclopropenoid fatty acids (CPFA) exert toxic and other adverse effects in a variety of animals (1). Rainbow trout (*Salmo gairdneri*), in particular, have been shown to be extremely sensitive to the effects of these fatty acids. Several investigators have reported changes in cellular morphology and metabolism (2-6) in trout, in response to dietary CPFA. Generally, these changes have been characterized in liver by the development of unique "fibers" within the cellular cytoplasm and by accumulation of lipid globules. With continued feeding, these cytoplasmic alterations were shown to increase and appeared limited to the rough-surfaced endoplasmic reticulum (6). More recently, it was shown that these fatty acids decreased the levels of microsomal cytochrome P-450 (7).

In order to investigate the possibility that CPFA alter the composition of membrane proteins, the microsomal protein components of hepatocytes of fish fed different levels of CPFA were investigated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

All fish used in this study were one-year-old Mt. Shasta strain rainbow trout (*Salmo gairdneri*) raised at the Oregon State University Food Toxicology Laboratory. Fish were fed ad libitum diets containing 50 and 300 ppm CPFA in the form of methyl sterculate (8) for 140 days with appropriate controls fed CPFA-free diets. Three fish from each group were removed at random and livers from freshly killed trout were perfused with ice-cold 0.9% NaCl, combined, and homogenized in 4 vol of 0.25 M sucrose 0.01 M Tris HCl, pH 7.4, with 5 passes in a motor driven Teflon pestle glass homogenizer. Duplicate samples were prepared in exactly the same manner. The homogenates were centrifuged at 12,000 x g for 30 min. Microsomes were prepared from the 12,000 x g supernatant by centrifugation at 105,000 x g for 90 min. The microsomal pellets were washed by resuspension in ice-cold-buffer and centrifuged for an additional 60 min at 105,000 x g.

Following this procedure the washed microsomes were suspended in 1 ml of SDS sample buffer (0.05 M Tris HCl, 1% SDS, 0.002 M EDTA, 0.1 M β-mercaptoethanol, 10% glycerol, pH 6.8) and placed in a boiling water bath for 1 min to solubilize proteins. Aliquots were taken and the macromolecular components were separated on 12.5% polyacrylamide slab gels (0.05 cm x 7 cm) as described by Laemmli (9). Application vol of samples was between 2 μl and 17 μl, depending on protein concentration. Protein molecular weight (MW) standards were obtained as a mixture from BioRad (2 mg/ml/protein). The markers were diluted 2:1 with SDS sample buffer and placed in a boiling water bath for 1 min prior to use. The standard proteins were as follows: phosphorylase B (94,000 MW), bovine serum albumin (68,000 MW), ovalbumin (43,000 MW), carbonic anhydrase (30,000 MW), soybean trypsin inhibitor (21,000 MW), and lysozyme (14,300 MW). Protein MW were confirmed on gels of 8, 12.5 and 15.0% polyacrylamide. Following electrophoresis, the gels were protein-specific stained with 0.2% coomassie Blue R 250 in 45%

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methanol, 10% acetic acid for 15 min and destained 1 hr in 45% methanol, 10% acetic acid.

Bio-Gel P-150 was equilibrated for 48 hr in 0.05 M Tris HCl pH 6.8 0.1% SDS, 0.002 M EDTA, 0.1 M β-mercaptoethanol, 10% glycerol. An LKB 2137 column (1.5 cm x 35 cm) was packed with the Bio-Gel P-150 and the above buffer was used as the running buffer. The flow rate was 2 ml/hr, and samples were applied in 0.8 ml of SDS buffer. All columns were done in duplicate. The data shown in Figure 1 represents a typical elution pattern. Following gel filtration, aliquots were taken directly from column fractions, absorptions read at 280 nm with a Beckman DB spectrophotometer and prepared for analysis on polyacrylamide gels.

Protein concentrations were determined using the BioRad protein assay and lysozyme was used as a standard (10). Differences in liver weights were analyzed by a one-way analysis of variance (11).

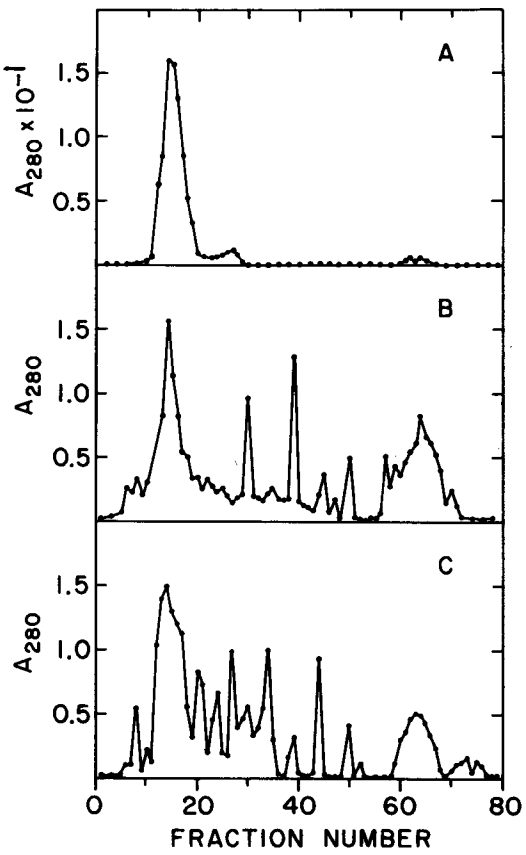

FIG. 1. Bio-Gel P-150 separation of SDS solubilized microsomal protein; A: control; B: 50 ppm CPFA exposure; and C: 300 ppm CPFA exposure. The arrow indicates the elution position of the putative CPFA "induced" protein.

RESULTS

Differences in liver weights between control and experimental fish were found after 140 days of feeding CPFA. The mean liver weights of the controls, 50 ppm CPFA, and 300 ppm CPFA groups were (gm): 1.04, 0.55, and 0.52, respectively. The liver weights of both experimental groups were significantly lower than those fed the control diet (p value = 0.008).

Polyacrylamide gel electrophoresis of microsomal proteins from control fish and microsomal proteins from fish treated for 20 weeks at 50 and 300 ppm CPFA are shown in Figure 2. In control microsomes, a minimum of 20 proteins were observed with five major bands resolved between 43,000 and 93,000 dalton MW. These are numbered 1-5 in Figure 2. Several changes in the protein pattern were observed in response to CPFA feeding. The most notable changes were an overall reduction of membrane proteins between 43,000 to 93,000 daltons and the increase of a band at 41,500 daltons. The intensity of this band increased with an increase in the levels of CPFA treatment (Fig. 2). Figure 1 shows the Bio-Gel

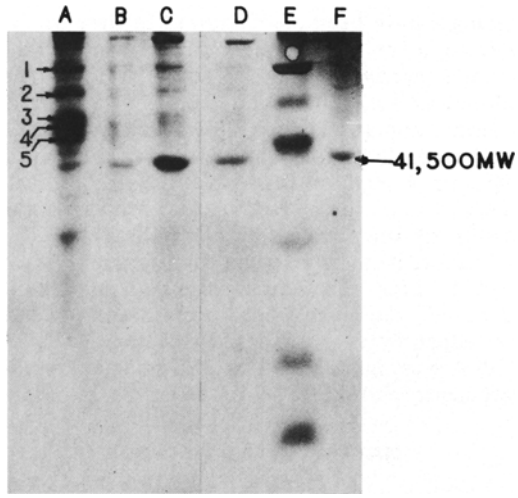

FIG. 2. SDS-PAGE separation of microsomal proteins from A: control animal; B: 50 ppm CPFA exposure; C: 300 ppm CPFA exposure; D: 50 ppm CPFA exposure; E: protein standards in order of decreasing molecular weight; phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme; F: 50 ppm CPFA exposure. Between 25 and 50 μg of protein was applied to each well. The arrow marks the putative CPFA "induced" protein at 41,500 MW.

P-150 elution pattern for the SDS solubilized microsomal proteins from untreated controls and CPFA-treated trout. Control proteins were resolved into one major peak, and only trace amounts of low MW components were observed (Fig. 1A). In contrast, microsomal proteins from CPFA-treated trout were remarkably different from controls (Fig. 1B,C). The elution pattern of these proteins were characterized by a complex mixture of low MW components; they were most pronounced in the sample prepared from 300-ppm-treated fish.

Electrophoresis of the partially purified proteins revealed a complex pattern of separation from control microsomes (Fig. 3A). Proteins common to control were clearly absent or significantly reduced in the treated samples (Fig. 3B,C). The 41,500 subunit was particularly obvious in fractions 13 and 14 of the 300-ppm CPFA treatment (Fig. 3C) and would account for the increased peak shoulder observed in Figure 1C.

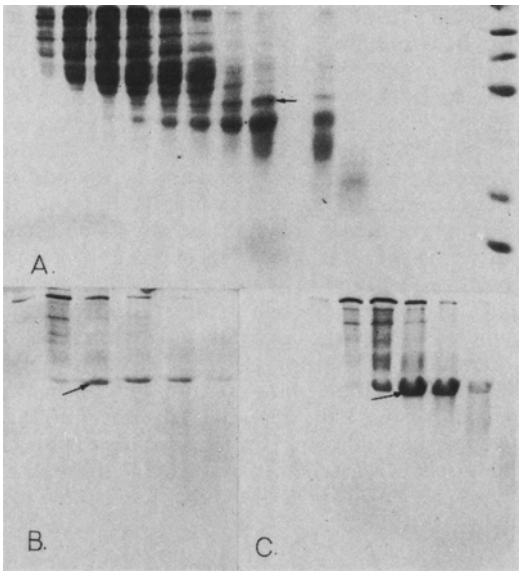

FIG. 3. SDS-PAGE separation of Bio-Gel P-150 fractionated microsomal proteins. A: Fractions 7 through 17 from control column; B: fractions 7 through 12 from 50 ppm CPFA exposure column; C: fractions 7 through 15 from 300 ppm CPFA exposure column. The arrows indicate the location of the puntative CPFA "inducted" protein at 41,500 MW.

DISCUSSION

The detection of altered proteins in liver microsomal membranes of CPFA treated fish is consistent with previous reports which have shown CPFA-induced changes in the histology and biochemical behavior of liver cells. A general degradation of parenchymal cells with fibrous inclusions and lipid globules has frequently been reported as characteristic in studies with light microscopy (2-4). Upon ultrastructural examination, livers from CPFA-treated fish were found to contain grossly altered membranes and the effect appeared to be confined to degranulation of the rough-surfaced endoplasmic reticulum (6). Prolonged CPFA treatment at high levels (200 ppm for 12 weeks) resulted in a dissection of membrane complexes to the extent that the endoplasmic reticulum was distributed in small patches surrounded by aggregates of membrane and rosettes of glycogen.

Our results showed a reduction in a number of SDS extractable membrane proteins with CPFA treatment (Fig. 3). This suggests either a depressed rate of protein synthesis in concert with normal turnover of proteins causing the loss of normal constituents, or facilitation in protein turnover exceeding replacement rates. On the basis of previous reports, the possibility of reduced protein synthesis is favored. Malevski et al. (12) reported significantly lower liver protein concentrations in CPFA-treated fish after 4 days of feeding, with liver protein concentration remaining consistently lower throughout the 54-day period of their study. When incorporation of ^{14}C-labeled amino acids into liver protein of CPFA-treated and CPFA-free trout were compared, these investigators found a significant decrease in specific activity of protein in CPFA-treated fish. Other investigators have noted a reduction in both cytosolic and membrane-bound enzyme activity in dietary studies when CPFA were present (6,13). Recently (7,14), it was shown that CPFA reduced the concentration of liver microsomal cytochrome P-450 as well as the activity of NADPH cytochrome c (P-450) reductase. The results of our study show a clear reduction in membrane protein components with a MW range corresponding to the above proteins. For example, under denaturing conditions of SDS-PAGE, it has been reported that rat liver cytochrome P-450 separates as a single polypeptide with MW between 48,000 and 55,300 (15-18) and NADPH cytochrome P-450 reductase as a single polypeptide between 74,000 and 79,000 MW (19-22). Elcombe and Lech (23) have also shown that trout hepatic hemoproteins P-450 have comparable MW to those found in rat microsomes.

The most striking observation in our study was the relative increase in the amount of the 41,500 MW protein in microsomal membranes

isolated from CPFA-treated trout. While its origin is unknown, we can speculate that it is either a stable protein, a product of protein degradation, or the result of selective synthesis induced by CPFA. We tentatively favor the possibility that it is the result of selective synthesis, for the following reasons. It would be unusual that a protein would be resistant to normal turnover enzymes; at the same time, there is considerable degradation of other proteins. Moreover, any protection against protease attack through membrane interactions would be minimal in cells showing extensive membrane disruption. Consideration of these arguments would also suggest that accumulation of a high MW degradataion product would be unlikely. Further studies will be required to verify the tentative conclusion that this protein is selectively synthesized in response to CPFA.

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Covalent Binding of Peroxidized Phospholipid to Protein: III. Reaction of Individual Phospholipids with Different Proteins[1]

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ABSTRACT

Various peroxidized phospholipids were reacted with proteins under N_2. In all cases, phospholipid is bound covalently to the proteins whose molecular size is increased. Both the amount of bound phospholipid and the increase in molecular size of the protein depends on the nature of the phospholipid. Ultraviolet (UV) absorption of the proteins is increased in qualitatively similar ways. Their difference spectra, which show a gradual increase in absorption from 400 nm toward shorter wavelength, differ from that of malonaldehyde-protein complexes. The various complexes of proteins and peroxidized phospholipids have similar fluorescence spectra showing two excitation maxima at 310–320 nm and at 340–350 nm, respectively, and emission maximum at ca. 400 nm. This is different from both fluorescence spectra of malonaldehyde-protein complexes and fluorescence spectra reported for proteins after reaction with peroxidized polyunsaturated fatty acids. Amino groups of the proteins are consumed in the reaction with peroxidized phospholipids. Blocking the amino groups decreases the binding of phospholipid considerably. Besides amino groups, other structures of the protein molecule react with the peroxidized phospholipids. The similar features of UV absorption, fluorescence, decrease of amino groups, and covalently bound phospholipid phosphorus of the various complexes suggest that they are formed by common type of reactions. The reactions seem to be different from those generally believed important between peroxidized lipid and protein. Important reacting species are compounds other than malonaldehyde.

INTRODUCTION

Most in vitro studies of the reaction between peroxidized lipids and proteins have used either methyl or ethyl esters of polyunsaturated fatty acids or the unesterified acids as model lipids. The results obtained indicate that the reaction causes extensive polymerization of proteins (1–3) as well as destruction of certain of their amino acids (3–7). The polymerization is believed to arise either by free radical chain polymerization (1,4,8,9) or by crosslinking by malonaldehyde (2,3), a product of peroxidation of polyunsaturated lipids (10). We recently studied the reaction between peroxidized phospholipid and protein using cardiolipin and albumin as model compounds (11,12). A considerable amount of peroxidized lipid binds covalently to the protein in this system with little or no polymerization. This is essentially different from the findings for the reaction between peroxidized polyunsaturated fatty acids and proteins. Our model system presumably is more representative of in vivo peroxidations than previously used systems because a phospholipid is used as model lipid, the type of lipid found in membranes in which in vivo peroxidations occur. However, because only one specific phospholipid was used, it is important to examine more generally the reaction between peroxidized phosphatides and proteins. This paper reports the results of that study.

[1]Preliminary report of this work was presented at the ISF/AOCS World Congress, New York, 1980.

MATERIALS AND METHODS

Proteins and Phospholipids

The following proteins were used: albumin (Kabi, Sweden; human); ovalbumin (Sigma); γ-globulins (Sigma, bovine, Cohn fraction II), and ribonuclease (Sigma, from bovine pancreas). The phospholipids were obtained as follows: phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from egg yolk (13). Phosphatidylserine (PS) was prepared from ox brain (14) and converted to its sodium salt (15). The phospholipids, which were chromatographically pure, were stored at −20 C in dry state in brown glass ampoules sealed under nitrogen.

Preparation of Phospholipid Suspensions

Phospholipid suspensions were prepared using a MSE 150 Watt Ultra Sonicator equipped with the exponential probe, end diameter 1/8". The phospholipid was pipetted into a 2 × 9 cm centrifuge tube and solvent was evaporated in a stream of N_2. Then, the dry lipid was wetted by adding a little diethyl ether (ca. 0.1 μl/μg of lipid P) and water was added with

simultaneous vigorous shaking into a Whirlimixer. After removal of the ether by bubbling with N_2, sonication was done under nitrogen at maximal energy output. Temperature was kept below 15 C by cooling in an ice bath. Homogeneous, opalescent suspensions were obtained within 1 hr of sonication.

Peroxidation of Phospholipids and Subsequent Incubation with Protein

Phospholipid suspensions were peroxidized in Warburg vessels (13–14 ml size, 80 oscillations/min) at 30 C in an oxygen atmosphere. The process was catalyzed by addition of Cu $(CH_3COO)_2$ and $FeCl_3$ or Cu $(CH_3COO)_2$ alone, as stated under the individual experiments, and ultraviolet (UV) irradiation with a 125 W mercury lamp (11) throughout the oxidation period. The peroxidized phospholipids were incubated with protein (pH 7.0–7.5) for 16–24 hr under N_2, at final concentrations of 600–650 μg P/ml and 6.7 mg protein/ml, respectively.

Phosphorus Analysis

Phosphorus was determined either by Bartlett's method (16) or by a modified microprocedure (17) as previously described (11).

Separation of Protein and Lipid by Gel Filtration in the Presence of Sodium Deoxycholate

As previously described (12) using the method of Helenius and Simons (18) in modified form, samples containing 10–15 mg of protein and lipid corresponding to 900–1,400 μg P were gel-filtered onto columns for which dimensions are given under the individual experiments. Flow rate was 17–18 ml/hr. V_o and V_t were determined from elution vol of Blue dextran and LiCl, respectively.

Preparation of Albumin-Malonaldehyde Complexes

Malonaldehyde obtained by acid hydrolysis of tetraethoxypropane (19) was incubated with albumin under N_2 overnight at 30 C, pH 7.4, at final concentrations of 0.14 M and 12.3 mg/ml, respectively. The complex was isolated as described for the protein-peroxidized phospholipid complex.

Protein Analysis

Protein was determined according to Lowry et al. (20).

Concentrating Protein Solutions

Protein eluted from columns was concentrated for subsequent analyses by pressure filtration in an Amicon 12 ml cell using a Diaflo XM 50 filter.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-Electrophoresis)

SDS-electrophoresis was performed as previously described (12) using a 7% running gel for electrophoresis of albumin complexes and a 5% gel for γ-globulin complexes. Gels were stained with 0.085% colloidal Coomassie Blue in 20% trichloroacetic acid (21).

Ultraviolet Absorption Spectra

UV absorption of proteins after reaction with peroxidized phospholipids was recorded with a Unicam SP 800 B spectrophotometer.

Measurement of Amino Groups by Fluorescamine

Amino groups were measured by fluorimetry after reaction with fluorescamine (22). Samples of 15–40 μg protein in 2.0 ml 0.18 M borate (pH 9.2) containing 2% SDS were heated for 1.5 min in a boiling water bath. After cooling to room temperature, 500 μl fluorescamine in dioxane (0.40 mg/ml) was added with stirring into a Whirlimixer. Fluorescence was measured 15 min later on an Aminco-Bowman spectrophotofluorometer with the excitation wavelength set at 390 nm and the emission wavelength set at 480 nm. Peroxidized lipids react with protein forming products which are themselves fluorescent (3). This fluorescence was negligible in the assay. Neither tubes from the store of this department nor tubes that have been soaked overnight in chrome-sulfuric acid or concentrated nitric acid could be used in the assay. Contaminants of unknown nature and origin can cause false readings, sometimes by several hundred percent. Disposable culture tubes, 16 × 100 mm, catalog number 114115 from Rudolf Brand, D-6980 Wertheim, Germany, are suitable, giving a low and constant background; thus, 12 determinations gave an average relative fluorescence of 0.60 ± 0.07, and 12 determinations of 23.4 μg of albumin gave an average of 5.94 ± 0.10. Disposable tubes made by other manufacturers probably can be used, as well.

Measurement of Fluorescence

Fluorescence of samples in buffer used for gel filtration was recorded with an Aminco-Bowman spectrophotofluorometer. Slit arrangement was: 1 = 2 mm; 2 = 4 mm; 3 = 2 mm; 4 = 2 mm; 5 = 4 mm; 6 = 2 mm; 7 = 2 mm. Emission spectra were recorded with excitation set

at maxima, and excitation spectra were recorded with emission set at maximum. Standardization was done on quinine sulfate in 0.1 N H_2SO_4 (1 μg/ml) with emission and excitation at 450 and 350 nm, respectively, by adjusting the sensitivity to obtain a reading of 2.00.

RESULTS

Analysis of the Complexes by Gel Filtration, Phosphorus Analysis, Amino Group Analysis and SDS-Electrophoresis

Figure 1 shows three effects of the reaction of peroxidized lecithin with albumin. Phosphorus is bound covalently to albumin as can be concluded on the basis of A, B and D. Molecular size of the monomer fraction (main peak in A) is increased as indicated by a slight decrease of its K_{av} value, comparing A with B and C. Polymerization occurs, increasing the amount of polymers from 10% in native albumin (B and C) to 28% (A). The polymer fraction corresponds to the minor peak eluted over K_{av} intervals of 0.0 to 0.2 (A) and 0.05 to 0.2 (B and C), respectively. The polymeric nature of these fractions was shown by SDS-electrophoresis. The number of mol. lecithin bound/mol albumin varies from 1 to 8 (A). Albumin that eluted in the K_{av} interval of 0.20–0.42 (albumin monomer) was pooled for amino group analysis (A). Amino groups were decreased by 22%. Because human serum albumin contains 59 amino groups/molecule (23), 13 amino groups are blocked per molecule by the

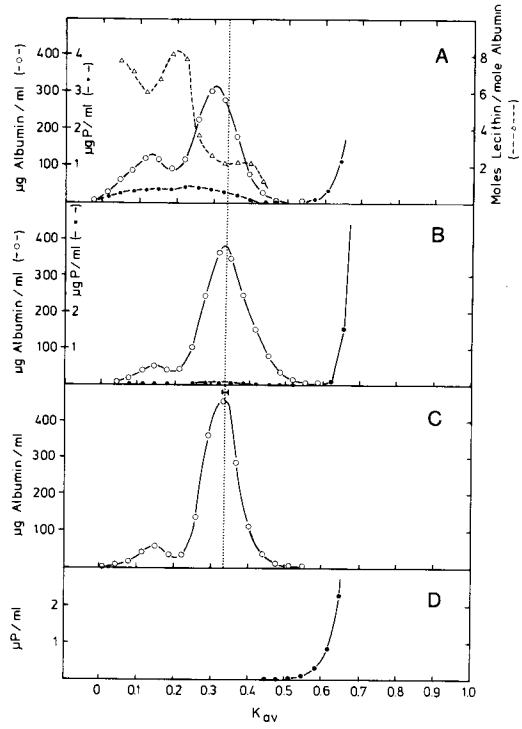

FIG. 1. Gel filtration of albumin on a 2.5 X 63 cm G 200 column after reaction with peroxidized lecithin (A). Oxidation (1.69 mol O_2/mol lecithin) was performed under constant UV irradiation at 5 ppm (7.93×10^{-5} M) of Cu^{++} and 5.45×10^{-4} M of Fe^{+++}. B refers to an experiment performed under the same conditions as A, except that UV irradiation was done under N_2. C refers to native albumin, and D shows the elution of peroxidized lecithin that had been incubated under conditions similar to A except that protein was omitted. The vertical, dotted line corresponds to the average of 5 determinations of K_{av} for native albumin, and the bar in C is equal to the double standard deviation.

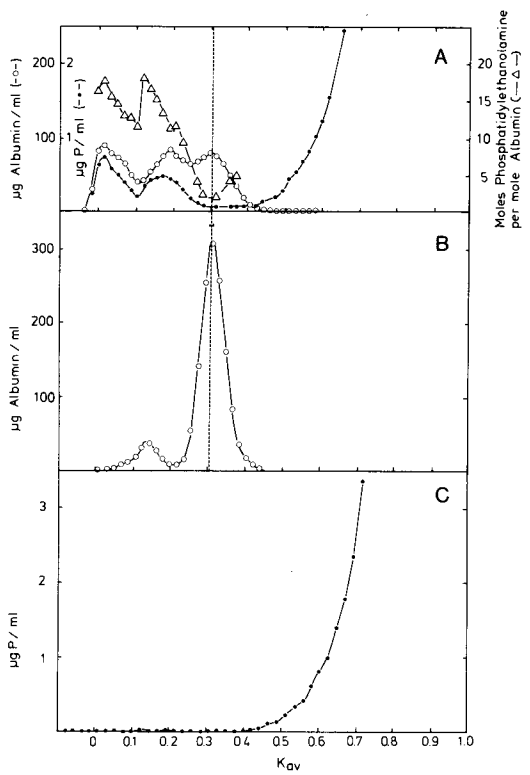

FIG. 2. Gel filtration of albumin on a 2.5 X 154 cm G 200 column after reaction with peroxidized phosphatidylethanolamine (1.7 mol O_2/mol phosphatidylethanolamine) is shown in A. Oxidation was performed under constant UV irradiation at 4.8 ppm (7.57×10^{-5} M) of Cu^{++}. B refers to native albumin and C shows elution of peroxidized phosphatidylethanolamine that was incubated under conditions similar to A, except that protein was omitted. The vertical, dotted line corresponds to the average of 4 determinations of K_{av} for native albumin, and the bar shown in B is equal to the double standard deviation.

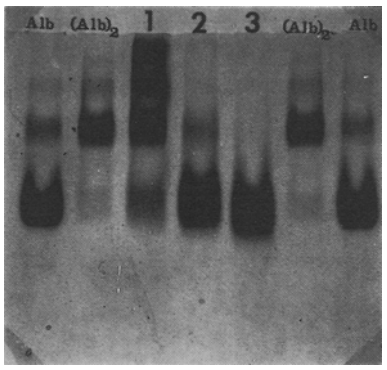

FIG. 3. SDS-electrophoresis of albumin after reaction with peroxidized phosphatidylethanolamine. Alb and $(Alb)_2$ refer to native albumin and albumin dimer. Samples 1–3 represent the first, middle and last third of the eluted protein of Fig. 2A corresponding to the following K_{av} intervals: −0.03 to 0.10; 0.10 to 0.24 and 0.24 to 0.41, respectively. Each sample contains 50 μg of protein.

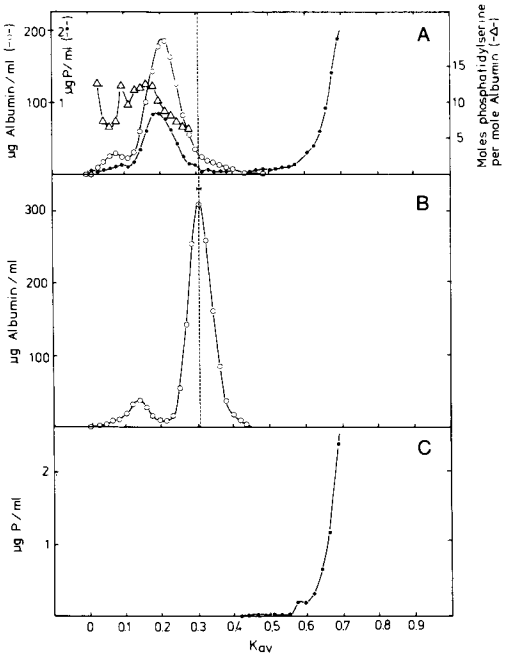

FIG. 4. Gel filtration of albumin on a 2.5 cm × 154 cm G 200 column after reaction with peroxidized phosphatidylserine (1.4 mol O_2/mol phosphatidylserine). Oxidation was performed under constant UV irradiation at 20 ppm (3.65×10^{-4} M) of Cu^{++}. B refers to native albumin and C shows elution of peroxidized phosphatidylserine that was incubated under conditions similar to A, except that protein was omitted. The vertical, dotted line corresponds to the average of 4 determinations of K_{av} for native albumin, and the bar in B is equal to the double standard deviation.

reaction. However, based on phosphorus and protein analyses, only 3.4 mol of lecithin are bound on the average/ mol albumin. Reaction between peroxidized cardiolipin (3.1 mol O_2/mol cardiolipin) and γ-globulin caused binding of 51 mol of cardiolipin and blocking of only 26 amino groups/mol of γ-globulin. Blocking 97–98% of the amino groups of albumin by maleylation (24) prior to the reaction with peroxidized cardiolipin (2.6 mol O_2/mol) diminishes the covalent binding by 85% (from 23 to 4.0 mol cardiolipin/mol albumin).

The reaction of peroxidized PE with albumin likewise causes covalent binding of phosphorus to albumin and increases its molecular size (Fig. 2). However, the effects differ quantitatively from those caused by peroxidized lecithin. The number of mol PE bound/mol albumin varies from ca. 2 for the later eluted albumin to ca. 17 for the earlier eluted (A). The elution profile which shows three peaks (A) indicates major increases in molecular size for ca. two-thirds of the protein. SDS-electrophoresis showed that the two peaks eluted over K_{av} intervals of 0.10 to 0.24 and 0.24 to 0.41 both consist of albumin monomer, whereas the earlier eluted peak (K_{av} interval of −0.02 to 0.10) which exhibits a shoulder consists of polymers of albumin with the dimer constituting by far the most important species, as shown by SDS-electrophoresis (Fig. 3). Polymers amount to 31% of the total. Thus, the reaction has caused polymerization of some 20% of the albumin. The complex eluted over K_{av} interval of 0.24 to 0.41 contains 12 amino groups less than native albumin per mol. However, because the amino group content of peroxidized PE itself may vary from 0 to 1 group/mol, this figure is a minimum value for the protein amino groups blocked by the reaction.

When peroxidized PS reacts with albumin, the effects, again, are covalent binding of phosphorus and increased molecular size as indicated by the decrease in elution vol (Fig. 4). From 6 to 12 mol (on the average 8.1) of PS are bound per mol of albumin (A). On the average, the complex contains 16 amino groups less than native albumin/mol. This, however, is a minimum value for the protein amino groups blocked by the reaction. SDS-electrophoresis of the protein corresponding to K_{av} intervals of −0.05 to 0.11 and 0.11 to 0.32 (A) shows that the earlier eluted pool, which constitutes 9% of the total eluted protein, consists almost exclusively of albumin dimer. This is equal to the dimer content of native albumin. The later eluted pool consists of monomer. Thus, the reaction does not cause polymerization.

The reaction of peroxidized PE with γ-globulin also causes covalent binding of phosphorus to this protein and increases its molecular size (Fig. 5). From 10 to 60 mol of PE become bound covalently/mol of γ-globulin, and molecular size is increased as indicated by the earlier elution of γ-globulin after the reaction (compare A with B). About 20% of the γ-globulin is polymerized by the reaction. This is found by SDS-electrophoresis as shown in Figure 6. Material eluted over K_{av} intervals of −0.06 to 0.08 and 0.08 to 0.19, samples 1 and 2, respectively, (Fig. 6) are polymers of γ-globulin. Material eluted over K_{av} interval of 0.19 to 0.38 is γ-globulin monomer (sample 3 of Fig. 6). It is thus observed that the monomer is eluted earlier than native γ-globulin, indicating an increase in its molecular size. when γ-globulin is reacted under similar conditions with peroxidized cardiolipin (3.1 mol O_2/mol cardiolipin), half of it is polymerized, and the monomeric fraction is eluted at a K_{av} value of 0.2 (range 0.15–0.25) as compared to a K_{av} of 0.32 for native γ-globulin.

Ultraviolet Absorption

UV absorption of albumin is increased after reaction with various peroxidized phospholipids and malonaldehyde (Fig. 7). The difference spectrum of albumin-malonaldehyde complex shows maxima at ca. 280 and 400 nm. The difference spectra of the complexes of peroxidized phospholipids and albumin show increase in UV absorption over a wide wavelength range. The complexes of albumin with lecithin and PE show maxima at ca. 280 nm but none at 400 nm. The other two complexes show no maximum. The difference spectrum of the albumin-cardiolipin complex, however, has a shoulder from 255 to 295 nm. The difference spectrum of the albumin-PS complex shows an almost evenly increasing absorption. The difference spectra for complexes between a given phospholipid and proteins other than albumin were similar to that of the complex between the phospholipid and albumin. Thus, the difference spectra of ovalbumin-PE complex and γ-globulin-PE complex resemble that of the albumin-PE complex and the difference spectra of ribonuclease-cardiolipin complex and γ-globulin-cardiolipin complex resemble that of the albumin-cardiolipin complex.

Fluorescence

Figure 8 A–C show fluorescence spectra of complexes formed by the reaction of albumin with peroxidized PE, peroxidized PS and malonaldehyde, respectively. The complexes of albumin with the two peroxidized phospholipids have similar fluorescence spectra. Two excitation maxima are observed at 315–320 and 345–350 nm, respectively. Emission maximum is at 400–415 nm. The same fluorescence spectra were observed for other complexes of proteins and peroxidized phospholipids. The complexes examined were albumin-lecithin; albumin-cardiolipin; γ-globulin-PE; ribonuclease-cardiolipin, and ovalbumin-PE. The fluorescence intensities of these complexes were comparable to those shown in Figure 8A and B. The complex of albumin with malonaldehyde has excitation maximum at 405 nm and emission maximum at 465 nm. Similar fluorescence spectra were observed for complexes of malonaldehyde with other proteins (ribonuclease, ovalbumin and γ-globulin).

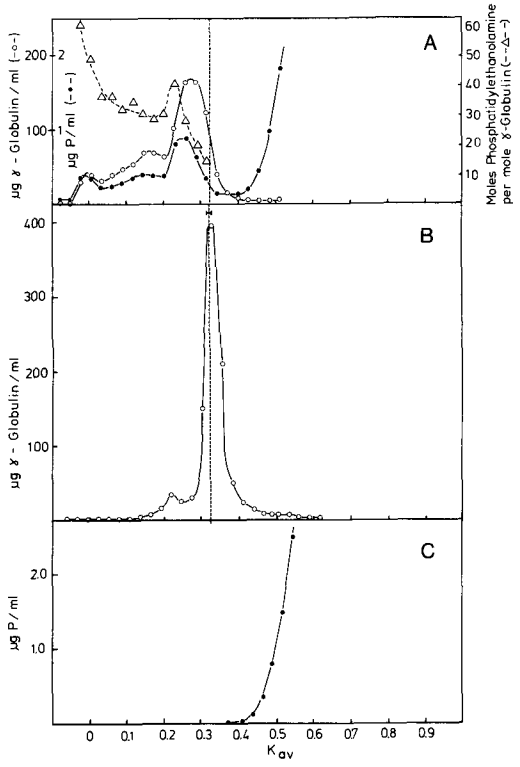

FIG. 5. Gel filtration (A) of γ-globulin on a 2.5 × 98 cm Sephacryl S-300 Superfine column after reaction with peroxidized phosphatidylethanolamine (1.48 mol O_2/mol phosphatidylethanolamine). Oxidation was performed under constant UV irradiation at 4.6 ppm (7.13×10^{-5} M) of Cu^{++}. B refers to native γ-globulin and C shows elution of peroxidized phosphatidylethanolamine that was incubated under conditions similar to A, except that protein was omitted. The vertical, dotted line shows the average of 4 determinations of K_{av} for native γ-globulin, and the bar in B is equal to the double standard deviation.

DISCUSSION

The gel filtration experiments show that molecular size of monomeric complexes of proteins an peroxidized phospholipids is always increased over that of the native protein. In addition to the space-filling of the bound lipid, unfolding of the proteins may also contribute to the increase in molecular size. The increase caused by different phospholipids varies. Lecithin, e.g., causes the smallest increase, whereas PE and PS cause considerably greater increases. Using a previously reported calibration curve (12), the increase caused by lecithin corresponds to ca. 10,000 daltons, whereas that caused by peroxidized PS corresponds to ca. 40,000 daltons. Also, the number of mol phospholipid bound/mol protein depends on the phospholipid. Thus, on the average, only 3.4 mol lecithin, whereas 11 mol of PE and 9 mol PS are bound/mol of albumin (Figs. 1A, 2A and 4A, respectively); 51 mol cardiolipin are bound to γ-globulin. These figures state the number of P-containing products that are bound, but other peroxidized products which do not contain phosphorus may also be important reacting species. Obviously, this is the case in the reaction of peroxidized lecithin with albumin, as only 3.4 atoms of lecithin phosphorus are bound/mol of albumin but 13 amino groups are consumed. Thus, at least 10 mol of peroxidized compounds which contain no phosphorus have reacted. Amino groups of albumin also are decreased after reaction with peroxidized PE and PS, as are amino groups of γ-globulin after reaction with peroxidized cardiolipin. The decrease in amino groups of proteins after reaction with peroxidized phospholipids is direct evidence that they are involved in the reaction. In support of this is the observation that blocking the amino groups of albumin by maleylation diminishes the binding of peroxidized cardiolipin. An electrostatic effect, however, may also be involved because maleylation increases the negative charge of the protein, and that expectedly will impede reaction with the negatively charged cardiolipin. Previous work indicates that the amino group of lysine residues of proteins react with peroxidized lipid (3, 5, 6, 25–27). Also, the observation that proteins fail to react with ninhydrin after incubation with peroxidized lipid (28, 29) indicates that amino groups are involved in the reaction. The reaction between peroxidized cardiolipin and γ-globulin causes 51 mol of cardiolipin to be bound/mol of γ-globulin, but only 26 amino groups are blocked. This indicates that other structures besides amino groups react; this possibility is supported by previous observations of destruction of other amino acids besides lysine in the reaction of peroxidized lipid with protein (4–6, 25–27).

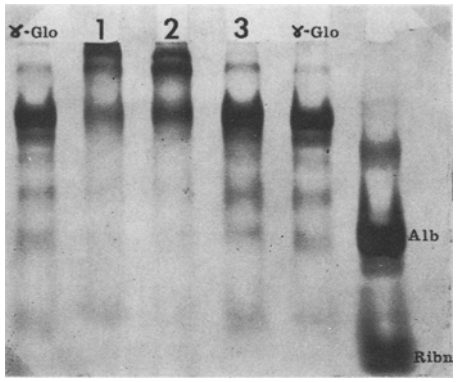

FIG. 6. SDS-electrophoresis of γ-globulin after reaction with peroxidized phosphatidylethanolamine. γ-Glo, Alb and Ribn are markers of γ-globulin, albumin and ribonuclease. Samples 1–3 represent protein eluted over the following K_{av} intervals of Fig. 5: −0.06 to 0.08, 0.08 to 0.19 and 0.19 to 0.38, respectively. Each sample contains 50 μg protein.

The amounts of peroxidized lipid bound to protein is an order of magnitude greater than observed in other model systems (1, 4, 8). In those investigations, ethyl linolenate-1-C^{14} (1), linolenic acid-1-C^{14} (4) and linoleic acid-1-C^{14} (8) were used as model lipids and the amount of bound peroxidized lipid was determined from the radioactivity of the protein. From 0.063 to 0.96 mol ethyl linolenate were bound/mol cytochrome c (1), and from 0.03 to 0.99 mol linolenic acid were bound/mol of cytochrome c (4). Binding of peroxidized linoleic acid to ribonuclease was even smaller, from 0.001 to 0.025 mol/mol (8). However,

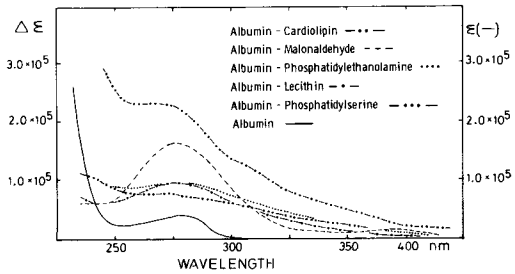

FIG. 7. Increase in UV absorption of albumin after reaction with peroxidized phospholipids or malonaldehyde. Difference spectra were recorded at 0.300 mg/ml. For comparison, the spectrum of native albumin is shown. The ordinate gives molar absorbance (ε) for native albumin, and increase in molar absorbance (Δε) for the various complexes.

the significance of these results is uncertain because only binding of those peroxidation products which contain the carboxylic carbon atom is measured.

Polymerization does not seem to be nearly as important in our model systems as in previously used systems. In three of the reactions, polymerization amounted to ca. 20%, in one reaction no polymerization occurred, and only in the reaction between heavily peroxidized cardiolipin (3.1 mol O_2/mol) and γ-globulin was as much as 50% of the protein polymerized. In comparison, ribonuclease and cytochrome c are almost completely polymerized by ethyl linolenate peroxidizing, corresponding to 0.54 mol O_2/mol (1); the same applies to ribonuclease when it reacts with linolenic acid peroxidizing, corresponding to 0.2 mol O_2/mol (2). Thus, even though considerably higher degrees of oxidations were used by us, the extent of polymerization is much less. This indicates an essential difference between the reactions of the present model system and the reactions studied in systems for which polyunsaturated fatty acids are used as model lipid.

It is general knowledge that peroxidized lipid causes discoloration of proteins, but no detailed studies are available of UV absorption of proteins after reaction with peroxidized lipid. The reason for this is that no method has been available for solubilizing the covalent protein-lipid complexes. However, it turns out that the complexes are solubilized by the detergent sodium deoxycholate (11, 12). The difference spectra of the various complexes (Fig. 7) show that the increase in UV absorption of the proteins caused by their reaction with peroxidized lipid occurs over a wide range of wavelengths. Probably, the spectra are summations of the spectra of several structures which conceivably comprise both structures formed in the reaction and structures in the peroxidation products being bound. The absorption maximum at 270–280 nm for the albumin-lecithin and albumin-PE complexes, and the shoulder in the same wavelength range for the albumin-PS and albumin-cardiolipin complexes probably are not indicative for a significant content of iminopropene structure. This is partly because no additional maximum is observed at ca. 400 nm, and partly because the fluorescence of the complexes are different from that of albumin-malonaldehyde complex.

The similarity of the fluorescence spectra of the various complexes between proteins and peroxidized phospholipids indicates a common fluorescent structure. Because the spectra are essentially different from the fluorescence spectra of malonaldehyde-protein complexes, fluorophors also will be different. Thus, while the fluorophor of malonaldehyde-protein complexes is substituted 1-amino-3-iminopropene formed by the reaction of one molecule of malonaldehyde with two amino groups (3, 30), some compounds other than malonaldehyde probably are produced in the peroxidation of phospholipids and through reaction with protein forms a fluorophor with excitation

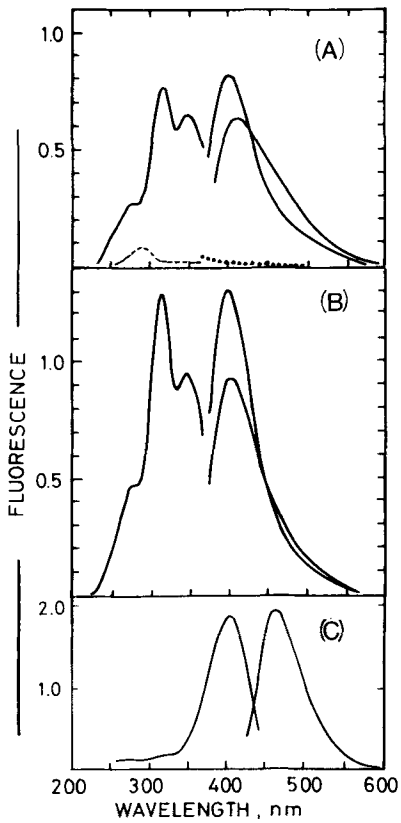

FIG. 8. Fluorescence spectra of the complexes of peroxidized phosphatidylethanolamine-albumin (A), peroxidized phosphatidylserine-albumin (B), and malonaldehyde-albumin (C). The peroxidized phosphatidylethanolamine-albumin complex is that of Fig. 2A eluted over K_{av} range of −0.02 to 0.41. On an average, it contains 10 mol phosphatidylethanolamine/mol albumin. The complex of peroxidized phosphatidylserine and albumin is that of Fig. 4A eluted over K_{av} range of −0.05 to 0.32. On an average, it contains 9.4 mol of phosphatidylserine/mol albumin. The malonaldehyde-albumin complex is that described under Ultraviolet Absorption (Fig. 7). All spectra were recorded at 0.300 mg albumin/ml and the same apparatus sensitivity. The broken-line curves in A are recordings of native albumin. The two emission spectra shown for each of the phospholipid-protein complexes correspond to excitation at their two maxima.

maxima at 315–320 and 345–350 nm, and emission at 400–415 nm.

The oxidation conditions used in this work are severe. This probably ensures that most possible effects of peroxidative damage of proteins are displayed. On the other hand, severe oxidation conditions are likely to exaggerate effects and possibly expose effects which are undetectable at milder oxidation conditions, and our results should be read with this qualification. The diversity of peroxidative effects observed in our model system indicate the number of parameters which should all be measured when a biological material is examined for peroxidative damage. Increase, then, in either molecular size, phosphorus content, UV absorption or fluorescence of its protein suggests peroxidative damage.

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α-Tocopherol and Serum Lipoproteins

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ABSTRACT

Twenty-six patients with clinically confirmed mammary dysplasia and five age-matched controls were treated with α-tocopherol, 600 mg/day. Serum samples collected on the 21st day of the menstrual cycle were analyzed for cholesterol in lipoprotein fractions, isolated by a combination of precipitation and ultracentrifugation techniques. Eighty-five percent of patients showed objective and subjective remission from disease following therapy. In mammary dysplasia patients, the ratio of serum cholesterol/high density lipoprotein cholesterol was higher than those in age-matched controls, an abnormality which was corrected by α-tocopherol therapy. Furthermore, as a result of therapy, high density lipoproteins increased and ester cholesterol associated with low density lipoproteins decreased. The results suggest that α-tocopherol may serve as an effective agent in treating patients with benign breast disease, as well as correct the inherent abnormality in serum cholesterol distribution in mammary dysplasia patients.

INTRODUCTION

Serum tocopherol is carried primarily by the lipoproteins. Studies on the distribution of tocopherol in humans have shown that low density lipoproteins (LDL), principally the Sf 3-9 fractions, carry 41-58% of the total serum tocopherol whereas high density lipoproteins (HDL) carry 29-37% (1). Several workers have also recognized an interrelationship between serum tocopherol levels and circulating lipoproteins in blood (1,2) and with plasma cholesterol and age (3). Tocopherol is claimed to have a role in the prevention and cure of a variety of human cardiovascular diseases including atherosclerosis (4), and has been used to reduce the blood lipids in hyperlipemic individuals (3,5).

Barclay et al. (6) found that LDL was elevated and HDL depressed in women with breast cancer and their relatives. Very litttle work has been done in correlating the changes in serum lipids and lipoproteins with the incidence of breast cancer and its progression. Similarly, we have limited knowledge regarding these levels in mammary dysplasia (M.D.) patients who are at increased risk for breast cancer. α-Tocopherol, a noncaloric dietary factor, has been used by physicians for over a decade in treating M.D. This study, which is part of our ongoing investigation in patients with M.D., is designed to determine whether patients with M.D. have abnormal lipid and lipoprotein levels, and whether treatment with tocopherol would correct any of these observed aberrations.

MATERIALS AND METHODS

Five control patients without underlying breast or other endocrine pathology and 26 patients with documented (M.D.), either on the basis of mammography or previous biopsy, were enrolled in the study after informed consent was obtained. None of the patients had any history of gynecologic disease or endocrine dysfunction and none were on any hormonal or vitamin therapy. All subjects had regular menstrual cycles. After this screening, a complete clinical history was obtained followed by physical and breast examinations. Fasting blood samples were obtained between 8:30-9:30 a.m. on day 21 of each menstrual cycle over a period of four months. The subjects consumed their usual diets during this period and serum tocopherol levels were determined to monitor adherence to therapy. During the first month, the patients and controls received placebo and during the second and third months they were given dl-α-tocopherol acetate, 300 international units (IU), twice daily. Blood was allowed to clot at 4 C, centrifuged and the serum was separated. Serum tocopherol concentrations were determined by a well established procedure (7).

Very low density lipoproteins (VLDL) and LDL were isolated together by precipitation with heparin and 2 M $MnCl_2$ (8) and reconstituted in 2 M NaCl. Further addition of reagents to the supernatant did not result in the formation of more precipitate. Titration of control sera with the volume of reagents used showed that the amount of reagents was sufficient to precipitate quantitatively the LDL. The HDL_2 and HDL_3 fractions were isolated by sequential

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preparative ultracentrifugation in a Beckman Model L2-50 ultracentrifuge using a Ti-50 rotor for 36 hr at 40,000 rpm at 4 C (9). Preliminary experiments using sera from normal volunteers showed that HDL_2 and HDL_3 isolated by this method did not differ from HDL_2 and HDL_3 isolated by ultracentrifugal isolation of VLDL and LDL at dl 1.063 g/ml followed by sequential preparative ultracentrifugal isolation of HDL_2 and HDL_3. The total and free cholesterol in the lipoprotein fractions and serum were measured by the enzymatic method of Allain et al. (10), using a reagent kit from Biodynamic Division of Bohringer-Manheim Co. Cholesterol values obtained by the enzymatic method were in close agreement with (correlation coefficient 0.984) those obtained by procedures of the lipid research clinics (11) and those with other methods (12,13). Because sedimentation of manganese chloride reagent in the cuvets in an autoanalyzer can give a slightly higher value, this problem was avoided by washing the spectrophotometer cuvet in the manual method.

Statistical analysis was performed using Student's t-test. Differences in the means were considered significant when the value of p was less than 0.05. The study was done in a double-blind fashion neither the physician nor the patient knew whether the patient was on placebo or tocopherol at any time. Serum samples were coded and laboratory analysis for each test was done under identical conditions. The single laboratory technician performing the tests was unaware of the code. Clinical response to the therapy was graded by both subjective (patient) and objective (physician) analysis. Subjective improvement at each examination was noted using our previous criteria (14). Objective changes were recorded by a single examining physician. Criteria included overall tenderness, size and location of the lesions and measurement of discrete lesions when detected.

RESULTS

The results from normal control subjects were compared with those from patients, before and after therapy. The treatment group was also divided into responders and nonresponders to correlate clinical response with any alteration in lipid and lipoprotein levels. The serum tocopherol levels (mg/dl) increased from 1.62 ± 0.20 to 2.60 ± 0.39 and from 1.37 ± 0.11 to 2.66 ± 0.21 in controls and patients, respectively. The responders and nonresponders increased from 1.46 ± 0.11 to 2.77 ± 0.23 and 0.90 ± 0.17 to 2.04 ± 0.26, respectively.

The ratio of total serum cholesterol (STC) to HDL is presented in Table I. Before treatment, the ratio of STC to HDL was significantly more in M.D. patients than in controls (4.28 ± 0.31 vs 3.75 ± 0.16, $p<0.1$ with all patients and 4.40 ± 0.33 vs 3.75 ± 0.16, $p<0.05$ with responders). The ratios in nonresponders and controls were similar. α-Tocopherol administration lowered the ratios in M.D. patients (total and responders) to pretreatment control values. Such lowering of ratio was evident in nonresponders, also. Although the treatment appeared to increase the ratio in controls, such increase from 3.75 ± 0.16 to 5.01 ± 1.11 was not statistically significant. Studies by others have stressed the importance of using HDL_2 or apo A-I values rather than total HDL in assessing the protective role of high density lipoproteins. Changes in STC-to-HDL_2 were similar to changes in STC-to-HDL ratios.

Table II presents the values (mg/dl) of actual

TABLE I

Ratio of Serum Total Cholesterol to HDL and HDL_2 Cholesterol before and after Therapy

Ratio		Controls (n=5)	Patients: Total (n=26)	Patients: Responders (n=22)	Patients: Nonresponders (n=4)
$\frac{STC}{HDL}$[a]	B.T.	3.75 ± 0.16	4.28 ± 0.31	4.40 ± 0.33[b]	3.72 ± 0.90
	A.T.	5.01 ± 1.11	3.88 ± 0.24	4.03 ± 0.28	3.22 ± 0.12
$\frac{STC}{HDL_2}$	B.T.	11.86 ± 2.88	13.45 ± 2.31	14.17 ± 2.62	10.61 ± 6.15
	A.T.	13.19 ± 4.65	10.91 ± 1.49	12.05 ± 1.62	6.38 ± 0.28

Values are expressed as mean ± standard error of the mean; n = number of subjects.

[a]STC = serum total cholesterol, in mg/dl; HDL = high density lipoprotein; HDL_2 = high density lipoprotein fraction floating at 1.125 g/ml density; b = patient value significantly ($p<0.05$) different from corresponding control value; B.T. = before therapy; A.T. = after therapy.

TABLE II

Levels of Total and Free Cholesterol in Serum and Lipoprotein Fractions before and after Therapy

			Patients	
	Controls (n=5)	Total (n=26)	Responders (n=22)	Nonresponders (n=4)
S-TC	B.T. 182.60 ± 17.00	177.18 ± 6.00	178.78 ± 6.78	170.00 ± 14.53
	A.T. 207.60 ± 11.95	191.18 ± 4.69[a]	196.33 ± 4.90	168.00 ± 6.08
HDL-TC	B.T. 49.20 ± 5.21	45.98 ± 3.37	44.14 ± 3.16	54.25 ± 12.52
	A.T. 48.00 ± 3.14	52.93 ± 3.32[a]	53.03 ± 4.06[a,b]	52.50 ± 0.21
(VLDL+LDL)-TC	B.T. 136.75 ± 14.35	131.56 ± 6.91	136.50 ± 8.37	114.25 ± 6.00
	A.T. 163.50 ± 23.17	129.39 ± 6.40[b]	134.21 ± 7.68	112.50 ± 6.63[b]
HDL_2-TC	B.T. 22.75 ± 8.87	18.00 ± 3.87	15.75 ± 2.75	27.00 ± 1.00
	A.T. 20.75 ± 5.35	20.00 ± 2.23	18.00 ± 2.23	28.00 ± 2.00
HDL_3-TC	B.T. 22.25 ± 6.83	20.70 ± 3.10	20.13 ± 3.41	23.00 ± 10.00
	A.T. 23.25 ± 3.96	26.00 ± 1.26[a]	26.00 ± 1.57[a]	26.00 ± 1.00
(VLDL ± LDL)-FC	B.T. 36.60 ± 4.97	40.04 ± 1.78	41.50 ± 2.09	33.25 ± 2.06
	A.T. 57.60 ± 5.65[a]	47.17 ± 2.70[a,b]	49.28 ± 3.30[a]	34.75 ± 1.65[b]

Values are expressed as mean ± standard error of the mean; n = number of subjects. TC = total cholesterol, in mg/dl; FC = free cholesterol, mg/dl; S = serum; HDL = high density lipoprotein; (VLDL+LDL) = very low density lipoprotein plus low density lipoprotein; HDL_2 = high density lipoprotein fraction isolated at 1.125 g/ml density; HDL_3 = high density lipoprotein fraction isolated at 1.21 g/ml density.

[a]After treatment value, significantly ($p<.05$) different from pretreatment value.

[b]Patient value significantly ($p<.05$) different from corresponding control value.

total and free or unesterified cholesterol in serum or lipoprotein fractions for controls and patients, before and after therapy. α-Tocopherol administration increased the serum cholesterol levels significantly (from 177.18 ± 6 to 191.18 ± 4.69, $p<.05$) only in M.D. patients and not in nonresponders or controls. At the same time, there was a concurrent increase in the HDL fraction in the M.D. patients but not in nonresponders or controls. The table further shows that the increase in the HDL_3 fraction was relatively more than in the HDL_2 fraction.

Total cholesterol level in the atherogenic VLDL+LDL fraction in controls was similar to that in patients (total and responders but not in nonresponders) before treatment. There was no significant alteration in these levels in any of the groups as a result of treatment, although at the end of treatment period, the patient values were significantly lower than the control values. In spite of a lack of change in the total cholesterol in VLDL+LDL, the free cholesterol of VLDL+LDL increased significantly in the patients (total and responders but not nonresponders) and also in controls, as a result of treatment with α-tocopherol.

DISCUSSION

Pharmacologic quantities of α-tocopherol has been used by physicians for over a decade in the treatment of patients with M.D. Remission of disease with a clinical response rate of 85% is demonstrated in this study. As reported by Yang and Desai (15), a linear rise in serum tocopherol due to oral intake of α-tocopherol has been observed, the concentration of which may depend on several factors such as dosage, duration of treatment, age and species. Uniform elevation of serum tocopherol in both controls and patients showed that there are no differences in absorption and transport of this vitamin in both groups. However, the effect of different doses of this vitamin on the efficacy of treatment is unknown.

Before treatment, the patients, in general, had lower levels of α-tocopherol than controls; this difference was more pronounced in nonresponders. Although both controls and patients responded uniformly to the administration of α-tocopherol, the nonresponders attained a relatively lower level, suggestive of a defect in the absorption of α-tocopherol by nonresponders.

Tocopherol has been investigated in the treatment of hyperlipidemia by numerous investigators. Hypolipemic and hyperlipemic subjects have relatively lower and higher blood levels of α-tocopherol than controls, respectively (3,15,16). α-Tocopherol concentration in blood has been shown to be highly correlated with cholesterol concentrations (3,15,17,18) and total lipids (19). Ingestion of α-tocopherol was shown to have no significant effect on total cholesterol concentrations in normal subjects (17). However, in hyperlipemic subjects, α-tocopherol ingestion had a dose-dependent lowering effect on serum cholesterol

concentrations (5,15). Furthermore, studies (18) with rabbits and rats demonstrated that α-tocopherol deficiency was associated with an increased level of serum cholesterol, attributed to a deficiency in lecithin cholesterol acyl transferase activity. These studies in humans and animals suggest that supplementation of the diet with α-tocopherol can cause a reduction in the serum cholesterol concentrations, especially in subjects with a higher-than-normal level of cholesterol.

Our subjects, both controls and patients, had serum cholesterol levels well within the normal range for women of this age group (ages 25-35). While administration of α-tocopherol did not alter the serum cholesterol concentration of nonresponders, it increased the levels in patients by 8% and to a somewhat greater extent (by 10%) in responders. These increases were statistically significant. An increase by 14% was also observed in the control group. This increase was not statistically significant, confirming earlier observation by others (17); it is also possible that statistical significance was not obtained due to population size and larger variation in values.

An increase in serum total cholesterol, even by 10%, is an undesirable side effect. However, it is now well known that diet- or drug-induced small fluctuations in total serum cholesterol are not true indicators of increased or decreased risk of atherosclerosis. One should look at the levels of atherogenic LDL and protective HDL for meaningful interpretation. Our data show that, although there is an increase in serum cholesterol as a result of α-tocopherol therapy, increase is due to a rise in the protective HDL-cholesterol fraction and not to any increase in the atherogenic LDL cholesterol. In fact, there is a slight decrease in the LDL cholesterol levels in the patients. While α-tocopherol therapy seems to be clearly beneficial to the M.D. patient by increasing the HDL cholesterol and decreasing the LDL cholesterol, the data does not indicate similar trends in the control group. Although statistically not significant, controls do show an increase in LDL cholesterol and no alteration in HDL levels, strongly suggesting the need for a detailed study with a larger number of controls before beneficial or harmful effects of α-tocopherol in normal subjects can be claimed.

When the free cholesterol concentrations are considered, a much clearer picture is seen. A highly significant rise ($p<0.01$) in the free cholesterol associated with VLDL+LDL due to α-tocopherol therapy is observed in the control and patient groups, whereas nonresponders show no change. Because total cholesterol is the sum of free and ester cholesterol, α-tocopherol therapy decreases the ester cholesterol, the form that is deposited in the arteries. Thus, α-tocopherol appears to change the composition of atherogenic VLDL+LDL beneficially, not only in the M.D. patients but also in the normal control subjects. In the M.D. patients, α-tocopherol appears to be beneficial by altering the VLDL+LDL composition as well as by increasing the protective HDL fraction.

M.D. has been considered to be a premalignant condition, in view of the increased risk of developing breast cancer (19) in such patients. Investigations from Sloan Kettering Memorial Institute (20,21) have established that not only cancer patients, but also their relatives, have higher LDL and lower HDL levels. Surgical removal of tumor or medical treatment corrects this abnormality. If M.D. is truly a precancerous condition, the chances of M.D. developing into a neoplasm may be decreased by the ingestion of α-tocopherol by favorably altering their lipid composition.

In conclusion, α-tocopherol therapy appears to be an excellent mode of treatment for M.D. Along with the clinical improvements in these patients, changes in the lipid and lipoproteins were observed. In normal subjects, α-tocopherol ingestion may have a beneficial role by increasing the free cholesterol and altering the composition of LDL. Studies are in progress to determine whether the observed effects of α-tocopherol are applicable in males also, and to elucidate the mechanism by which the alteration in lipid levels are brought about by this vitamin.

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Effects of Feeding Chenodeoxycholic Acid on Metabolism of Cholesterol and Bile Acids in Germ-Free Rats

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ABSTRACT

The aim of this investigation was to study the influence of chenodeoxycholic acid administration on cholesterol and bile acid synthesis in germ-free rats. Seven rats were fed a basal diet and 2 groups of 4 rats received the same diet supplemented with 0.4 and 1% chenodeoxycholic acid, respectively. After 6 weeks, feces were collected in one 3- and one 4-day pool for analysis of cholesterol and bile acids. When the sampling period was finished, the rats were killed and the liver microsomal fractions isolated. The activities of HMG CoA reductase and cholesterol 7α-hydroxylase were determined, the 7α-hydroxylase by a mass fragmentographic method. The 2 dominating bile acids in the untreated rats were cholic acid and β-muricholic acid. During treatment with chenodeoxycholic acid, 60–70% of this bile acid was converted into α- and β-muricholic acid, indicating a high activity of the 6β-hydroxylase. The excretion of cholic acid was almost completely inhibited and the 7α-hydroxylase activity was decreased ca 75% in the rats fed 1% chenodeoxycholic acid. The activity of the hepatic HMG CoA reductase was unchanged. The fecal excretion of cholesterol increased 2–3 times. An accumulation of cholesterol was seen in the rats treated with 1% chenodeoxycholic acid, which was probably a result of the decreased catabolism of cholesterol to bile acids.

ABBREVIATIONS

TLC, thin layer chromatography; TLC, gas liquid chromatography; HMG, 3-hydroxy-3-methylglutaryl; HMG CoA reductase, mevalonate: NADP oxidoreductase, EC 1.1.1.34; cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholanoic acid; α-muricholic acid, 3α, 6β, 7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α, 6β, 7β-trihydroxy-5β-cholanoic acid.

INTRODUCTION

Germ-free rats accumulate cholesterol in the liver to a considerably higher extent than conventional rats (1, 2). One reason seems to be more efficient absorption of cholesterol from the intestine (3). The synthesis of cholesterol in the liver and the catabolism of cholesterol bile acids are both decreased (4–8). These rats have an enlarged pool size of bile acids, particularly of cholic acid. The bile acids have a low fractional turnover rate (2, 3, 9, 10) as a result of a more efficient reabsorption of bile acids from the intestine (3) and of the lack of bacterial degradation.

In a series of investigations, we studied cholesterol and bile acid metabolism in germ-free rats. Cholesterol feeding is associated with an inhibition of hepatic cholesterol synthesis (8). Simultaneously, the catabolism of cholesterol to bile acids, especially to chenodeoxycholic acid, is increased (2, 8). Interruption of the enterohepatic circulation of bile acids by cholestyramine treatment causes a several-fold increase in the synthesis both of cholesterol and bile acids (11). Whether bile acid feeding also influences cholesterol metabolism in germ-free rats is unknown.

Since cholic acid is the dominating bile acid in germ-free rats, it was considered of interest to feed chenodeoxycholic acid and study its influence on cholesterol and bile acid metabolism in these animals. In this study, we administered chenodeoxycholic acid to germ-free rats and determined the activities of HMG CoA reductase and 7α-hydroxylase, rate-determining enzymes in cholesterol and bile acid syntheses. In addition, we measured the daily fecal excretion of cholesterol and bile acids. We did not try to preform comparative studies in conventional rats since, in these animals, bile acids and cholesterol are metabolized to a spectrum of metabolites by the intestinal microorganisms, which makes a quantitative analysis more complicated.

The structures of the bile acids mentioned in this paper are depicted in Figure 1.

MATERIALS

Glutaryl-[3-^{14}C] HMG CoA (sp act 20

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μCi/mg) DL[5-^{3}H](N)mevalonic acid (dibenzylethylene-diamine salt, sp act 25 μCi/mg), [4-^{14}C]cholesterol (sp act 145 μCi/mg), [1,2-^{3}H]cholesterol (sp act, 110 μCi/mg), and [24-^{14}C]cholic acid (sp act 138 μCi/mg) were purchased from New England Nuclear Corp., Boston, MA. The radioactive compounds were analyzed by thin layer chromatography (TLC) and were found to be more than 98% pure. The radioactively labeled HMG CoA was diluted with unlabeled material obtained from P-L Biochemicals, Inc., Milwaukee, WI, to yield a specific radioactivity of 145 μCi/mg. Before use, [4-^{14}C]cholesterol was purified by chromatography on a column of Al_2O_3 (grade III; Woelm Eschwege, Germany).

Unlabeled DL-mevalonic acid lactone, NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and chenodeoxycholic acid were obtained from Sigma Chemical Co., St. Louis, MO.

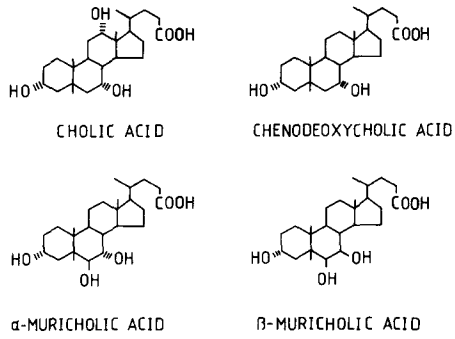

FIG. 1. Structures of the major bile acids in germ-free rats.

METHODS

Animals and Preparation of Microsomes

Germ-free male rats of the Sprague-Dawley strain weighing ca. 350 g were used. They were reared according to the technique of Gustafsson (12, 13) and were fed a standardized diet ad libitum with 10% (w/w) arachis oil as the source of fat; the diet contained 0.004% (w/w) cholesterol (8). Seven animals were given the basal diet and 4 animals received the basal diet supplemented with either 0.4 or 1% (w/w) chenodeoxycholic acid. When the rats had been fed the experimental diets for 6 weeks, feces were collected in one 3-day and one 4-day pool and were stored at −20 C until analyzed. This long feeding time was chosen since germ-free rats have a very long transit time of intestinal contents and, following a change in diet, it will take several weeks until a new steady-state in cholesterol and bile acid metabolism is reached (14). During the experimental period, the control rats gained, on an average, 16% of their body weight. The rats fed 0.4% chenodeoxycholic acid, on the other hand, gained less than 1% and those fed 1% chenodeoxycholic acid only ca. 2% of their body weight. This was correlated to a diminished food intake. No other untoward side effects were observed in the treated animals. The reason is not quite obvious for the reduced intake of food in the animals fed chenodeoxycholic acid. To our knowledge, no such effect has been reported from corresponding feeding experiments in conventional rats.

When the sampling period was completed, the rats were killed by decapitation immediately after they had been taken out of the germ-free isolators at 9 am. The livers were removed quickly and chilled on ice, and were then homogenized in 4 vol of a medium containing 0.3 M sucrose, 0.075 M nicotinamide, 0.002 M EDTA and 0.02 M mercaptoethanol using a Potter-Elvehjem Teflon-glass homogenizer equipped with a loosely fitting pestle. The concentration of cholesterol was determined as described by Hanel and Dam (15). The homogenate was centrifuged at 20,000 × g for 15 min. The microsomal fraction was isolated by centrifuging the 20,000 × g supernatant at 100,000 × g for 70 min. The microsomes were washed with homogenizing medium once and centrifuged again at 100,000 × g for 30 min. One part of the microsomal fraction was suspended in 0.17 M phosphate buffer (pH 7.2 and containing 0.034 M mercaptoethanol) to a vol corresponding to 10% (w/v) homogenate. The protein concentration was determined by the Lowry et al. method (16). This microsomal fraction was used for the assay of HMG CoA reductase activity.

Another part of the microsomal fraction was suspended in 0.1 M phosphate buffer (pH 7.0 and containing 0.028 M nicotinamide). The protein concentration (16) and cholesterol content (15) of the microsomes were determined. This microsomal fraction was used for the assay of 7α-hydroxylase activity.

Assay of HMG CoA Reductase Activity

The assay has been described recently (4) and was similar to that described by Shefer et al. (197). The complete system contained in a vol of 0.85 ml: 0.2 ml of microsomal fraction; phosphate buffer, pH 7.2, 100 mM; NADP, 3 mM; glucose-6-phosphate, 10 mM; glucose-6-phosphate dehydrogenase, 5 enzyme units; $MgCl_2$, 3 mM; mercaptoethanol, 22 mM; [3-^{14}C]-HMG CoA, 0.2 mM. The incubation

was done for 15 min at 37 C. It was stopped by the addition of 0.1 ml of 5 M HCl. Tritium-labeled and unlabeled mevalonic acids were added as internal standards and the incubation mixture was further shaken for 30 min at 37 C. The mixture was extracted and further subjected to TLC and the radioactivity of mevalonic acid lactone was determined.

Assay of 7α-Hydroxylase Activity

To a mixture of 3 ml of microsomal fraction, 2 ml of buffer, and 5 μmol of NADPH was added 10 μg of [4-^{14}C] cholesterol, dissolved in a suspension of 0.1 ml of buffer containing 3 mg of Tween 80 (18). This mixture was incubated for 15 min at 37 C and incubation was terminated by addition of 20 vol of chloroform/methanol (2:1, v/v). Just before addition of chloroform/methanol to the incubation mixture, ^{2}H-labeled 5-cholestene-3β, 7α-diol (3 μg) was added as internal standard (cf. 18, 19). The chloroform phase was analyzed by TLC. Conversion of the trace amount of added [4-^{14}C] cholesterol into 5-cholestene-3β, 7α-diol was determined by radioscanning of the TLC plate. The mass of 5-cholestene-3β, 7α-diol formed from endogenous cholesterol was determined by means of combined gas liquid chromatography-mass spectrometry (GLC-MS) of the appropriate chromatographic fraction using an LKB instrument equipped with a 1.5% SE-30 column (19).

Analysis of Fecal Bile Acids and Neutral Steriods

Feces were disintegrated in water and refluxed in 70% (v/v) aqueous ethanol for 2 hr. After filtration, the residue was refluxed in chloroform/methanol (1 1, v/v) for 1 hr and the extracts were combined.

An aliquot was further hydrolyzed with 1 M KOH in 50% aqueous ethanol for 10 hr at 110 C. The saponification mixture was extracted with petroleum ether, which was discarded. The mixture was then acidified and extracted with ethyl acetate. The ethyl acetate phase was washed with water until neutral and the solvent was evaporated. [24-^{14}C] Cholic acid was used as an internal standard for the correction of losses during the extraction procedure. The residue of the extract was methylated, silylated and analyzed by GLC using 1% Hi Eff 8BP as the stationary phase.

Another aliquot of the combined extracts was hydrolyzed with 1 M KOH in 50% aqueous ethanol for 1 hr and extracted with petroleum ether. The petroleum ether phase was evaporated. [1,2-^{3}H] Cholesterol was used as an internal standard to correct for losses during the extraction procedure. The residue of the extract was silylated and analyzed by GLC using 1% Hi Eff 8BP as the stationary phase.

RESULTS

Liver Cholesterol Concentrations and HMG CoA Reductase Activity

As Table I demonstrates, feeding with 0.4% chenodeoxycholic acid did not significantly affect the cholesterol concentration of liver homogenate. Feeding with 1% chenodeoxycholic acid, on the other hand, increased the cholesterol content ca. 50%. In the microsomal fraction, the cholesterol concentration was about the same in all 3 groups of animals.

The HMG CoA reductase activity in untreated rats was 24.8 ± 2.4 pmol/mg protein/min (Table I). Feeding with chenodeoxycholic acid did not significantly change the enzyme activity.

7α-Hydroxylase Activity

The endogenous formation of 5-chole-

TABLE I

Influence of Chenodeoxycholic Acid Feeding on Concentration of Cholesterol in Liver Homogenate and Microsomes, and on HMG CoA Reductase and Cholesterol 7α-Hydroxylase in Liver Microsomes from Germ-Free Rats

Treatment	Number of rats	Cholesterol concentration of homogenate	microsomes	HMG CoA reductase	7α-Hydroxylase
		mg/ml		pmol/mg protein/min	
None	7	0.82 ± 0.04[a]	0.13 ± 0.01	24.8 ± 2.4	4.1 ± 0.7
0.4% Chenodeoxycholic acid	4	0.89 ± 0.03	0.13 ± 0.01	26.4 ± 1.8	3.3 ± 0.7
1% Chenodeoxycholic acid	4	1.24 ± 0.02[b]	0.16 ± 0.02	29.4 ± 3.5	0.9 ± 0.3[c]

[a]Values are the means ± SEM.

[b]Significantly different from untreated rats, $P<0.001$, with Student's t-test.

[c]Significantly different from untreated rats, $P<0.02$, with Student's t-test.

TABLE II

Influence of Chenodeoxycholic Acid Feeding on the Daily Fecal Excretion of Bile Acids and Cholesterol in Germ-Free Rats

Treatment	Number of rats	Cholic acid	Chenodeoxycholic acid	α-Muricholic acid (mg/kg body weight)	β-Muricholic acid	Total bile acids	Cholesterol
None	7	8.2 ± 1.9[a]	Trace	Trace	6.5 ± 0.8	15.1 ± 2.2	9.8 ± 0.9
0.4% Chenodeoxycholic acid	4	Trace	23.8 ± 3.1	25.4 ± 8.1	35.3 ± 4.2	84.4 ± 8.9	16.4 ± 1.5[b]
1% Chenodeoxycholic acid	4	Trace	72.7 ± 5.9	53.5 ± 3.5	52.6 ± 3.9	178.8 ± 11.8	26.2 ± 2.7[b]

[a]Values are the means ± SEM.

[b]Significantly different from untreated rats, $P<0.001$, with Student's t-test.

stene-3β, 7α-diol was 4.1 ± 0.7 pmol/mg protein/min in the untreated rats (Table I). Treatment with 0.4% chenodeoxycholic acid did not significantly decrease the 7α-hydroxylase activity. In the rats treated with 1% chenodeoxycholic acid, the formation of 5-cholestene-3β, 7α-diol was decreased by ca. 75%.

Fecal Excretion of Bile Acids and Cholesterol

The 2 dominating bile acids in feces from untreated rats were cholic acid and β-muricholic acid (Table II). Only trace amounts of chenodeoxycholic acid and α-muricholic acid were found. In the 2 groups of rats treated with chenodeoxycholic acid, only trace amounts of cholic acid were found. The total excretion of the bile acids was 84.4 ± 8.9 mg/kg body weight in the rats treated with 0.4% bile acid. Chenodeoxycholic acid, α-muricholic acid and β-muricholic acid constituted 28, 30 and 42% of the fecal bile acids, respectively. During the treatment with 1% bile acid, the daily excretion of total bile acids was 178.8 ± 11.8 mg/kg body weight. The percentage composition of chenodeoxycholic acid, α-muricholic acid and β-muricholic acid was 41, 30 and 29%.

The amount of cholesterol excreted/day in the untreated animals was 9.7 ± 0.9 mg/kg body weight (Table II). Treatment with 0.4% chenodeoxycholic acid increased the excretion of cholesterol by ca. 70% and, in the rats treated with 1% chenodeoxycholic acid, the excretion was increased by ca. 170%.

DISCUSSION

The 2 dominating fecal bile acids in germ-free rats are cholic acid and β-muricholic acid (5, 7, 8). Cholic acid is, together with chenodeoxycholic acid, primarily formed in the liver from cholesterol. Cholic acid is not further metabolized. Chenodeoxycholic acid, on the other hand, is hydroxylated in 6β-position to yield α-muricholic acid (for a review, see ref. 20). The α-muricholic acid is further converted to β-muricholic acid, probably with 3α, 6β-dihydroxy-7-keto-5β-cholanoic acid as an intermediate. Since germ-free rats have no microbial enzymes, all these transformations of chenodeoxycholic acid to β-muricholic acid must occur in the liver.

During treatment of germ-free rats with chenodeoxycholic acid, this bile acid and α-muricholic acid became major bile acids in feces. If all chenodeoxycholic acid ingested was quantitatively metabolized to β-muricholic (i.e., 6β-hydroxylation of 6–7 mg chenodeoxycholic acid/kg body weight/day). In the rats treated

with 1% chenodeoxycholic acid, more than 100 mg of the bile acid was 6β-hydroxylated, thus confirming the previous finding that germ-free rats have a very high 6β-hydroxylase activity (21). It may be mentioned that an induction of the 6β-hydroxylase has previously been shown to occur in conventional rats during long-term treatment with chenodeoxycholic acid (22).

In conventional rats, bile acid synthesis is regulated homeostatically by the enterohepatic circulation the bile acid pool. Intraduodenal infusion of taurochenodeoxycholic acid to rats with a bile fistula results in a marked reduction of bile acid biosynthesis (23, 24). Similarly, feeding taurochenodeoxycholic acid to intact rats depresses bile acid synthesis (25). Conflicting reports, however, have been published concerning the effect of taurochenodeoxycholic acid on the cholesterol 7α-hydroxylase (25–27). In germ-free rats, chenodeoxycholic acid treatment led to a marked inhibition of cholic acid formation. Feeding 1% chenodeoxycholic acid also inhibited the 7α-hydroxylase activity. The slight accumulation of cholesterol in the livers of treated rats can be ascribed to the lowered activity of the cholesterol 7α-hydroxylase, i.e., a relative decrease in the capacity of the liver to remove excess cholesterol by converting it to bile acids.

In conventional as well as germ-free rats, hepatic cholesterol synthesis appears to be regulated predominantly by dietary cholesterol transported to the liver by lipoproteins of intestinal origin, especially chylomicron remnants (8, 26, 28–31). The role of bile acids in the regulation of HMG CoA reductase is uncertain. According to some studies, bile acids inhibit HMG CoA reductase in the liver (25, 26, 32). Other investigations indicate that bile acids do not directly influence hepatic HMG CoA reductase but may have an effect on the flow of cholesterol within or to the liver (33, 34). In germ-free rats, chenodeoxycholic acid treatment did not lead to a decreased HMG CoA reductase activity. One explanation for this finding might be that germ-free rats already have a low HMG CoA reductase activity (4).

The fecal excretion of cholesterol was higher in the chenodeoxycholic-acid-treated rats than in the untreated rats, and may result from several factors: first, there may be an increased secretion of biliary cholesterol. Such increased secretion can be expected since the liver synthesis of cholesterol, as judged from the HMG CoA reductase activity, was unchanged and the catabolism of cholesterol to bile acids was depressed. Second, chenodeoxycholic acid feeding may have resulted in an impaired cholesterol absorption. Germ-free rats have more efficient absorption of cholesterol compared to conventional rats, which has been ascribed to the higher concentration of bile acids in the intestine (3). The high concentration of cholic acid may be of particular importance, because this bile acid has been shown to promote cholesterol absorption more than chenodeoxycholic acid (35). In the treated rats, chenodeoxycholic acid together with its metabolites almost completely replaced cholic acid, which might have impaired cholesterol absorption. Third, the possibility exists of an increased secretion of cholesterol from the intestinal wall into the intestine.

Finally, it could be argued that the differences obtained between control rats and treated rats resulted from the diminished food intake in the chenodeoxycholic-acid-treated animals. However, the effects on cholesterol and bile acid metabolism were more pronounced in the rats fed the higher dose of chenodeoxycholic acid than in those fed the lower dose, although the weight curves were identical in the 2 groups of rats. This strongly contradicts the theory that the effects should depend only on differences in food intake.

In summary, this study demonstrates that treatment with chenodeoxycholic acid inhibits bile acid synthesis in germ-free rats. The hepatic synthesis of cholesterol, as judged from the HMG CoA reductase activity, is not depressed, which leads to an accumulation of cholesterol in the liver.

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Sterol Biosynthesis in the Oyster, *Crassostrea virginica*

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ABSTRACT

Sterol biosynthesis in the oyster, *Crassostrea virginica*, was examined by injection of [2-^{14}C] acetate and [2,3-^{3}H] lanosterol. The oyster incorporated [2-^{14}C] acetate into squalene, 4,4'-dimethylsterols, 4-monomethylsterols, cholesterol, desmosterol, isofucosterol, and 24-methylenecholesterol, and also converted [2-^{3}H] lanosterol to cholesterol. Therefore, the oyster was concluded to synthesize cholesterol, desmosterol, isofucosterol, and 24-methylenecholesterol from acetate via squalene, probably using the lanosterol pathway.

Many species of marine mollusks have been studied in relation to sterol-synthesizing ability (1,2). Generally, most gastropods are regarded as capable of synthesizing sterols from lower units, except the whelk, *Buccinum undatum* (3,4). However, some aspects of sterol biosynthesis in the pelecypods are not agreed upon. Fagerlund and Idler (5) first demonstrated the incorporation of [2-^{14}C] acetate into sterols in the mussel, *Mytilus californianus*, and the clam, *Saxidomus giganteus*, whereas Salaque et al. (6) observed no incorporation of [2-^{14}C] mevalonate into sterols in the oyster, *Ostrea gryphea*. Later, contradictory results were also obtained with sterol biosynthesis even in the closely related species of bivalves such as the mussels (5,7–9).

On the other hand, nutritional studies have shown that growth of oysters is markedly affected by the species of dietary algae (10–12). Generally, faster growth of the oyster, *Crassostrea virginica*, is attained on the diets containing 3 or 4 species of algae. Interestingly, however, Epifanio and coworkers have observed that *Thalassiosira pseudonana* (Chrysophyta) or *Isochrysis galbana* (Haptophyta) gave a high growth rate as compared to controls (13–15).

Cholesterol is as likely to be indispensable for normal growth of the oyster, *C. virginica*, as in other animals. However, both *T. pseudonana* and *I. galbana* contained cholesterol in very small quantities in their tissues (16). Accordingly, the question rises whether the oyster, *C. virginica*, synthesizes cholesterol and/or dealkylates a dietary source of C-28 and C-29 sterols to cholesterol.

This investigation examines the biosynthesis of sterols from [2-^{14}C] acetate and [2-^{3}H] lanosterol in the oyster, *C. virginica*, as a part of understanding the nutritional aspects of sterols.

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MATERIALS AND METHODS

Chemicals and Radioactive Measurements

Reference sterols were isolated from the oysters in another experiment (17). [2-^{14}C]-Sodium acetate (sp act–48.0 mCi/mmol) was purchased from the International Chemical and Nuclear Corporation (California). [2-^{3}H] Lanosterol (sp act–3.0 mCi/mmol) was synthesized from unlabeled lanosterol by the method of Thompson et al. (18). Radioactivity was measured with a Tricarb 6 liquid scintillation counter Model 500p using a toluene solution of PPO (0.6%) and POPOP (0.02%) as a scintillator.

Administration of Radioactive Precursors to Oysters

Oysters, 0.83–1.1 g in fresh meat weights, were used in this study. These oysters were spawned in April 1979, maintained on *T. pseudonana* and *I. galbana* in the greenhouse at the Marine Studies Laboratories, University of Delaware, and transported to this laboratory September 11, 1979. Twelve specimens of the oysters were carefully pierced at the shell (pore size, 1 mm) by a dental drill, and injected with a total of 250 μCi of [2-^{14}C] acetate dissolved in diluted sea water (salinity, 1.0%) using a microsyringe. Similarly, a total of 7.7 μCi of [2-^{3}H] lanosterol suspended in diluted sea water containing 5% (v/v) Tween 80 was injected into 12 oysters. The oysters were held in a carboy (20 ℓ) with aeration at 20 C. During the holding period, 15 ℓ of the sea water was exchanged with fresh sea water every 3 days. Six oysters were taken from the carboy for analysis 7 and 17 days after injection of radioactive precursors.

Chromatography

Thin layer chromatography (TLC) on Silica Gel G was done with chloroform/methanol (98:2). TLC on 20% (w/w) $AgNO_3$-impregnated Silica Gel $HF_{254+366}$ was performed to

separate the individual sterols with the solvent systems, chloroform (ethanol-free) or hexane/benzene (1:4). Sterols on TLC plates were located under UV-light after spraying Rhodamine 6 G in acetone and then eluted with ether. Neutral and polar lipids were separated by column chromatography on Silica Gel 60 with chloroform/methanol. Column chromatography on 20% (w/w) $AgNO_3$-impregnated silicic acid (Bio-Sil®A, 100–200 mesh; Bio-rad Laboratories, California) was conducted using hexane/benzene as described previously (19). Column chromatography on lipophilic Sephadex, which was prepared from Sephadex LH-20 by the method of Ellingboe et al. (20), was used to separate the individual sterols differing from each other only in the carbon number of the side chain (21). Gas liquid chromatography (GLC) was performed on a Glowall Chromalab Model A-110 equipped with an argon ionization detector and a glass column (1.8 m × 3.4 mm) packed with 3.0% SE-30 at 245 C (22).

Extraction, Separation and Identification of Radioactive Metabolites

Lipids were extracted from the oysters with chloroform/methanol/water (2:2:1) (23). To check for the presence of radioactive squalene and steryl esters, 10% of the lipid was subjected to column chromatography and analyzed by TLC on Silica Gel G with chloroform. The remainder of radioactive lipids was saponified with 10% ethanolic KOH, and the unsaponifiable matter was subjected to TLC on Silica Gel G with chloroform/methanol (98:2) to determine the proportional distribution of radioactivity among 4,4′-dimethyl, 4-monomethyl- and desmethylsterols. Desmethylsterols were acetylated with dry pyridine/acetic anhydride (1:1, v/v) at room temperature and the acetate derivatives were chromatographed on a $AgNO_3$-silicic acid column. The radioactive steryl acetates from $AgNO_3$-column chromatography were further separated into the individual components by argentation TLC and Sephadex column chromatography. Finally, the identification of radioactive metabolites was confirmed with a constant sp act (dpm/mg) during successive crystallizations with authentic sterols (24).

RESULTS

After injection of either [2-^{14}C]acetate or [2-^{3}H]lanosterol, lipids were extracted from the tissues of oysters and separated into the lipid fractions (Table I). The oysters incorporated effectively the injected [2-^{14}C]acetate into lipids; the percentage incorporation of [2-^{14}C]acetate into lipids was 8.3 and 5.9% after 7 and 17 days, respectively. The apparent decrease in the percentage incorporation of [2-^{14}C]acetate into lipids between 7- and 17-day incubation seems to imply that the injected acetate was metabolized actively in the oysters and probably excreted as expired carbon dioxide.

TLC of radioactive neutral lipids on Silica Gel G indicated that the zone corresponding to steryl esters was labeled. To confirm the incorporation of radioactivity into steryl esters, the steryl ester fraction was saponified and the presence of radioactive free sterols was checked by TLC. TLC of the unsaponifiable matters on Silica Gel G indicated that squalene, 4,4′-dimethylsterols, 4-monomethylsterols and desmethylsterols were labeled in both 7- and 17-day incubations. In this TLC, however, 4,4′-dimethylsterols and 4-monomethylsterols were not separated clearly from the ketosteroids such as cholest-4-en-3-one, which was regarded as an intermediate of bioconversion of Δ^5-sterols to Δ^7-sterols in the starfish (25, 26) and also in the primitive mollusk (27). Hence, the 4,4′-dimethylsterol and 4-monomethylsterol fractions were acetylated and analyzed by TLC on Silica Gel G with chloroform. The results of TLC showed that 4,4′-dimethylsterols and 4-monomethylsterols, but not cholest-4-en-3-one and 5α-cholestan-3-one, were labeled. In both 7- and 17-day incubations, more than 90% of radioactivity in sterols was associated

TABLE I

Yields of Lipid Fractions from Oysters after Injection of [2-^{14}C]Acetate

Fraction	Holding period (days)	
	7	17
Oysters, total wt of fresh meat	6,600[a]	5,300
Lipids	39.6 (0.60)	27.1 (0.51)
Unsaponifiable matter	13.0 (0.20)	6.4 (0.12)
Sterols	3.2 (0.048)	2.2 (0.042)

[a]Weight in mg, values in parentheses expressed as % fresh weight.

with desmethylsterols (Table II).

To examine the incorporation of radioactivity into the individual components of desmethylsterols, the radioactive desmethylsterols were acetylated, chromatographed on $AgNO_3$-silicic acid, and separated into 25 fractions (Table III). The highest radioactivities were recovered in the following fractions: fractions 10–13 containing cholesteryl, 24-methylcholesteryl, and 24-ethylcholesteryl acetates, fraction 18 containing mainly 24-norcholesta-5, 22-dienyl, 22-dehydrocholesteryl, and desmosteryl acetates, fraction 19 containing desmosteryl and isofucosteryl acetates, and fractions 20–23, 24-methylenecholesteryl acetate alone. Fractions 10–13 were hydrolyzed and chromatographed on a lipophilic Sephadex column after addition of ca. 5 mg each of campesterol and β-sitosterol. The results of Sephadex column chromatography showed that

TABLE II

Incorporation of Radioactivity into Lipid Fractions after Injection of [2-^{14}C] Acetate into Oysters

Fraction	7-day incubation			17-day incubation		
	Radioactivity (dpm)	Sp act (dpm/mg)	Incorp. (%)	Radioactivity (dpm)	Sp act (dpm/mg)	Incorp. (%)
Lipids	3,860,000	94,500	8.3	2,590,000	95,600	5.9
Fatty acids	1,780,000	66,900	3.8	1,590,000	76,800	3.6
Unsaponifiable matters	553,000	42,500	1.2	370,000	57,800	0.84
Squalene	3,700	–	0.008	2,400	–	0.05
Total sterols	303,000	94,700	0.65	137,000	62,300	0.31
Esterified sterols	7,880	–	0.17	10,000		0.023
Free sterols	295,000	–	0.63	127,000		0.29
4.4'-Dimethylsterols	(3.9%)[a]	–	–	(2.9%)		
4-Monomethylsterols	(2.4%)	–	–	(1.6%)		
Desmethylsterols	(93.7%)	–	–	(95.5%)		

[a]% Distribution of radioactivity in free sterols.

TABLE III

$AgNO_3$-Column Chromatography of the Radioactive Desmethylsterols Isolated from the Oysters 7 and 17 days after Injection of [2-^{14}C] Acetate[a]

Fraction no.	Radioactivity (dpm) Incubation period		Sterols (as acetates) detected in each fraction[b]
	7 days	17 days	
1–6	18	41	–
7–9	530	150	–
10–13	54,400	26,900	Cholesterol (4.3 mg), 24-methylcholesterol (0.40 mg), and 24-ethylcholesterol (0.37 mg)
14	10,000	3,160	Cholesterol (0.81 mg), 24-ethylcholesta-5, 22-dienol (0.20 mg), and 24-methylcholesta-5, 22-dienol (0.18 mg)
15	4,160	980	24-Methylcholesta-5, 22-dienol (2.0 mg) and cholesterol (0.32 mg)
16	3,840	760	24-Methylcholesta-5, 22-dienol (0.41 mg), 22-dehydrocholesterol (0.11 mg), and cholesterol (0.28 mg)
17	3,560	2,140	22-Dehydrocholesterol (0.25 mg), 24-methylcholesta-5, 22-dienol (0.05 mg), and 24-norcholesta-5, 22-dienol (0.01 mg)
18	68,000	17,900	Desmosterol (0.30 mg), 22-dehydrocholesterol (0.01 mg), and 24-norcholesta-5, 22-dienol (0.46 mg)
19	85,700	21,400	Isofucosterol (0.44 mg) and desmosterol (0.21 mg)
20–23	18,700	8,740	24-Methylenecholesterol (1.35 mg)
24, 25	100	15	–

[a]To the radioactive desmethylsterols, 0.5 mg of desmosterol and 10 mg of oyster sterols (from another experiment) were added and acetylated. The steryl acetates were chromatographed on 30 g of 20% $AgNO_3$-silicic acid and eluted in 50-ml fractions with 100 ml each of 0, 10, 15, 20, 25, 27, 29, 33, 36, 50, 60, 75 and 90% benzene in hexane.

[b]Data from 7-day incubation.

TABLE IV

Recrystallization of Radioactive Metabolites Isolated from the Oysters Injected with [2-^{14}C]Acetate

	Specific activity (dpm/mg)			
	Metabolites			
Crystallization[a]	[^{14}C] Cholesterol	[^{14}C] Desmosteryl ac.	[^{14}C] Isofucosteryl ac.	[^{14}C] 24-Methylenecholesteryl ac.
Initial	2,480	11,330	3,700	1,400
1st	2,510	9,310	2,500	1,250
2nd	2,520	8,050	1,810	1,130
3rd	2,505	7,930	1,950	1,250
4th	–	8,010	1,820	1,140

[a]Crystallized from methanol.

radioactivity was associated with cholesterol, but not campesterol and β-sitosterol. Finally, to the radioactive cholesterol, ca. 10 mg of nonradioactive cholesterol was added and crystallized from methanol successively. The sp act of crystals remained constant during the last 3 crystallizations (Table IV). Therefore, cholesterol was concluded to be synthesized from acetate in the oyster, *C. virginica*. Fractions 14, 15, and 16 gave significant radioactivity, but the TLC of these fractions on 20% $AgNO_3$-Silica Gel HF 254+366 with chloroform (ethanol-free) indicated that radioactivity was due to contaminating cholesteryl acetate, but not 24-ethylcholesta-5,22-dienyl, 24-methyl-cholesta-5,22-dienyl, and 22-dehydrocholesterylacetates. The $AgNO_3$-TLC of fractions 17 and 18 also showed that radioactivity was associated with demosteryl acetate, but not 22-dehydrocholesteryl or 24-norcholesta-5,22-dienyl acetates. The identification of radioactive desmosteryl acetate was confirmed by a constant sp act during crystallization after addition of 10 mg of nonradioactive desmosteryl acetate (Table IV). These data demonstrated that the oyster was capable of synthesizing desmosterol from acetate but not able to synthesize 22-dehydrocholesterol or 24-norcholesta-5,22-dienol. The TLC of fraction 19 on 20% $AgNO_3$-Silica Gel $HF_{254+366}$ with hexane/benzene (1:4) showed that both isofucosteryl acetate and desmosteryl acetate were labeled significantly. The radioactive isofucosteryl acetate was crystallized after addition of ca. 10 mg of nonradioactive isofucosteryl acetate. The sp act of radioactive isofucosteryl acetate reached a constant value after a 50% decrease during crystallizations. Radioactive 24-methylene cholesteryl acetate was further purified by 20% $AgNO_3$-TLC with hexane/benzene to remove the possible contaminants such as cholesta-5,7-dienyl acetate. Finally, to the radioactive 24-methylene cholesteryl acetate, ca. 10 mg of nonradioactive 24-methylene cholesteryl acetate was added and recrystallized from methanol. The synthesis of 24-methylenecholesterol from acetate was confirmed by recrystallization to a constant sp act (Table IV).

Next, to obtain evidence of the intermediary role of lanosterol in cholesterol biosynthesis, the oysters were injected with [2-^{3}H]lanosterol and incubated for 7 days. The unsaponifiable matter (7.6 mg) yielded radioactive desmethylsterols (2.0 mg; 1,350,000 dpm). The radioactive desmethylsterols were diluted with 20 mg of unlabeled carrier cholesterol, and cholesterol was isolated by $AgNO_3$-column chromatography and Sephadex column chromatography. The crystallization of radioactive cholesterol with added carrier showed an initial sp act of 15,000 dpm/mg in the first recrystallization and remained constant at 12,000 dpm/mg during the next 3 crystallizations, confirming the bioconversion of lanosterol to cholesterol in the oyster. We were unable to determine whether desmosterol, 24-methylenecholesterol, or isofucosterol was labeled in this experiment.

As already stated, the oyster, *C. virginica*, was found to synthesize cholesterol, desmosterol, isofucosterol, and 24-methylenecholesterol presumably via squalene and lanosterol from [2-^{14}C]acetate.

In addition to the tracer experiments, the variation in the sterol composition of oysters during starvation was investigated. The results are given in Table V. During starvation, the proportions of 24-methylcholesta-5,22-dienol and 24-methylenecholesterol decreased, whereas that of cholesterol increased. This suggests that the first 2 sterols are primarily of dietary origin.

TABLE V

Variation in the Sterol Composition of Oysters during Starvation

Sterol	% Composition			
	Starvation period (days)			
	0	10	16	21
Cholesterol	12.6	23.7	27.6	35.1
24-Methylesta-5, 22-dienol	60.1	51.9	55.5	45.0
24-Methylenecholesterol	27.3	24.4	16.9	19.9
24-Ethylcholesta-5, 22-dienol	t[a]	t	t	t
24-Ethylcholesterol	t	t	t	t

[a]Less than 0.1%.

DISCUSSION

This study showed that the oyster, *C. virginica,* incorporated the injected [2-^{14}C]-acetate into squalene, 4,4'-dimethylsterol, 4-monomethylsterols, cholesterol, desmosterol, 24-methylenecholesterol and isofucosterol. Also, the oyster was shown to convert the injected [2-^{3}H]lanosterol to cholesterol. However, [2-^{14}C]acetate was not incorporated into the C_{26}, C_{27}, C_{28} and C_{29}-sterols with $\Delta^{5,22}$-bonds. These results demonstrated that the oyster, *C. virginica,* apparently possesses the ability for de novo synthesis of cholesterol, desmosterol, 24-methylenecholesterol and isofucosterol via squalene and lanosterol. However, a large proportion of radioactivity in desmethylsterols was associated with desmosterol even 7 and 17 days after injection of [2-^{14}C]acetate. This suggests that, in the oyster, the Δ^{24}-sterol reductase may act as a regulating enzyme in cholesterol biosynthesis. As to the sea urchin, *Echinus esculentus,* Smith and Goad (28) have also observed that [2-^{14}C]-mevalonate was readily incorporated into lanosterol and desmosterol, but only to a small extent into cholesterol.

Another interesting finding in our study is that the oyster apparently synthesized 24-methylenecholesterol and isofucosterol. Generally, plants elaborate C_{28}- and C_{29}-sterols from lower molecules, and the biosynthetic mechanisms for formation of C-24 alkylated sterols are well established as reviewed by Goad et al. (29). In the biosynthesis of C_{28}- and C_{29}-sterols, methionine acts as the methyl donor with a Δ^{24}-sterol as the substrate, and the 24-methylene compound formed is then converted to the 24-ethylidene compound. We assume that 24-methylenecholesterol and isofucosterol detected as radioactive metabolites in the oyster, *C. virginica,* are probably formed from demosterol by the mechanism just described. However, it is still questionable whether the oyster itself possess the enzyme systems for alkylation of C_{27}-sterol to C_{28}- and C_{29}-sterols. These activities could still be attributed to contaminating algae or other microorganisms. Fagerlund and Idler (30) first demonstrated the biosynthesis of the C-24 alkylated sterol, 24-methylenecholesterol, in the clam, *Saxidomus giganteus.* Later, the biosynthesis of 24-methylenecholesterol and some C_{28}- and C_{29}-sterols was pointed out in the several pelecypods, *M. edulis* (8, 31), *Anodunta cygnea, Cardium edulis,* and *Mya arenaria* (9).

On the other hand, the sterol composition of the oysters used in our study was unique as compared with that of wild oysters (17, 32, 33). The oysters contained 24-methylcholesta-5, 22-dienol and 24-methylenecholesterol as the predominant components, but cholesterol in a lesser amount. The unique sterol composition of the oysters is possibly ascribed to the dietary algae used for the artificial culture. The decrease in the proportions of 24-methylcholesta-5, 22-dienol and 24-methylenecholesterol during starvation suggested that the two sterols were of dietary origin. The decline in % sterol during a 17-day starvation is in constrast to an increase in % sterol noted during a 37-day starvation (34).

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Lipoproteins of Fetal and Newborn Calves and Adult Steer: A Study of Developmental Changes[1]

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ABSTRACT

Serum lipoproteins in fetal and newborn calves were characterized and compared with those of adult animals. Fetal calf serum contains only low density (LDL) and high density (HDL) lipoproteins; the LDL is the major lipoprotein class. Fetal LDL are ca. 26.0 nm diameter and are morphologically unusual in that particles form linear aggregates or "chains" in which LDL have flattened, parallel sides. These particles contain only apolipoprotein B and are high in polar lipids. Fetal HDL consist of 8.2-nm, round particles which contain large amounts of cholesteryl ester thus suggesting an active lecithin: cholesterol acyltransferase system in the fetal state. The major protein in fetal HDL is apolipoprotein A-I (80%); however, another component with a molecular weight (MW) of ca. 9,000 is also present. Newborn calves show a 5-fold increase in HDL concentration. These particles are 9.0 nm spherical particles and they contain mainly apolipoprotein A-I although C-apolipoproteins are also present; the lipid and apolipoprotein composition of newborn HDL is similar to that of adults. Newborn calves possess very low density (VLDL) lipoproteins which have a mean diameter of 61 nm and are similar in size and composition to those of adult animals; their apolipoprotein composition is principally apolipoprotein B, although C-apolipoproteins and apolipoprotein A-I are also present. The LDL of neonatal and adult animals are similar in morphology, chemical composition and apolipoprotein content. In both instances, LDL are round particles ca. 19.0 nm diameter which contain less polar lipids than the fetal animal. Apolipoprotein B is the major protein in newborn LDL, but adult LDL additionally contains a protein of 27,000 MW which probably represents apolipoprotein A-I from overlapping α-migrating particles in this region. The altered morphology and composition of fetal LDL, together with the lack of VLDL, suggest that the LDL particles may be synthesized de novo.

INTRODUCTION

Comparative studies of fetal calf, newborn calf and adult steer lipoproteins can provide insight into the genesis and metabolism of the major plasma lipoproteins, including high density (HDL), low density (LDL) and very low density (VLDL) lipoproteins. In addition, information on neonatal calf lipoproteins will also help to define the physical and chemical properties of lipoproteins routinely added to tissue culture systems in which 10–20% fetal or newborn calf serum is required to establish viable cultures. An early observation by Brown et al. (1) indicated that a component of density <1.21 g/ml in fetal calf serum was able to partially inhibit cholesterol synthesis in fibroblast cultures; this observation suggests that lipoproteins in fetal calf serum may interact with cells. In this study, the morphology, distribution and chemical composition of fetal and newborn calf lipoproteins were determined in order to characterize the major lipoprotein species in prenatal and postnatal animals. The lipoproteins in these young animals are compared with that of mature steers.

[1]Preliminary data was presented at the American Oil Chemists' Society Meeting, 1979.

EXPERIMENTAL PROCEDURES

Serum Samples

Because most tissue culture studies utilize commercially available fetal or newborn calf serum, we examined two different batches of frozen fetal and newborn calf serum obtained from Grand Island Biological Co., Grand Island, NY. Samples of fresh, near-term, fetal calf serum were obtained through the courtesy of Dr. Culbertson, University of California, Davis; two of these samples were pooled sera from several animals and the other consisted of serum from a single animal. Three samples of fresh, newborn calf serum (1 to 9 days postpartum) as well as blood from four mature steers were also obtained from the University of California, Davis. Lipoprotein distribution, composition, and morphology of Gibco frozen sera were identical with those of fresh sera; hence, data were combined for all fetal calf sera and newborn calf sera.

Analyses

Distribution of lipoprotein components were determined by analytic ultracentrifugation as described by Lindgren (2).

Lipoprotein fractions of $d<1.006$ g/ml (VLDL), d 1.006–1.063 g/ml (LDL) and d

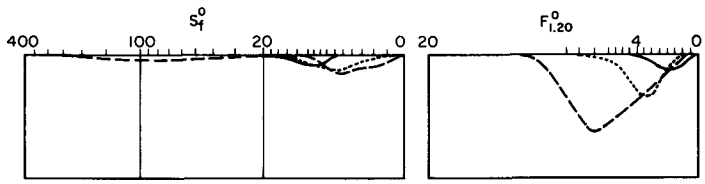

FIG. 1. Analytic ultracentrifugal distribution patterns of fetal calf (——), newborn calf (-----) and adult steer (– – –) lipoproteins. The flotation region of $F^0_{1.20}$ corresponds to the HDL distribution while S^0_f 0–20 corresponds to that of LDL and S^0_f 20–400 to that of VLDL.

1.063–1.21 g/ml (HDL) were isolated by sequential preparative ultracentrifugation (3). Lipoprotein protein was determined by the method of Lowry et al. (4) and phospholipid according to the method of Bartlett (5). Cholesterol and triglyceride were determined enzymatically (6, 7). Apolipoprotein composition was determined by polyacrylamide gel electrophoresis done on samples dilipidized with tetramethylurea (TMU) according to the procedure of Kane (8) or solubilized by sodium dodecylsulfate (SDS) according to Weber and Osborn (9). Relative intensities of Coomassie blue staining bands were estimated by densitometry.

Electron microscopy was done on negatively stained (2% sodium phosphotungstate, pH 7.4) samples which had previously been dialyzed against 0.13 M ammonium acetate buffer, pH 7.4. Negatively stained preparations were immediately examined with a JEM 100C (JEOL Co., Tokyo, Japan) electron microscope. All size determinations were done by measuring only free-standing particles on micrographs.

RESULTS

Lipoprotein Distribution

Representative analytic ultracentrifugal patterns of fetal calf, newborn calf, and mature steer lipoproteins are seen in Figure 1. The concentrations of LDL and HDL calculated from these data are summarized in Table I. Fetal calf serum is characterized by extremely low concentrations of HDL ($F^0_{1.20}$) and LDL (S^0_f 0–20) whereas VLDL (S^0_f 20–400) are not apparent. Newborn calf serum also contains low concentrations of LDL. The large variation in LDL concentration of fetal and newborn calf sera cannot be accounted for by errors in analytic ultracentrifugation (less than 10% in this case) but rather appear to be due to biological variation. In contrast to fetal calf serum, however, small quantities of VLDL (9.9 mg/100 ml ± 0.3 SD) can be isolated from newborn calf when d< 1.006 g/ml fractions are concentrated 36-fold. Although LDL are slightly decreased in newborn sera compared to fetal sera, the HDL concentration is elevated 5-fold. The HDL/LDL ratio in the newborn is, therefore, 5.45 compared to 0.80 for fetal calves. All classes of lipoproteins are evident in adult steer (Fig. 1). The VLDL are present in very low concentrations (21.1 mg/100 ml ± 6.7 SD) and, as shown, the HDL form the bulk of the lipoprotein mass. There is a 2-fold increase in HDL and LDL mass in the steer compared to the newborn (Table I); however, the steer HDL/LDL ratio is 4.97 which is similar to that of newborn calves.

Analytic ultracentrifugal patterns of the three types of sera also show differences in the mean flotation rates of the HDL and LDL. These differences suggest that the size and density of the LDL and HDL may be altered by developmental changes. Fetal calf HDL is characterized by a slow moving peak with a mean flotation rate of $F^0_{1.20}$ 2.0 ± 0.4 SD, whereas LDL has a fast moving peak with a mean value of S^0_f 8.9 ± 0.3 SD. The peak flotation rate of newborn calf HDL ($F^0_{1.20}$ 3.2 ± 0.5 SD) is somewhat higher than that of fetal calf but is consistent with the slightly larger diameter of newborn HDL as determined by electron microscopy (Table II). In contrast, the LDL flotation peak of newborn calves is decreased to S^0_f 4.9 ± 0.5 SD which suggests that these LDL particles are smaller and more

TABLE I

Serum Concentration of LDL and HDL Derived from Analytic Ultracentrifugal Data

	LDL (mg/100 ml)	HDL (mg/100 ml)
Fetal calf	43.8 ± 18.6 (5)[a]	34.5 ± 9.4 (5)
Newborn calf	30.7 ± 20.6 (5)	167.4 ± 37.2 (5)
Steer	73.4 ± 18.0 (4)	365.2 ± 30.2 (4)

[a]Numbers in parenthesis indicate number of samples analyzed.

dense than fetal calf LDL. HDL of steer have a mean flotation rate of $F^{\circ}_{1.20}$ 6.9 ± 0.3 SD which is faster than either fetal or newborn calf HDL. The LDL flotation peak (S°_{f} 5.3 ± 0.3 SD) of steer, on the other hand, is similar to that of newborn calves.

Electron Microscopy

The electron microscopic morphology of fetal calf, newborn calf and steer lipoproteins are seen in Figure 2 whereas particle size of the major lipoprotein fractions is summarized in Table II. Fetal calf serum is unique in that no VLDL particles are detectable in the d<1.006 g/ml fraction. However, VLDL of newborn and adult animals are very similar in size and structure (Table II and Fig. 2). In addition to round, 60-nm particles, both VLDL fractions contain large, flattened structures (Fig. 2) which may represent surface remnant material or particles rich in saturated fatty acids. Similar structures have been noted in bovine intermediate density lipoproteins enriched in saturated fatty acid (D. Puppione, personal communication). Fetal calf LDL are distinct from both newborn and steer LDL, in that numerous particles appear to have flattened, parallel sides; the long and short axis of such particles are 29.8 and 18.1 nm, respectively. Fetal LDL with round profiles have a mean diameter of 26 nm (Table II) which is ca. 27% greater than round particles in both newborn calf and steer LDL. The smaller size of LDL from newborn and mature animals (Table II) is consistent with their slower flotation rates. HDL of both fetal and newborn calves are fairly homogeneous spherical particles less than 10 nm diameter (Table II). Steer HDL have a mean diameter of 12.6 nm which is 40% larger than that of the newborn calf. The larger particle diameters of steer HDL are commensurate with their faster flotation rate and increased lipid core content (Table III).

Chemical Composition and Apolipoprotein Distribution of Lipoprotein Fractions

The protein and lipid composition of the major lipoprotein classes from fetal calf, newborn calf and steer are summarized in Table III. No measurable quantity of fetal calf VLDL was detected; however, VLDL isolated from both newborn calf and steer were similar in composition. Triacylglycerol is the major constituent of the VLDL in both groups of animals; moreover, these particles also appear to be enriched in protein. The most notable difference between newborn calf and steer VLDL composition is the higher percentage of cholesteryl ester in the newborn.

TABLE II

Electron Microscopic Size (nm) of Fetal, Newborn and Mature Bovine Lipoproteins

	Mean particle diameter ± SD		
	VLDL	LDL	HDL
Fetal	ND	26.0 ± 2.0	8.2 ± 0.6
Newborn	61.3 ± 16.1	19.4 ± 2.5	9.0 ± 0.9
Steer	60.2 ± 23.9	18.8 ± 4.6	12.6 ± 1.4

[a]ND = not detectable.

LDL in fetal, newborn and mature animals were rich in cholesteryl ester and poor in triacylglycerol (Table III). Triacylglycerol was present in only negligible quantities in fetal calf LDL. The polar lipids, phospholipid and unesterified cholesterol, were elevated in fetal calf LDL as compared with either newborn or steer.

The HDL of fetal, newborn and mature animals show decreasing protein content with increasing age; on the other hand, cholesteryl ester increases during development (Table III). The changes in surface and core lipid components are quite pronounced when the polar-to-nonpolar lipid ratios between fetal and mature HDL are compared, i.e., 1.16 and 0.68 respectively. The low protein content (38%) and high lipid core content (47%) of the steer HDL is consistent with the observed lower density and higher flotation rate of these HDL.

The tetramethylurea-soluble apolipoproteins of the various lipoprotein fractions are seen in Figure 3. Precipitated apolipoprotein B was the only identifiable apolipoprotein in fetal calf LDL. Insoluble apolipoprotein B was present in VLDL and LDL of newborn calves and mature animals; calf and steer VLDL additionally show apolipoprotein A-I as well as minor bands which migrate to positions similar to mammalian C-apolipoprotein. A protein of 27,000 MW presumed to be apolipoprotein A-I is also found in steer LDL and probably reflects the presence of α-migrating particles in the LDL density range. Previous work of Puppione et al. (10) showed that α-migrating particles of d 1.04–1.06 g/ml can, indeed, be isolated from cow serum.

Apolipoprotein A-I is the major protein of HDL from all three developmental stages. In tetramethylurea-solubilized samples, this protein appears to be a double band. However, on SDS polyacrylamide, the material migrates as a single band of 27,000 Daltons (data not shown). In addition to apolipoprotein A-I, a single, fast

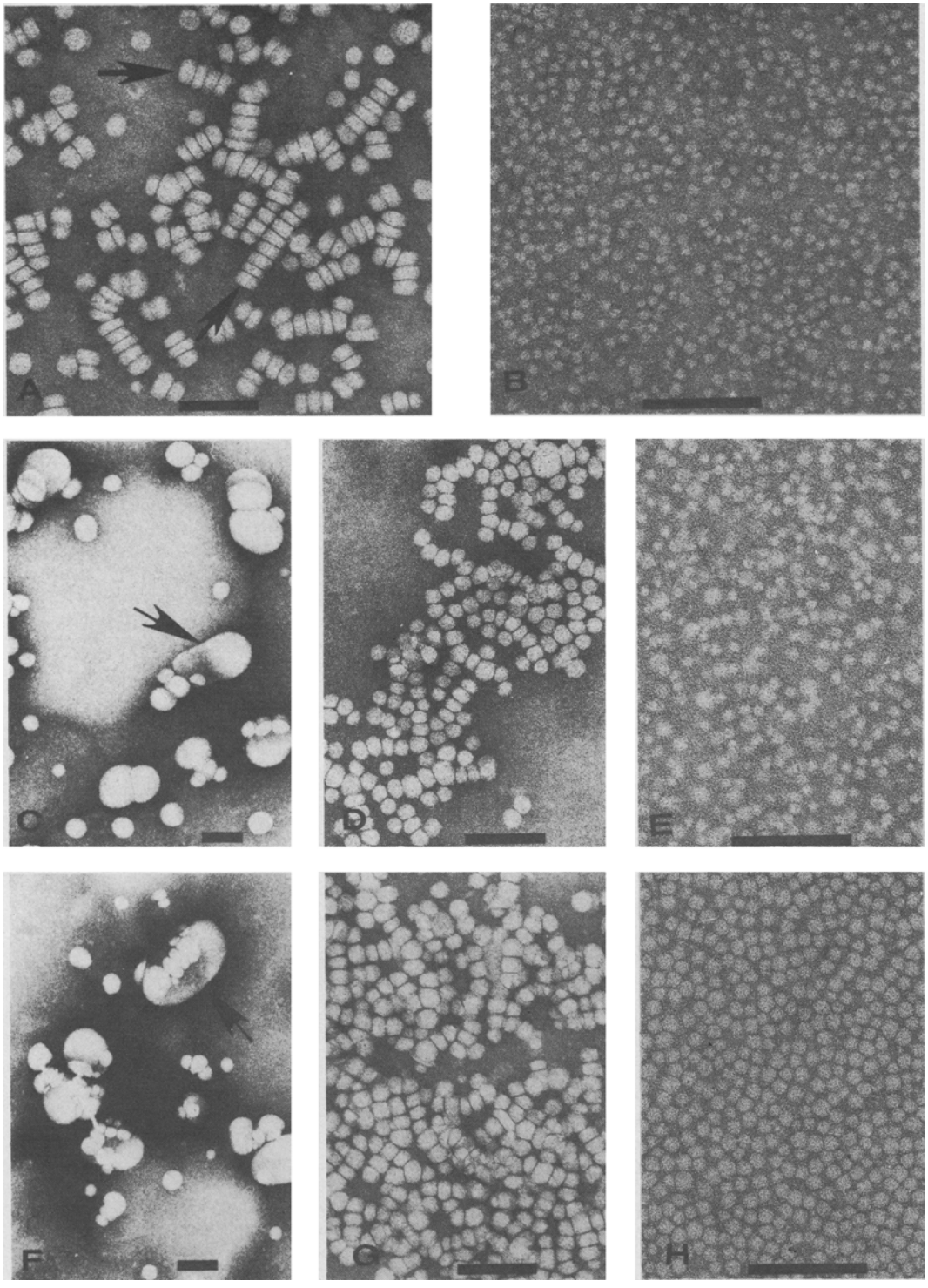

FIG. 2. Micrographs of negatively stained lipoproteins from fetal, newborn and adult animals. A: fetal LDL. Note chaining LDL (arrows) with parallel sides; B: fetal calf HDL; C: newborn calf VLDL. Arrow indicates unusually shaped particle; D: newborn calf LDL; E: newborn calf HDL; F: adult VLDL. Arrow indicates unusual particle morphology. G: adult LDL; H: adult HDL. Bar markers represent 100 nm.

TABLE III

Composition of Various Lipoprotein Classes from Fetal Calf, Newborn Calf and Mature Steer

Sample	Fraction	Protein	Percentage weight of component[a]			
			Phospholipid	Unesterified cholesterol	Cholesteryl ester	Triacylglycerol
Fetal	HDL	50.3 ± 2.4	23.8 ± 1.1	3.0 ± 0.7	22.9 ± 1.2	0.25 ± 0.5
	LDL	22.5 ± 1.2	26.6 ± 3.6	10.3 ± .7	40.3 ± 2.6	0.4 ± 0.2
Newborn	HDL	45.1 ± 2.7	20.6 ± 1.7	2.7 ± 0.3	29.0 ± 4.8	0.2 ± 0
	LDL	30.3 ± 2.8	22.3 ± 3.5	7.7 ± 0.7	37.1 ± 4.0	3.4 ± 1.1
	VLDL	24.6 ± 2.4	17.9 ± 2.6	6.7 ± 2.1	9.8 ± 1.6	41.1 ± 3.3
Steer	HDL	37.5 ± 1.6	22.4 ± 0.7	2.8 ± 0.4	37.3 ± 1.4	0.05 ± 0.2
	LDL	26.0 ± 3.1	21.6 ± 4.9	7.7 ± 0.8	39.9 ± 4.6	6.4 ± 3.5
	VLDL	22.5 ± 3.6	21.5 ± 5.5	6.9 ± 1.0	2.4 ± 1.6	46.8 ± 8.1

[a]The total weight of each component was taken as the sum of phospholipid, unesterified cholesterol, cholesteryl ester, triacylglycerol and protein. The values given are means ± SD from 5 separate samples of fetal calf serum and 4 samples each of newborn and steer sera.

migrating band which accounts for 20–25% of the total Coomassie blue staining material is also present in fetal HDL (Fig. 3). Electrophoresis on SDS polyacrylamide gels indicated that this faster band has an approximate MW of 9,000 Daltons. The latter component is also present in newborn and mature animals but to a lesser degree. Newborn calves and steers also have additional C-protein bands (Fig. 3) not seen in the fetal condition. Furthermore, in more developed animals, the total C-proteins account for only 9–15% of the total HDL protein.

DISCUSSION

The present studies on the distribution of lipoproteins in fetal calves show that total serum lipoprotein concentration is ca. one-fifth that of adult animals. Triacylglycerol-rich VLDL appear to be lacking in the fetus; however, their synthesis at very low levels together with their rapid utilization cannot be entirely ruled out. A deficiency of VLDL (11) and extremely low concentrations of VLDL (12–14) have also been reported for human cord blood samples.

Low density lipoproteins are the major lipoproteins of fetal calf. However, after birth, with the onset of feeding there is an immediate shift to HDL as the major lipoprotein species. A similar alteration in the pattern of lipoprotein distribution has been noted in the fetal rat (15), in which LDL predominate before birth and in which HDL become elevated postnatally. In contrast, human cord blood is characterized by a predominance of HDL (12, 16), whereas LDL become the major lipoprotein class shortly after birth (17).

Fetal calf LDL possess an unusual morphology in which particles tend to form chains with individual particles that have parallel, flattened sides. The physical and chemical properties responsible for their unusual morphology are unknown; however, the higher content of both phospholipid and unesterified cholesterol may be partially responsible for the unusual shape. LDL of fetal calf contain only B protein whereas postnatal and mature animals contain apolipoproteins A-I and C in the equivalent lipoprotein fraction. Because both the physical and chemical properties of fetal LDL differ from those of more mature animals and because no VLDL-like particles can be identified in their sera, it is tempting to specu-

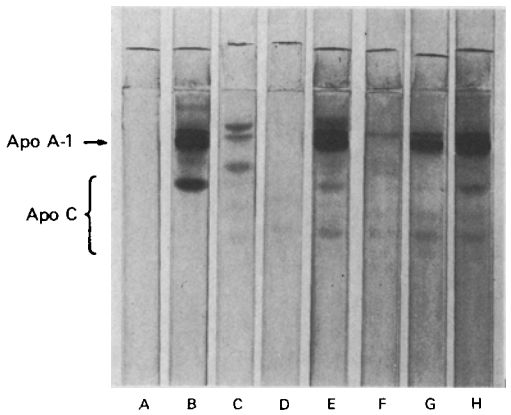

FIG. 3. Tetramethylurea solubilized apolipoproteins on 7% polyacrylamide gels; 25–35 μg protein were loaded onto each of the gels. Apolipoprotein A-I (apoA-I) typically consists of a double band and C-apolipoproteins (apoC) consist of several bands. A: fetal LDL; B: fetal HDL; C: newborn VLDL; D: newborn LDL; E: newborn HDL; F: adult VLDL; G: adult LDL; H: adult HDL.

late that these particles may represent nascent LDL secreted by the fetal liver.

HDL of fetal calf are small, spherical particles similar in size to human HDL_3. The fact that the HDL are round, and not discoidal, together with the fact that cholesteryl ester is the major core lipid, suggest that lecithin: cholesterol acyltransferase is active in the fetal state. Preliminary experiments have, indeed, identified this enzyme system in fetal serum. The small size of the HDL may be a function of VLDL deficiency. It has recently been shown in human plasma that phospholipid and cholesterol are transferred from VLDL to HDL_3 during lipolysis, thus increasing the size of the particles to those of HDL_{2a} or HDL_{2b} (18, 19).

Apolipoproteins of VLDL and LDL in the bovine species have not been previously described. It is not surprising, however, that they contain apolipoproteins B and C which are commonly associated with the less dense lipoprotein classes in other animals. As indicated earlier, the presence of apolipoprotein A-I in steer LDL suggests the presence of large, less dense HDL particles in this region.

Apolipoprotein A-I is the major protein in HDL from fetal calves as well as from more mature animals. These animals do not contain apolipoprotein A-II which is the second major apolipoprotein in human HDL. Similar observations were previously made by Jonas (20) and Forte et al. (21) for mature bovine HDL. The C-apolipoproteins seen in newborn calf and mature animals are similar to those previously described by others (20, 22). It is interesting that both newborn calves and steers contain multiple C-apolipoprotein bands whereas fetal calves possess a single band in rather large amounts. It remains to be determined whether this apolipoprotein possesses lipolytic properties or whether it plays a unique role in lipid transport in the fetal state. The fact that additional apolipoprotein C bands appear in VLDL and HDL of newborn calves suggests, furthermore, that feeding stimulates the synthesis of additional C-apolipoproteins, some of which may be derived from intestinal cells.

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Lactoperoxidase-Catalyzed Iodination of Arachidonic Acid: Formation of Macrolides

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ABSTRACT

In the presence of iodide and hydrogen peroxide, lactoperoxidase catalyzed the conversion of arachidonic acid into several iodinated products; the major one was previously identified as an iodo-δ-lactone. Two minor and less polar products have now been characterized as 15-iodo-14-hydroxyeicosatrienoic acid, ω-lactone and 14-iodo-15-hydroxyeicosatrienoic acid, ω-lactone, on the basis of ^{125}I incorporation, mass spectrometry, proton magnetic resonance spectroscopy and chemical modifications.

INTRODUCTION

Lipid iodination has been the subject of a number of studies with two different emphases. It has been shown that the thyroid lipids of dogs on high iodine intake contain olefin diiodides (1–3). Other studies demonstrated that lactoperoxidase-catalyzed iodination of intact cells labels several classes of membrane lipids in addition to cell surface proteins (4–9). In both cases, iodination presumably involves the covalent binding of iodine to fatty acids either by addition to double bonds or substitution for hydrogen. Arachidonic acid is metabolized through a variety of pathways, all of which involve primarily dioxygenation reactions. In tissues such as the thyroid gland, which contain an iodide peroxidase, iodination might provide an alternative pathway for arachidonic acid metabolism. We have thus investigated the iodination of arachidonic acid catalyzed by lactoperoxidase and identified the major product as an iodo-δ-lactone (10). In this paper, we report the structure of two less abundant products of this iodination reaction.

MATERIALS AND METHODS

Materials

(5, 6, 8, 9, 11, 12, 14, 15-^{3}H) Arachidonic acid (60–100 Ci/mmol) and ^{125}I (15.8 mCi/μg) were purchased from New England Nuclear, Boston, MA, and from Amersham, Arlington Heights, IL, respectively. Arachidonic acid (purity >99%) was obtained from Nu-Chek-Prep, Elysian, MN. Lactoperoxidase (EC 1.11.1.7) from milk (58 purpurogallin units/mg) and methimazole (1-methylimidazole-2-thiol) were purchased from Sigma Chemical Co., St. Louis, MO.

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Incubation of *Arachidonic Acid* with Lactoperoxidase

Lactoperoxidase (2.9 μg/ml or 0.17 purpurogallin units/ml), [^{3}H]arachidonic acid (5–50 μg/ml, 0.05 μCi/ml), [^{125}I]KI (0.4 mM, 0.05 μCi/ml) and H_2O_2 (0.36 mM) were stirred in phosphate buffer (0.1 M, pH 7.4) for 30 min at 20 C. After addition of sodium thiosulfate, the reaction mixture was extracted with two vol of ethyl acetate.

Liquid Chromatography

Silicic acid column chromatography was performed in 1.5 cm-diameter glass columns packed with a slurry in chloroform of Porasil A (35–75 μ particles: Waters Associates, Milford, MA) (column height: 18 cm). Elution was performed with chloroform (90 ml), chloroform/methanol (95:5, v/v:60 ml) and methanol (30 ml). Three-ml fractions were collected and aliquots were counted in solid and liquid scintillation counters for ^{125}I and ^{3}H, respectively. Reversed phase-high pressure liquid chromatography (RP-HPLC) was performed on a μBondapak C_{18} column (3.9 × 300 mm, 10 μm particles: Waters Associates, Milford, MA). The injector (model U6K) and the pump (model 6000A) also were from Waters Associates. The samples were injected, dissolved in 50 μl methanol. Elution was performed with methanol/water (80:20, v/v) and the flow rate was 1 or 2 ml/min.

Gas Chromatography and Mass Spectrometry

Gas chromatographic analysis was performed on a Varian 2100 instrument with flame ionization detection, using a 1% OV-1 column isothermally at 185 C. Equivalent chain lengths were determined by reference to methyl esters of saturated fatty acids. Mass spectra scanning and selected ion monitoring were performed on a Hewlett-Packard combined gas chromato-

graph-quadrupole mass spectrometer (Model 5982A); 1% OV-1 columns (1 m × 2 mm) were used at 190 C with helium as carrier gas (flow rate: 30 ml/min). The injection port temperature was 250 C and the electron energy was 70 eV.

Chemical Modifications and Derivatizations

Reduction by lithium aluminum hydride ($LiAlH_4$): 10 μg of material was dissolved in 0.1 ml THF and 1 mg $LiAlH_4$ was added; after flushing with N_2, the reaction mixture was heated at 60 C for 72 hr and then diluted with water and extracted with ethyl acetate. Catalytic hydrogenation: 10 μg of material was dissolved in 0.5 ml ethanol to which 1 mg platinum oxide was added; hydrogen gas was bubbled for 2 min, after which the reaction mixture was diluted with water and extracted with diethyl ether. Trimethylsilyl ether derivatives were obtained by reaction with excess bis-trimethylsilyl-trifluoro-acetamide (BSTFA) in pyridine.

Proton Magnetic Resonance Spectroscopy

Proton magnetic resonance spectra were recorded on a JEOL FX-90Q Fourier transform spectrometer operated at 90 MHz. The sample was dissolved in deuterochloroform and tetramethylsilane was used as internal reference.

RESULTS

Incubation of arachidonic acid with lactoperoxidase in the presence of iodide and hydrogen peroxide resulted in the formation of several iodinated products; five peaks of coeluting ^{3}H-and ^{125}I-radioactivities were resolved by silicic acid column chromatography (10). The major product was identified previously as 6-iodo-5-hydroxy-eicosatrienoic acid, δ-lactone (10). A minor peak of radioactivity (X) eluted earlier than the iodo-δ-lactone in the void volume of the column. The yield of this product represented 10% of the yield of the iodo-δ-lactone, which itself amounted to 10-20% of the added arachidonic acid. During further purification by RP-HPLC on a μBondapak C_{18} column, ^{3}H- and ^{125}I-radioactivities coeluted again. The retention volume was 44.2 ± 2.8 ml (mean ± SD, n = 5) compared to 23 ml for the iodo-δ-lactone (solvent: methanol/water, 80:20, v/v; flow rate: 1 or 2 ml/min). The peroxidase inhibitor methimazole inhibited the generation of both the iodo-δ-lactone and compound X in a similar range of concentrations (10–100 μM; not shown).

Gas chromatographic analysis of underivatized component X showed a single peak of equivalent chain length C-21.8 (OV-1), compared to C-23.9 for the iodo-δ-lactone. The electron ionization mass spectrum of this material was indistinguishable from that of the iodo-δ-lactone (Fig. 1A) (10); it showed a molecular ion at m/z 430 and a prominent peak at m/z 303 (M-127), produced by the loss of iodine, which is the typical fragmentation of alkyl iodides (11). This suggested that component X and the iodo-δ-lactone could be isomers. After catalytic hydrogenation, a single peak of equivalent chain length C-19 (OV-1) was observed during gas chromatography. Its electron ionization mass spectrum showed a molecular ion at m/z 310, like the spectrum of the hydrogenated iodo-δ-lactone (Fig. 1B) (10). The shift from a prominent peak at m/z 303 to a molecular ion at m/z 310 could be explained by the saturation of three double bonds and the substitution of hydrogen for iodine. This substitution would be expected to increase the volatility of the compound and would thus explain the decrease of the GC-retention time observed after hydrogenation. The mass spectrum of hydrogenated material X differed from that of the hydrogenated iodo-δ-lactone by the lack of a base peak at m/z 99, characteristic of δ-lactones (12), and by the presence of ions at m/z 239 (M-71) and m/z 225 (M-85) (10). Ions at m/z 125, 111, 97, 83 and 69 are likely to be generated by the sequential loss of CH_2 units from a saturated hydrocarbon chain.

After reduction of the hydrogenated material X with $LiAl\ H_4$ followed by silylation, gas chromatography revealed a major peak of equivalent chain length C-22.5 (OV-1). The mass spectrum showed characteristic ions at m/z 387, 373, 187 and 173 (base peak) and was thus consistent with the expected fragmentation pattern of a mixture of 1, 14 and 1, 15 diols, resulting from the reduction by $LiAlH_4$ of a mixture of 14-hydroxy- and 15-hydroxy-ω-lactones (Fig. 2). The relative intensity of these ions suggested that the 15-hydroxy-ω-lactone was slightly more abundant than its 14-isomer. By comparison, a similar treatment (hydrogenation, $LiAlH_4$, silylation) of the iodo-δ-lactone provided a compound having the same equivalent chain length C-22.5 (OV-1); its mass spectrum showing major fragment ions at m/z 313 and m/z 247 was consistent with the structure of a 1, 5 diol.

The proton magnetic resonance spectrum of material X purified by RP-HPLC was consistent with a mixture of 14-iodo-15-hydroxyeicosatrienoic acid, ω-lactone and 15-iodo-14-hydroxyeicosatrienoic acid, ω-lactone. It revealed the following peaks: 0.89 ppm (tr, 3H, C_{20}), 1.28 ppm (brs, 6H, C_{17}, C_{18}, C_{19}), 1.65

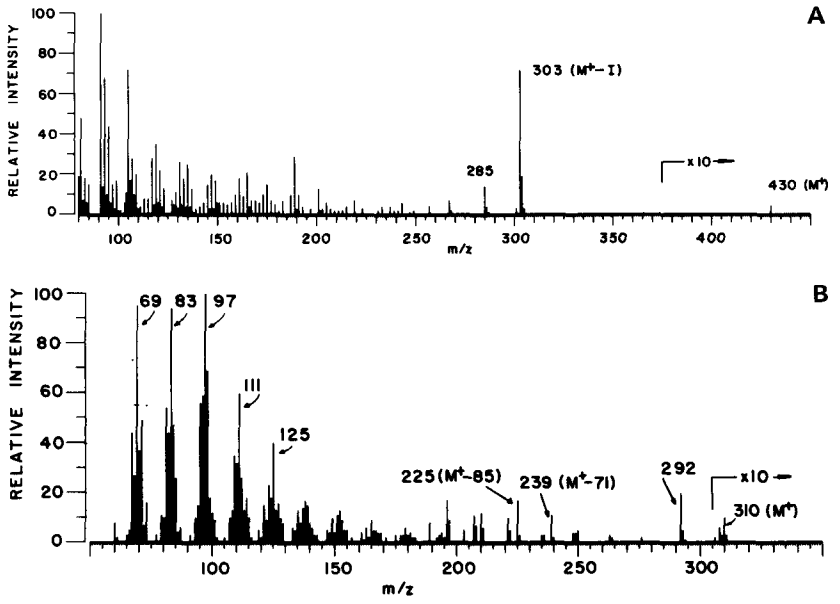

FIG. 1. (A) Electron ionization mass spectrum of component X, the minor product of lactoperoxidase-catalyzed iodination of arachidonic acid, eluted in the void volume of the silicic acid column. (B) Electron ionization mass spectrum of hydrogenated component X. An explanation for the existence of ions at m/z 225 and 239 is depicted in Fig. 2.

ppm (m, 4H, C_3, C_{16}), 2.0–2.5 ppm (m, 6H, C_2, C_4, C_{13}), 2.5–2.8 ppm (m, 4H, C_7, C_{10}), 4.14 ppm (tr of d, C_{14} or C_{15}, proton on iodine-bearing carbon), 4.46 ppm (tr of d, C_{15}, proton on alcohol-bearing carbon), 4.87 ppm (d of tr, C_{14}, proton on alcohol-bearing carbon) and 5.39 ppm (m, 6H, C_5, C_6, C_8, C_9, C_{11}, C_{12}). An homonuclear decoupling study supported the presence of the iodine and the hydroxyl function on vicinal carbons.

DISCUSSION

The major product of lactoperoxidase-catalyzed iodination of arachidonic acid is 6-iodo-5-hydroxyeicosatrienoic acid, δ-lactone (10). This transformation is analogous to the well-known reaction of alkaline iodolactonization (13–15). It is known that β, γ and γ, δ unsaturated carboxylic acids can be converted into iodo-γ-lactones and δ, ϵ unsaturated acids into iodo-δ-lactones (14). We have now observed the formation of 15-iodo-14-hydroxy-eicosatrienoic acid, ω-lactone and 14-iodo-15-hydroxyeicosatrienoic acid, ω-lactone. These compounds are macrolides which could also be called 15-iodo-eicosatrien-14-olide and 14-iodo-eicosatrien-15-olide. It is likely that these macrolides are formed by a mechanism similar to the formation of the iodo-δ-lactone (Fig. 3).

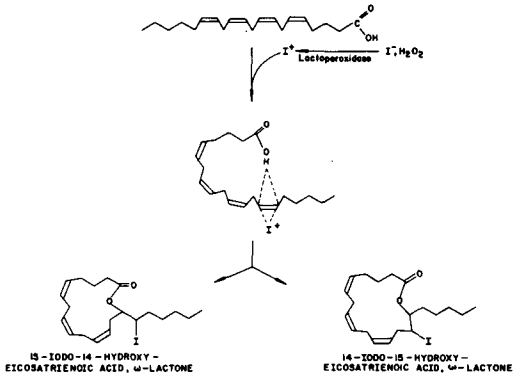

FIG. 3. Tentative scheme of arachidonic acid transformation into iodinated macrolides.

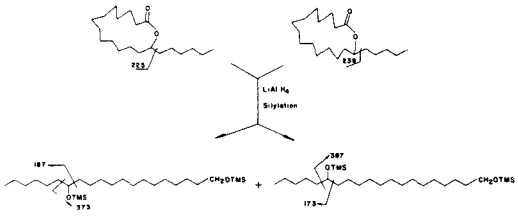

FIG. 2. $LiAlH_4$ reduction of hydrogenated component X: gas chromatographic-mass spectrometric analysis of the main product. The major fragmentation pattern is described and interpreted.

Corey et al. (15) have previously observed the spontaneous transformation of peroxyarachidonic acid into 14,15-epoxyeicosatrienoic acid by intramolecular oxygen transfer. These investigators concluded that a 15-membered ring may be energetically favorable compared to smaller structures. The formation of 15- and 16-membered iodinated macrolides could be explained by similar considerations and probably illustrates a tendency of arachidonic acid to adopt a J-like configuration. Further studies will determine if these macrolides are formed in intact cells and what could be their biological activity.

ACKNOWLEDGMENTS

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Separation of the Apoprotein Components of Human Very Low Density Lipoproteins by Ion-Paired, Reversed-Phase High Performance Liquid Chromatography

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ABSTRACT

A number of crude apolipoprotein samples isolated from human very low density lipoproteins (VLDL) were analyzed by reversed phase high performance liquid chromatography. The mobile phase consisted of a 1% solution of the polar ion-pairing reagent triethylammonium phosphate. A slow, nonlinear gradient of acetonitrile (37–42%) was used to elute the apolipoproteins. The order of elution was as follows: apolipoprotein C_X, apolipoprotein C-I, apolipoprotein C-III_2, apolipoprotein C-III_1, apolipoprotein C-III_0 and apolipoprotein C-II. This order is consistent with the known polarity of the proteins, i.e., the most nonpolar, apolipoprotein C-II, was the last to be eluted, whereas apolipoprotein C-I, with the lowest nonpolar surface area eluted first. The recovery of the individual apolipoproteins was 80–95% and the individual peaks were characterized by amino acid analysis, UV absorption spectra and chromatography of pure protein standards.

ABBREVIATIONS

VLDL, very low density lipoproteins; HDL, high density lipoproteins; C-apolipoproteins, lower molecular weight apolipoprotein constituents of VLDL; HPLC, high performance liquid chromatography.

INTRODUCTION

Recently the scope of reversed phase high performance liquid chromatography (HPLC) was extended to peptides and proteins by the use of mobile phases containing polar ion-pairing reagents (1–4). The rapid and highly efficient separations, which are a feature of this chromatographic technique, have great potential for the analysis of complex mixtures of proteins present in biological samples. In the past, the apoprotein constituents of human lipoproteins have been best analyzed by electrophoretic techniques, such as polyacrylamide gel electrophoresis (5–9) or isoelectric focusing (10–13). Neither approach is able to rapidly separate and quantitate all major components of human very low density lipoproteins (VLDL) and, therefore, we have investigated the application of HPLC to this separation problem. This report describes the development of a rapid and highly efficient HPLC separation of the C-apoproteins of VLDL. The procedure uses a μBondapak-alkylphenyl column with an aqueous mobile phase of 1% triethylammonium phosphate and acetonitrile gradients.

EXPERIMENTAL PROCEDURES

The analyses were performed on a Waters High Pressure Liquid Chromatograph equipped with a M-660 Solvent Programmer (Waters Associates, Milford, MA). A Schoeffel 770 UV Spectrophotometer was used to monitor the effluent at 220 nm. The column was a Waters μBondapak-alkylphenyl column (4 × 250 mm) containing 10-μ microparticulate silica particles with a surface coating of ca. 3 $\mu mol/m^2$, and a pore diameter of ca. 60 Å. After introduction of the reversed phase, any unreacted silanol groups are minimized by secondary capping with trimethylchlorosilane (Hancock and Sparrow, laboratory manual, in preparation).

Water was purified by passage through a deionizer, a carbon filter, and then careful distillation in a quartz still (Barnsted, Boston, MA). The mobile phase was prepared by addition of 10 ml of orthophosphoric acid (AR grade, J. T. Baker, Phillipsburg, NJ) to 1 ℓ of the purified water. The pH was adjusted to 3.2 with triethylamine (Aldrich, Milwaukee, WS) to form a ca. 0.17 M solution of the ion-pairing reagent. The reagent was then filtered in a stainless steel funnel (Hydrosol) fitted with a 47-mm Fluoropore (FH) 0.5-μ filter (Millipore, Bedford, MA), degassed with helium and used

[1]JTS is an Established Investigator of the American Heart Assn.

as solvent A. Acetonitrile was obtained from Burdick and Jackson (UV grade, Muskegon, MI), degassed with helium and used as solvent B.

The C-apolipoproteins were isolated from the chylomicrons and VLDL of Type V hyperlipidemic plasma by two ultracentrifugal flotations at d 1.006 g/ml, delipidation with ether/ethanol (3:1), gel filtration on Sephacryl S-200 in 3 M guanidine-HCl, 0.01 M Tris-HCl, pH 8.0, and finally desalting on Bio-Gel P-2 equilibrated with 0.10 M ammonium bicarbonate, pH 8 (11). The C-apoprotein mixture was dissolved in 1% triethylammonium phosphate, 6 M guanidine-HCl (BRL, Rockville, MD), pH 7.5. The sample was stored at −15 C. Immediately before chromatography, a 50–100-μl sample was treated with 10 μl of orthophosphoric acid to decrease the pH and ensure a large excess of the ion-pairing reagent.

After chromatography, the C-apolipoprotein samples were collected manually and cooled to 4 C. The pH was adjusted to 6 with triethylamine (5-20 μl) and the samples were then lyophilized. This procedure was done as quickly as possible to minimize any decomposition of the protein samples when exposed to acidic water/acetonitrile mixtures. The lyophilized samples were then sealed under vacuum with 0.5 ml of 6 N HCl, heated at 110 C for 24 hr and the amino acid composition was determined on a Beckman 117 Amino Acid Analyzer. Alternatively, the lyophilized samples were dissolved in 0.2 ml of 3 M guanidine/HCl solution and the UV spectrum measured on a Cary 15 Spectrophotometer.

RESULTS

Initial trial experiments indicated that the Waters μBondapak alkylphenyl column exhibited good selectivity for the different C-apolipoproteins and allowed elution of the proteins at relatively low organic solvent concentrations (Hancock and Sparrow, manuscript in preparation). Acetonitrile was chosen as the organic modifier, because Holmquist and Carlson (14) have shown that 50% aqueous acetonitrile is an efficient solvent for the C-apolipoproteins.

Shown in Figure 1 is the analysis on a μBondapak-alkylphenyl column of a mixture of apoC-I, C-II, and C-III_1 standards by an isocratic system. The mobile phase consisted of a 60:40 mixture of acetonitrile/0.1% phosphoric acid and the peaks were identified by the retention times of the individual standards. Although the separation was adequate, repeated injections showed that the recoveries were low and variable. The poor recoveries were probably caused by the high level of organic solvents necessary to elute the nonpolar apolipoproteins, as denaturation and precipitation of proteins can readily occur in nonaqueous solvents.

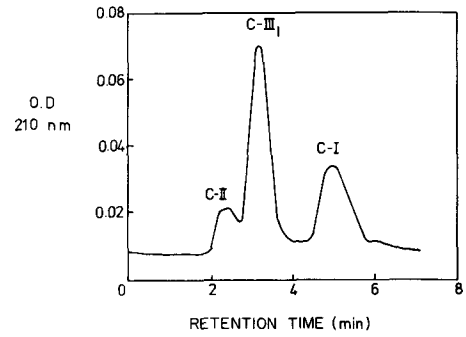

FIG. 1. The elution profile obtained for the isocratic elution of the apolipoproteins C-I, C-II and C-III_1 on a μBondapak-C_{18} column with a mobile phase which consisted of 0.1% phosphoric acid, pH 2.0/acetonitrile (40:60) and a flow rate of 1.5 ml/min.

In an effort to overcome these problems, the use of gradient analysis and a mobile phase containing triethylammonium phosphate was investigated. As shown in Figure 2, a shallow,

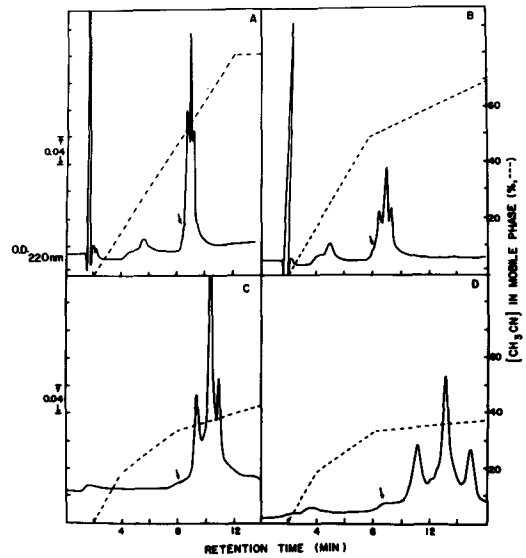

FIG. 2. The elution profiles obtained for a crude mixture of C-apolipoproteins (50 μg) on a μBondapak-alkylphenyl column with a mobile phase of 1% triethylammonium phosphate, pH 3.2. Chromatograms A to D demonstrate the effect of different acetonitrile gradients on the separation. The apoC-I peak is indicated by an arrow because it is present in low concentrations in this serum sample. The line (– – –) represents the program on the gradient former.

nonlinear gradient gave the best separation of the C-apolipoproteins. Indeed, the use of a 10-min concave gradient followed by a 20-min convex gradient gave a separation far superior to that obtained with a shallow, 2-hr linear gradient. The latter part of the convex gradient where the increase in organic content was nearly linear is shown in Figure 3. The crude C-apolipoprotein mixtures used in this separation were isolated using the procedure described in Experimental Procedures. To obtain good reproducibility between injections, it was necessary to follow the analysis by equilibration of the reversed phase with a high concentration of the organic modifier (2-min gradient to 80% acetonitrile, and 2 min at this concentration) and then equilibration with the aqueous phase (5-min gradient to 0% acetonitrile, followed by a 10-min equilibration). This analysis protocol was followed for all subsequent C-apolipoprotein chromatography.

Using the conditions described in Figure 3, almost all of the C-apolipoproteins were eluted over a narrow range of acetonitrile concentration (37–42%). The extreme sensitivity of the separation to solvent changes was further indicated by the observation of extremely broad peaks and low recoveries when the mixture was chromatographed using a slightly lower acetonitrile concentration (up to 40%). Conversely, rapid, steep acetonitrile gradients also gave low recoveries of the protein components. After such a gradient, followed by re-equilibration of the column with the aqueous phase (5-min gradient 80 to 0%), unidentified peaks were observed eluting when the reverse gradient again reached the crucial acetonitrile concentration. A possible explanation for this observation is that the protein can be precipitated with a rapid organic solvent gradient, with some of the precipitate redissolving at a later stage. If the analysis procedure just described is followed, however, less than 0.1% of a 100-μg injection of the apolipoprotein mixture is observed either during a subsequent re-equilibration, or a blank run after injection of 100 μl of 6 M guanidine/HCl.

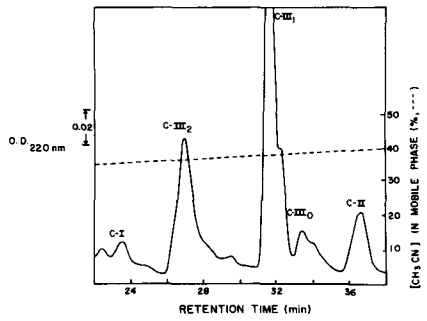

FIG. 3. The elution profile obtained for a crude mixture of C-apolipoproteins (100 μg) on a μBondapak-alkylphenyl column with a mobile phase of 1% triethylammonium phosphate and a flow rate of 1.5 ml/min. The gradient consisted of a 10-min concave gradient of 0–37% acetonitrile (number 2 on the Waters 660 Solvent Programmer) and 20-min convex gradient of 37–42% acetonitrile (number 8).

In an effort to detect and characterize minor components of the mixture, a somewhat larger sample (800-μg) was chromatographed on the column without a drastic loss in separation efficiency (Figs. 4A and B). The variation in the ratio of optical density to amino acid content can be caused by a number of factors, e.g., different proteins contain different amounts of residues which strongly absorb in the UV, small amounts of nonprotein contaminants may be present in the sample, and some UV absorbing impurities which eluted early in the gradient are always present in the mobile phase, i.e., the water and buffer salts. However, a blank gradient run under the same conditions did not show any significant optical density peaks in the region where the apolipoproteins eluted. For these reasons, it was concluded that optical density peaks in an elution profile must be further identified. In this study, all identification and quantitation was based on amino acid analytical data and not on optical density values. Amino acid analysis of the collected fractions indicated that the bulk of the apolipoproteins eluted between 37 and 42% acetonitrile in the gradient analysis. The amino acid analysis data are shown in Table I and the proposed identity of each fraction is given in Table II. The early eluting fractions (1-15) in the chromatogram contained small amounts of material which could not be identified from the amino acid analysis data. Other studies (not shown) with apoB and E samples suggested that this material may be due to apoB and E contaminants. Although fractions 16-17 and 18-19 are identified as apoC-I and apoC-III_2 in Table II, both samples were clearly contaminated with other protein(s). The tentative assignment of identity was confirmed by peak enhancement studies done by injecting an apolipoprotein mixture that had been enriched with either a pure sample of apoC-I or C-III_2. The amount of material in fractions 19 and 22, 23 was sufficient for determination of the UV spectrum. The observed maxima at 280 nm and minima at 250 nm indicated that both samples contain unmodified tryptophan residues.

In Table I, some of the fractions exhibit elevated levels of glycine and serine which have been found to be common contaminants in

amino acid hydrolysates (15). The elevated levels of aspartic acid are of unknown origin. However, the nonspecific contamination was not present in apolipoprotein mixtures which have been further purified before HPLC analysis (Table III). The impurities usually did not prevent identification if the retention times and the diagnostic residues listed in Table II were used. Although fraction 24 is only a shoulder on the trailing end of the large apoC-III_1 peak, the amino acid analysis of the minor peak is significantly different from that of apoC-III_1 (Table I). This clear difference shows that the separation efficiency of the column has not been significantly affected by the large sample load and that the analysis of the smaller peaks is not obscured by tailing of the earlier components of the mixture.

A possible contaminant present in partially purified serum proteins is serum albumin. Figure 5 shows the elution profile for a sample of this protein chromatographed under the same conditions as for the apolipoprotein samples. A broad peak between 26 and 36 min is observed, and thus albumin could be a significant contaminant in the analysis of the apolipoprotein fractions which are not purified on Sephacryl S-200 before HPLC analysis.

From other studies with partially purified apoVLDL and high density apolipoprotein (HDL) samples, it was observed that the presence of apo-E components in the C-apolipoprotein mixture greatly complicated the analytical separation. Isoelectric focusing studies (11, 13) have shown the presence of five apo-E polymorphs and such structural diversity could explain the complex HPLC elution profile observed in the early part of the analysis shown in Figure 4. In an attempt to determine the elution profile of just the C-apolipoprotein mixture, a sample with a much lower apoE content was examined. The elution profile is shown in Figure 6. In addition to the apolipoproteins C-I (fraction 5), C-II (fraction 8), C-III_1 (fraction 7), and C-III_2 (fraction 6), two small, early eluting peaks were observed with an amino acid analysis which fits reported values for apoC-$X_{1,2}$ (16). The presence of this apolipoprotein was not observed in the previous analysis (Fig. 4A,B) due to the presence of larger amounts of contaminants, which eluted in the same region of the gradient. The amino acid analysis of the pooled fractions is shown in Table III; comparison of these data with those of Table I show that very little contamination was observed in this separation. This improved separation suggests that the presence of apo-E polymorphs does, indeed, complicate the elution profile. The apoC-I, apoC-III_1, and

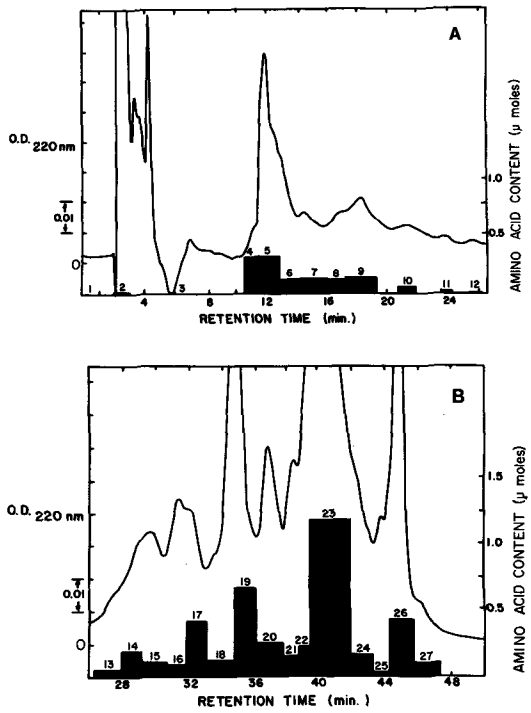

FIG. 4A, B. The elution profile of a mixture of C-apolipoproteins (800 μg) with the same chromatographic conditions used in Fig. 3 except that the time for the second gradient was increased to 30 min. The fractions were collected manually; each fraction corresponds to a change in UV absorbance. The samples were hydrolyzed to the constituent amino acids which were then quantitated by amino acid analysis. The sum of the amino acid values was expressed as the total protein content of the peak (solid bar).

apoC-III_2 peaks each contain a minor contaminant which apparently does not affect the amino acid compositional data as each analysis agrees well with the reported values for the respective apolipoproteins. The absence of tyrosine and histidine for apoC-I, histidine for apoC-II and isoleucine for apoC-III (each characteristic of the pure apolipoprotein) indicated that complete separation of the major components was achieved. The identity of the apoC-I contaminant is unknown, although other workers have reported an isomeric form of apoC-I (16). Kane et al. (7) have presented evidence for an additional isomeric form of apoC-III which contains one more sialic acid residue than apoC-III_2 and thus have named it apoC-III_3. The extra sialic acid residue would be expected to make apoC-III_3 slightly more polar than apoC-III_2 and thus could account for the peak eluting on the front edge of peak 6 in Figure 6. Shore and Shore (17) have charac-

TABLE I

Amino Acid Analysis Data for the Isolated Fractions from the Chromatography of a Crude C-Apolipoprotein Mixture (Figs. 4A and B)

Fraction no.[a]	4	5	9	14	16	17	18	19	20	21	22	23	24	26	E	CI	CII	CIII
Amino acid[b,c]																		
Asp	58	102	101	165	96	97	90	86	123	96	81	79	85	64	51	81	64	80
Thr	31	32	41	65	52	49	44	35	52	54	46	55	41	74	34	45	101	57
Ser	42	125	102	120	98	90	61	125	138	70	106	121	108	75	45	106	99	125
Glu	105	105	96	126	125	134	99	136	151	110	151	131	142	106	135	136	104	114
Pro	11	–[d]	–	–	–	12	–	–	–	–	21	17	14	–	13	15	26	23
Gly	48	31	58	72	45	185	20	73	26	27	29	36	160	31	51	15	28	34
Ala	62	69	49	76	60	94	56	66	85	80	60	103	77	43	78	45	75	114
Cys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Val	46	68	30	60	82	42	50	72	80	57	65	69	46	48	55	30	45	68
Met	6	13	10	10	19	13	21	21	25	12	16	27	10	26	5	15	21	23
Ile	6	12	14	6	5	23	13	23	16	0	2	2	21	14	13	45	10	0
Leu	41	60	84	53	108	69	85	75	81	60	59	55	74	94	90	91	92	57
Tyr	14	36	14	15	21	4	32	25	42	18	21	27	21	55	13	0	53	23
Phe	20	50	30	28	63	57	48	72	76	33	46	48	20	24	16	45	24	46
His	12	2	4	3	6	0	11	13	16	5	9	13	4	0	6	0	0	11
Lys	42	40	47	29	90	116	42	72	67	69	63	53	39	72	37	136	70	68
Arg	15	42	–	27	41	46	17	23	17	25	16	23	41	13	69	45	13	23

[a]The minor peaks did not give sufficient material for a calculable analysis and are not listed.

[b]Trp was not determined due to destruction during acid hydrolysis; Ser and Thr values are uncorrected.

[c]Expressed as mol/100,000 g protein.

[d]Absent or insufficient quantity to determine accurately.

TABLE II

Proposed Assignment for Proteins Eluted from the μBondapak-Alkylphenyl Column

Fraction no.	Amino acids (μmol)	% of total	Assignment	Comments
1–3	–	0	Nonprotein	Solvent impurities, guanidine-HCl
4–9	1.13	20	Unknown	Rich in Glu and Asp, some Tyr and Ile
10–12	.10	2	Unknown	Insufficient to characterize
13–15	.38	7	Unknown	Rich in Glu, Asp and Ser, some Tyr and Ile
16–17	.53	10	ApoC-I	Rich in Lys, trace of His and Tyr
18–19	.80	14	ApoC-III_2	Has His and Tyr
20	.26	5	Unknown	Has His and Tyr
21–23	1.55	28	ApoC-III_1	Has His and Tyr, no Ile
24	.18	3	Unknown	Rich in Glu and Ser; some Tyr and Ile
25, 26	.47	8	ApoC-II	Rich in Tyr, trace His
27	.10	2	Not apoC-II	Insufficient to characterize

terized apoC-III_0, a variant of apoC-III which does not contain sialic acid. It could be expected that such a variant would be slightly less polar and thus be eluted just after apoC-III_1, an observation which fits the observed elution profile in the trailing edge of peak 7 (Fig. 6). The insets in Figure 6 show the minor contaminants more clearly resolved from major peaks; the improved resolution was achieved by a decrease in sample loading.

Figure 7 shows the elution profile for the mixture of C-apolipoproteins obtained from the breakthrough volume of a DEAE-Sephadex column. The HPLC analysis indicated that this mixture contained predominantly apoC-I with some apoC-X and apoC-III_1, which is in agreement with previous observations (17). As is shown in Table III, this assignment was clearly supported by amino acid analysis data. Again, a small amount of material of unknown composition eluted near apoC-I.

Other workers have found preliminary evidence for a number of apoC-III variants in addition to apoC-$III_{0,1,2}$ such as apoC-III_x (14), apoC-III_3 (5), and three other uncharacterized proteins which chromatograph near apoC-III_2 (7, 11, 13). The HPLC analysis of a broad pool of apoC-III proteins from a DEAE-Sephadex column does support these claims of additional C-apolipoprotein heterogeneity. The elution profile (Fig. 8) and amino acid analysis data indicate that the apoC-III contains apoC-X (peaks 1 to 3), apoC-III_2 (peak 6) and apoC-III_1 (peak 7) as well as an unknown apoC-III variant (peak 5). In common with the other elution profiles, the apoC-III_2 peak contains a minor second component (see inset in Fig. 8). Apart from the minor apoC-X peaks, all other peaks have a composition which closely fits the values expected for apoC-III.

DISCUSSION

Although the introduction of polar ion-pairing reagents has allowed the successful chromatography of a number of pure protein standards (1–4, 18–23), few examples have been reported of the separation of proteins present in crude biological samples. To date, the only reports of biological isolations have involved the isolation of small peptide hormones and endorphin fragments (4, 20, 22), both of which seem particularly well suited to reversed phase chromatography. Apolipoproteins are known to be difficult proteins to purify, due to a strong tendency to undergo aggregation and to the presence of many closely related species in lipoprotein particles. In addition, the presence of large, nonpolar surface regions in apolipoproteins and the ability of these proteins to rapidly denature and be adsorbed at silanol groups present on glass surfaces has further complicated chromatographic studies. The development of a HPLC procedure for the isolation of these proteins would, therefore, indicate that the technique is likely to be widely applied to protein purification.

The isocratic separation of a mixture of apoC-I, apoC-II, and apoC-III_1 on a μBondapak-C_{18} column is shown in Figure 1. A normal phase, rather than a reversed phase, partition mechanism was suggested by the observations that a decrease in acetonitrile concentration in the mobile phase caused a decrease in retention time (opposite to what is observed for reversed phase separations). The elution order of apoC-II, apoC-III_1 and apoC-I is also in the order expected for the interaction of basic residues of the proteins with the acidic silanol groups of the column. The number of basic residues for the proteins is 83, 113, and 181 mol/100,000 g

TABLE III

Amino Acid Analysis Data for the Isolated Peaks from Chromatography of Partially Purified C-Apolipoprotein Mixtures (Figs. 6–8)

	ApoC without apoE					*Fractions from DEAE-Sephadex*								
						Breakthrough peak								
Fraction no.[a]	T[b]	5	6	7	8	T	2	6	7	T	1–3	5	6	7
Asp	81	82	80	81	73	81	70	81	79	80	60	91	96	95
Thr	63	33	61	48	58	34	45	38	54	46	30	55	180[d]	55
Ser	124	91	125	82	65	93	135	104	128	110	126	114	–	123
Glu	117	156	116	78	124	126	126	157	120	131	150	134	130	134
Pro	28	–[e]	26	16	–	11	26	–	20	–	–	28	15	22
Gly	38	32	36	46	58	24	128	26	30	43	150	144	45	45
Ala	113	38	112	102	60	51	58	46	1	108	55	114	105	90
Cys	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Val	78	28	73	58	36	30	26	29	64	75	18	68	72	72
Met	24	10	22	12	10	15	2	15	14	18	3	15	16	14
Ile	2	33	0	1	9	42	13	46	1	2	8	0	1	1
Leu	75	75	65	53	73	96	25	95	58	60	20	56	62	62
Tyr	41	3	31	22	36	3	19	2	24	20	10	19	24	25
Phe	58	56	50	37	18	51	13	49	40	36	9	44	52	51
His	11	2	10	10	1	2	18	1	11	10	21	11	14	15
Lys	87	131	73	60	60	126	34	136	64	69	21	65	79	77
Arg	27	49	24	24	18	51	16	49	28	21	15	22	25	25
Recovery yield[c]			97					85.5				83		

[a]Same details as for Table I.

[b]Total composition of mixture before HPLC.

[c]Expressed as a percentage of amount injected (from amino acid analysis data).

[d]Combined value for Thr and Ser.

[e]Absent or insufficient quantity to determine accurately.

of each protein, respectively (24). Although the μBondapak-alkylphenyl has secondary capping (see Experimental Procedures), the packing will still contain a significant number of free silanol groups. Because apolipoproteins are extremely sensitive to adsorption to glass surfaces, the number of residual silanol groups are presumably sufficient to allow a mixed mode separation resulting from both adsorption and partition effects. Not surprisingly, this separation was found to give poor recoveries (20–50%) and has subsequently been abandoned in favor of the more successful gradient procedure.

Rivier (4) has recently shown that triethylammonium phosphate forms a particularly useful mobile phase for the analysis of peptides and proteins. The success of this reagent can be attributed to the combined effect of triethylamine which deactivates silanol groups, the low pH which ensures that the silanol groups are protonated, and the high buffer concentration (0.17 M) which disrupts ionic interactions. The use of a 10-fold lower buffer concentration gives a similar elution profile for the C-apolipoprotein mixtures but the recoveries and peak shapes are poor. Such a dramatic effect suggests that the salt acts to prevent ionic interactions between the column and the protein samples. An additional mechanism operates as well, because dihydrogen phosphate is an effective ion-pairing reagent and associates with ammonium groups of the sample molecules (1, 25). Such a complex has much greater polarity than the internal ion pairs formed by the protein in the absence of phosphate, with the result that there is a large decrease in retention of the sample on reversed phase columns. For example, the C-apolipoproteins can not be eluted from the reversed phase column in the absence of a *polar* ion pairing reagent.

The successful use of this mobile phase, when combined with gradient elution on a μBondapak-alkylphenyl column, can be seen in Figures 2–4 and 6–8. An important feature was the use of a shallow gradient of organic modifier at relatively low flow rates (1.5 ml/min). Stein and coworkers have recently stressed the importance of using low flow rates for the analysis of large protein molecules due to the relatively low diffusion rates of these species (22). The results presented here would support this observation. Also, with the apolipoproteins, peak shape and recovery of sample were particularly sensitive to the nature of the mobile phase and the column. Such a precise requirement for optimal separation conditions was not observed by Stein (26), or by Nice and O'Hare (20) in a careful study of the reversed

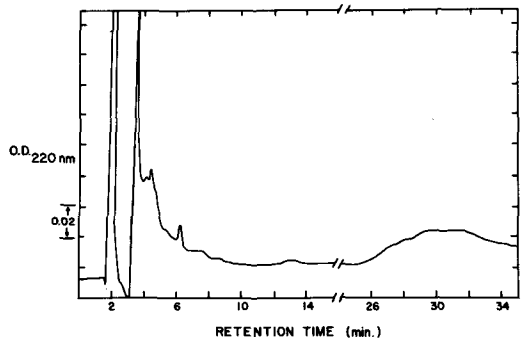

FIG. 5. The elution profile for human serum albumin using the conditions described in the legend to Fig. 4. A 0.12-mg sample was applied to the column.

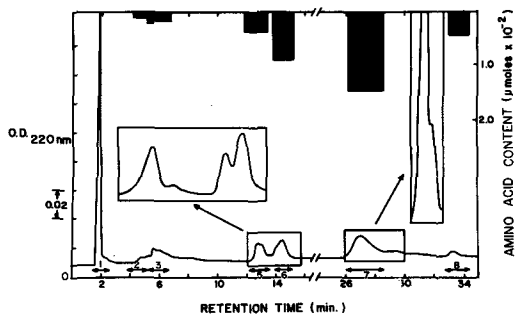

FIG. 6. The elution profile for a mixture of C-apolipoproteins which does not contain significant amounts of apo-E proteins. The chromatographic conditions used were the same as those described in the legend to Fig. 3, except that the initial gradient was 0 → 38% acetonitrile. The insets show the minor contaminants more clearly resolved from the major peaks; the improved resolution was achieved by a decrease in sample loading.

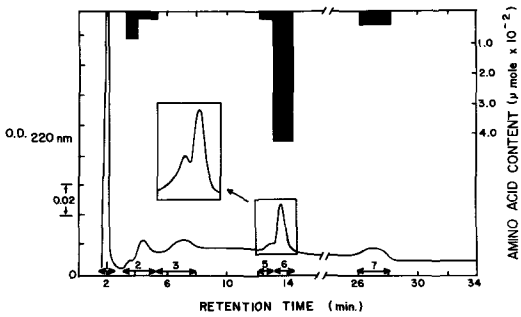

FIG. 7. The elution profile of a mixture of C-apolipoproteins from the breakthrough peak of a DEAE-Sephadex column; conditions as for Fig. 3.

phase chromatography of a variety of peptide and protein hormones. This difference probably reflects the wide variation in structure and function of different proteins and, unfortunately, demands that the researcher spend considerable time and effort developing a useful separation. The chromatography of the A-apolipoproteins, e.g., has quite different requirements for the optimal nature of the column and mobile phase, despite the close similarity in structure and function of the A- and C-apolipoproteins.

The elution order for the C-apolipoproteins shown in Figures 2–4 and 6–8 is consistent with a reversed phase separation mechanism, in that apoC-II which has the largest nonpolar surface area (24) is the most strongly retained. ApoC-I does not have any extended regions of nonpolar surface residues and is the first eluted, whereas apoC-III has an intermediate surface polarity (24) and retention on the column. The order of elution is converse to the basic amino acid content for the apolipoproteins (181, 113 and 83 mol/100,000 g of protein) and perhaps suggests that the observed order of elution is also influenced by the degree of hydrophilic ion-pairing of the dihydrogen phosphate anion with the ammonium ions of the protein molecules. ApoC-I, with the greatest content of basic residues, would form the most polar ion-paired complex and would thus be eluted first from the reversed phase column.

The recovery of the individual C-apolipoproteins was excellent and ranged from 83 to 97% as judged by amino acid analysis and UV absorption measurements of samples before and after chromatography. The carryover of material from one injection to the next was found to be less than 0.1%. The amount of the individual apolipoproteins in a single plasma sample, as measured by amino acid analysis was found to be consistent with previously determined values (Table IV). It should be noted that wide variations in the levels of the individual apolipoproteins in both normo- and hyperlipemic subjects have been observed by others (7–13; Hancock and Sparrow, manuscript in preparation).

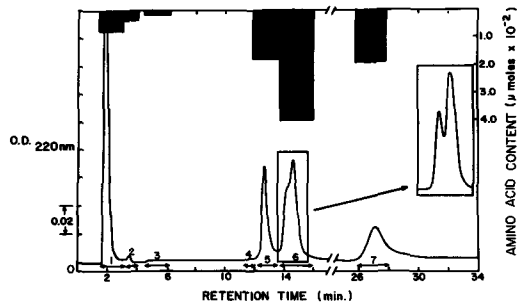

FIG. 8. The elution profile obtained for a mixture of apoC-III proteins; conditions as for Fig. 3.

A number of research groups have reported the presence of other minor C-apolipoproteins in delipidated VLDL (7,9,11–13, 16, 17). The apolipoprotein mixtures used in the present study were also analyzed by isoelectric focusing and were fractionated by DEAE-Sephadex

TABLE IV

Levels of C–Apolipoproteins in Normo- and Hyperlipemic Subjects

Apoprotein	Range of levels[a]	Levels from HPLC of a single hyperlipemic subject[b]
C–I	3.9–7.9	10
E	17–41.8	–[d]
C–II	10.6–20[c]	8
C–III_0	7–7.1	–[e]
C–III_1	23–51	28[e]
C–III_2	14–27	14
C–III_3	6.3–8	–[f]

[a]Expressed as a percentage of total apoC and E; the contribution of apoB is not included. Data from references 3, 4, 6, 8–10.

[b]As determined by amino acid analysis of fractions from the elution profile shown in Figure 4A, B after correcting for the average value of regions between fractions 9 to 13.

[c]Not including values which contain unresolved apoC–III_0.

[d]Unable to quantitate as material chromatographed as a number of heterogenous peaks.

[e]C-III_0 was not resolved from C-III_1 at the high sample loading used in this run.

[f]Either absent or converted to C-III_1 during our chromatographic purification techniques by spontaneous desialation (5).

chromatography. In each case, the amount of the individual apolipoprotein agreed well with the values listed in Table IV and the elution profiles shown in Figure 4A, B.

It is possible that some of the observed protein components were due to artifacts, such as carbamylation caused by the presence of isocyanates in urea containing buffers, by deamidation of asparagine and glutamine residues in basic or acidic solutions, or by aggregation. As described in Experimental Procedures, experimental conditions were chosen to eliminate such artifacts as much as possible. Urea was not used during purification of the apolipoprotein mixture used in the separation shown in Figure 4. Acidic conditions were avoided as the apolipoprotein samples were stored at neutral pH at −15 C. Aggregation was prevented by addition of a protein denaturant (3 M guanidine/HCl) in all storage buffers and in the HPLC injection sample. If a sample of apoC-III_1 was isolated from a gradient elution and reinjected, a single optical density peak was observed with the same retention time as an authentic sample of apoC-III_1. If this sample was not adjusted to a concentration of 3 M guanidine/HCl before reinjection, the same retention time was observed but the recovery decreased from 80 to 33%.

These studies suggest that aggregation is not the cause of the large number of minor constituents of C-apolipoproteins and a current research goal is to elucidate the structure of these minor proteins. In conclusion, reversed phase ion-paired HPLC has allowed the development of a rapid procedure for the separation of the complex mixture of C-apolipoproteins present in VLDL. It is planned to use this high efficiency separation to investigate the role of the C-apolipoproteins in the atherogenic process and to initiate structural studies of the uncharacterized components of the mixture.

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METHODS

The Use of SP2340 Glass Capillary Columns for the Estimation of the *trans* Fatty Acid Content of Foods[1]

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ABSTRACT

Glass capillary gas chromatography (GCGC) on 100-m and 60-m SP2340 columns was used for quantitation of the *trans* unsaturated fatty acids in shortenings and fast foods. The separation of the *cis* and *trans* octadecenoates on GCGC was evaluated by preparatory argentation thin layer chromatography. In addition, the *trans* content of shortening samples obtained by GCGC was compared to *trans* content determined by infrared analysis.

INTRODUCTION

Most margarines and shortenings are made from partially hydrogenated oils in order to improve their stability and create more desirable physical properties. This produces a large number of positional and geometric isomers of 18:1 and 18:2. *trans* Unsaturated fatty acids also can be formed by the action of bacteria during the digestion of fat in ruminant animals, which leads to the presence of small amounts of *trans* fatty acids in dairy products and certain meats (1). These fatty acids are thus a common constitutent of the American diet and are deposited in human tissue. Autopsy and biopsy tissues from human subjects contained as much as 14.4% *trans* unsaturated fatty acid in liver tissue and 12.2% in adipose tissue (2). There are a number of conflicting reports on the effects of *trans* unsaturated fatty acids. Some studies (3,4) suggested that *trans* unsaturated fatty acids cause an elevation of serum cholesterol. One study reported *trans* unsaturated fatty acids accentuated essential fatty acid deficiency (5). More recently, Enig et al. have implicated *trans* fatty acids in the causation of cancer (6). Alfin-Stater et al. (7) found no deleterious effects caused by hydrogenated fats in multigeneration studies in rats, and Mattson et al. (8) found no change in plasma cholesterol and triglyceride levels in men fed *trans* unsaturated fatty acids.

The interest in the nutritional effects of *trans* unsaturated fatty acids dates back to the early 1950s, but there is still little data on *trans*

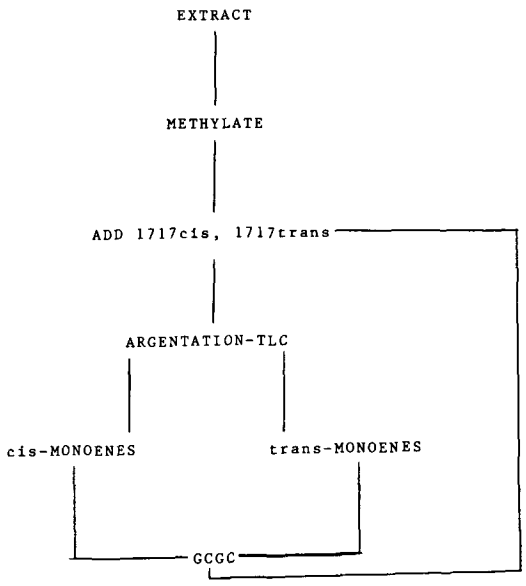

FIG. 1. Schematic of analytical procedure for analysis of *cis* and *trans* octadecenoates.

[1]This research conducted by the Science and Education Administration, USDA, on the commercial foods as reported in this paper was limited to analysis of their lipid compositions. The data are reported solely as factual information and are limited to the samples analyzed. No warranty or guarantee is made or implied that other samples of these products will have the same or similar composition. It is the policy of the USDA not to endorse those commercial products used in research over those that were not included in the research.

unsaturated fatty acids in foods except for margarines. Heckers and Melcher (9) analyzed the *trans* unsaturated fatty acid in German margarines, Beare-Rogers et al. (10) in Canadian margarines, and Carpenter and Slover (11) in selected U.S. margarines. The recent *Handbook* on the composition of fats and oils by the USDA (12) did not include information on *trans* content because reliable data were unavailable.

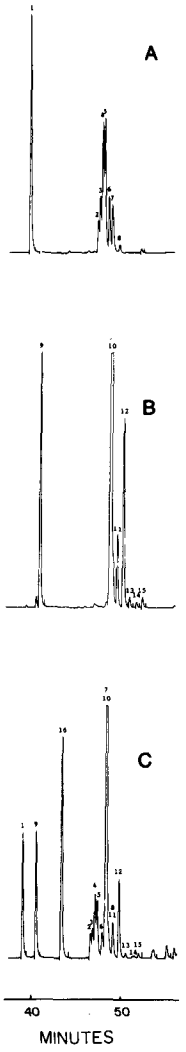

FIG. 2. Separation of the octadecenoate isomers of Crisco shortening. Column: SP2340, 100 m X 0.25 mm, glass; for conditions, see Methods. (A) *trans*-unsaturated FAME, after argentation TLC; (B) *cis*-unsaturated FAME, after argentation TLC; (C) Crisco FAME without argentation TLC. Peak identities: 1. 17:1ω7*t*; 2. 18:1ω12*t*; 3. 18:1ω9*t*; 4. 18:1ω?*t*; 5. 18:1ω7*t*; 6. 18:1ω1?*t*; 7. 18:1ω?*t*; 8. 18:1ω?*t*; 9. 17:1ω7*c*; 10. 18:1ω9*c*; 11. 18:1ω7*c*; 12. 18:1ω?*c*; 13. 18:1ω?*c*; 14. 18:1ω?*c*; 15. 18:1ω?*c*; 16. 18:0.

No simple procedure is available for the quantitation of small amounts of individual *trans* fatty acids in foods. Traditionally, *trans* unsaturated fatty acids were determined by either of 2 infrared (IR) spectrophotometric procedures (13,14) that do not quantitate individual fatty acids. Dutton and coworkers described ozonolysis procedures for determination of double bond position and geometric configuration in both monoenoic (15) and dienoic (16) fatty acids, but these procedures are complicated and time-consuming. Determination of *trans* unsaturated fatty acids by combination of preparative gas chromatography (GC) with either argentation thin layer chromatography (TLC) (17) or IR spectroscopy (18) also is laborious. A number of papers described the use of highly polar, temperature-stable cyanopropylsiloxane-packed columns. Conacher and Iyengar (19) found 80-85% agreement between the *trans* content of margarines analyzed on a 15′ Silar 10′C packed column and IR. Ottenstein et al. (20) and Perkins et al. (21) analyzed the *trans* unsaturated fatty acids of several margarines on 20′ packed OV-275 columns. Ottenstein et al. compared their GC data to the IR procedure of Allen (13) whereas Perkins used the AOCS IR procedure (14). Both reported reasonably good agreement with IR data.

The retention data of Heckers et al. (22) for various octadecenoate standards on Silar 10 C, OV-275 and other cyanosiloxane phases with packed columns indicated that separations of octadecenoates are incomplete. Furthermore, the chromatograms published by both Ottenstein et al. (20) and Conacher and Iyengar (19) showed only partial separation of peaks in the octadecadienoate region. Although octadecadienoates comprise only a small fraction of the total *trans* value in most foods, separation of peaks in this region is important since linoleate is the only octadecadienoate with essential fatty acid activity, and in foods its overestimation caused by incomplete separation is of nutritional concern (23). In addition, the feeding of *trans-trans* linoleate to rats was found to cause a decrease in prostaglandin biosynthesis (24).

Much of the early work with open-tubular, stainless steel columns was primarily qualitative (25-27). Recent technological advances in coated glass capillary columns have made glass capillary GC (GCGC) an ideal choice for the separation of individual fatty acid isomers. Jaeger et al. (28) reported fatty acid methyl esters (FAME) separation on FFAP and Ojan-

TABLE I

Octadecenoate Isomers in Selected Foods

Peak[a]	Fatty acid	Crisco shortening	Nabisco zweiback	Burger Chef cheeseburger	Beef[b]
		(g/100 g of food [wet wt])[c]			
2	18:1ω2*t*	0.71	0.07	0.02	0.01
3	18:1ω9*t*	1.40	0.11	0.04	0.02
4	18:1ω?*t*	3.55	0.24	0.13	0.16
5	18:1ω7*t*	3.26	0.24	0.24	0.03
6	18:1ω?*t*	1.36	0.11	0.04	0.01
7	18:1ω?*t*	1.39	0.13	0.07	0.01
8	18:1ω?*t*	0.18	0.02	0.02	Tr[d]
10	18:1ω9*c*	19.54	2.15	3.71	1.92
11	18:1ω7*c*	1.91	0.19	0.15	0.09
12	18:1ω?*c*	5.34	0.42	0.05	0.01
13	18:1ω?*c*	0.24	0.04	0.04	0.01
14	18:1ω?*c*	0.10	0.02	0.01	0.01
15	18:1ω?*c*	0.27	0.03	–	Tr[d]

[a]Peak numbers correspond to numbers in figures.

[b]Rib roast, separable lean.

[c]Average of 2 analyses.

[d]Tr, less than 0.01 g/100 g.

pera (29) on Silar 10 CP. Heckers et al. (30), von Dittmar et al. (31), Grob et al. (32), and Van Vleet and Quinn (33) have reported some success in separating octadecenoate isomers on 60M≤SP2340 capillary columns, but were unable to separate all 4 geometric isomers of 18:2ω6*cc*. None of these authors have used this technique for the quantitation of individual FAME in foods. Earlier, we reported on the suitability of SP2340 glass capillary columns for the quantitation of fatty acids in foods (34). In the present paper, we evaluate the use of SP2340 glass capillary columns for the quantitation of individual *cis* and *trans* monoenes and dienes in foods, and list the *trans* content of a variety of American foods.

EXPERIMENTAL PROCEDURE

Materials

We obtained methyl esters (names followed by code): vaccenate, 18:1ω7*c*; *trans*-vaccenate, 18:1ω7*t*; oleate, 18:1ω9*c*; elaidate, 18:1ω9*t*; petroselinate, 18:1ω12*c*; petroselaidate, 18:2ω-6*cc* from Nu-Chek-Prep, Inc. (Elysian, MN). The geometric isomers of linoleate: 9-*trans*-12-*trans*-octadecadienoate, 18:2ω6*tt*; 9-*cis*-12-*trans*-octadecadienoate, 18:2ω6*ct*; and 9-*trans*-12-*cis*-octadecadienoate, 18:2ω6*tc*; were a gift from Dr. J.D. Stuart, University of Connecticut, Storrs, CT.

Methods

A schematic of the experimental procedure used for analyzing the *cis* and *trans* octadecenoates in foods is given in Figure 1. Lipids were first extracted from foods using a modified chloroform/methanol procedure of Folch et al. (35). Fatty acids were methylated by a modified (34) BF_3-methanol procedure of Metcalfe et al. (36). About 6 mg of both 17:1ω7*c* and 17:1ω7*t* were added as internal standards to 100 mg of FAME. Part of this sample was taken for direct GCGC analysis. For all gas chromatographic work, we used a gas chromatograph, Hewlett-Packard Model 5840 (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector, an automatic liquid sampler, Hewlett-Packard Model 7671A, and an all-glass inlet splitter (Supelco, Inc. Bellefonte, PA). All SP2340 glass capillary columns were purchased from Quadrex Corp. (New Haven, CT). The gas chromatographic conditions were as follows: injection port and FID temperatures, 280 C; He flow 1 ml/min, split ratio 1/100. The column was temperature-programmed from 150 to 200 C at 0.4 C/min.

After part of the sample was taken for direct GCGC, the remainder was subjected to preparatory argentation TLC that separated the FAME into *cis* monoene and *trans* monoene fractions (37). Silica Gel G plates coated with 15% silver nitrate (Supelco, Inc.) were used for the separation. The plates were developed with a mixture of petroleum ether/diethyl ether (9:1) and the bands visualized with 0.2% dichlorofluorescein (37). The *cis* and *trans* fractions were scraped from the plates, dissolved in chloroform, centrifuged to remove the

TABLE II

Comparison of the *trans*-Octadecenoates Content of Four Foods, with and without Argentation TLC, on both a per Food and Fat Basis[a]

Sample	Fat content (%)	*trans* Content			
		Percentage of Food		Percentage of Fat	
		GCGC	Argentation-TLC and GCGC	GCGC	Argentation-TLC and GCGC
Crisco shortening	100.0	10.80	11.85	10.80	11.85
Nabisco zweiback	9.58	0.84	0.92	8.77	9.60
Burger Chef cheeseburger	15.51	0.47	0.56	3.03	3.61
Beef[b]	5.46	0.23	0.24	4.21	4.49

[a]Average of 2 analyses.

[b]Rib roast, separable lean.

precipitate, concentrated by evaporation, and analyzed by GCGC. Total *trans* contents of shortening samples determined by GCGC were compared to *trans* contents determined by the IR procedure of Allen (13).

RESULTS AND DISCUSSION

Chromatograms of the octadecenoate isomers of a Crisco sample before and after argentation TLC are shown in Figure 2. No *cis* octadecenoates overlap into the *trans* region; however, there are 2 *trans* peaks in the *cis* region, one under the 18:1ω9*c*, peak 7, and one under the 18:1ω7*c*, peak 8. The *cis* and *trans* TLC fractions were concentrated about 10-fold in order to detect isomers present in only trace quantities. Retention data of standards identified the peak as follows: peak 2, 18:1ω12*t*; peak 3, 18:1ω9*t*; peak 4, 18:1ω?*t*; peak 5, 18:1ω7*t*; and peaks 6, 7 and 8 were 18:1 *trans* isomers with ω numbers probably less than 7. *Cis* isomers with ω numbers higher than 9 would overlap into the *trans* region, but none was found. Analysis of the *cis* and *trans* octadecenoates determined by argentation TLC followed by SP2340 GCGC for Crisco and 3 food samples are reported in Table I. All samples are qualitatively similar but quantitatively different. Data for the total *trans* octadecenoates for these 4 samples analyzed by argentation TLC followed by GCGC and by GCGC alone appear in Table II. The agreement between direct GCGC (GCGC without argentation TLC) was for Crisco 91, for zweiback 91, for the cheeseburger 84 and for the beef 96% of that obtained when argentation TLC was used. Part of this difference can be accounted for by the overlap of the *trans* peaks 7 and 8 into the *cis* region and the incomplete resolution of peak 6 from the *cis* isomers.

Baseline separation of the standards of the geometric isomers of linoleate; *trans-trans*, *cis-trans trans-cis* and *cis-cis*, peaks 17, 18, 19 and 20 ad shown in Figure 3A was possible with the 100-m SP2340 glass capillary column using the same GCGC conditions as described for the separation of the 18:1 isomers. Sepa-

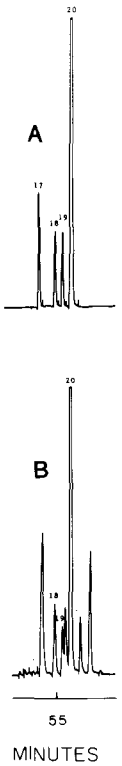

FIG. 3. Separation of the geometric isomers of linoleate. Column: SP2340, 100 m x 0.25 mm, glass; for conditions, see Methods. (A) reference standards; (B) Crisco shortening. Peak identities: 17. 18:2ω6*tt*; 18. 18:2ω6*ct*; 19. 18:2ω6*tc*; 20. 18:2ω6*cc*.

TABLE III

Geometric Isomers of Linoleate In Foods and Shortenings

Sample	Geometric isomers of 9,12-octadecadienoate (g/100 g of food)			
	trans-trans	*cis-trans*	*trans-cis*	*cis-cis*
A&P Dexo shortening	0.46	2.13	2.39	7.87
Crisco shortening	0.20	1.38	1.54	20.88
Giant shortening	0.21	1.68	1.97	22.50
Golden Fluffo shortening	0.08	0.61	0.72	12.95
Grand Union shortening	0.12	1.40	1.59	25.67
Beef	Tr	0.02	0.04	0.38
Burger King apple pie	0.04	0.20	0.21	1.61
Burger Chef cheeseburger	0.03	0.05	0.04	0.47
Gerber animal crackers	0.04	0.15	0.13	0.82
Nabisco zweiback	0.01	0.09	0.09	1.15

ration of the 18:2 fraction of Crisco from argentation TLC by GCGC, however, gave 7 peaks (Figure 3B). The first peak separated with approximately the same retention ratio as *trans-trans* 18:2ω6. In order to separate the geometric isomers of linoleate and be sure of their quantitation, all foods and shortenings listed in Table III were analyzed on both a 115-m SP2340 capillary column at 195 C and a 100-m OV-275 capillary column at 185 C.

The total *trans* unsaturated fatty acids in foods was estimated by adding the 18:1 and 18:2 *trans* unsaturated fatty acids. Because we had found the *trans-trans* 18:2ω6 content of foods and shortening to be very small, we chromatographed all samples listed in Tables IV and V at 150 C at 4 C/min to 200 C and ignored the contribution of *trans-trans* 18:2ω6 in our estimate of total *trans* content. The grams of fatty acids with *trans* unsaturation (monoene plus diene) for some shortenings are given in Table IV and for some fast foods in Table V. The data in Tables IV and V are calculated as g of *trans*-unsaturated fatty acid (as triglyceride)/100 g of food. Dividing this value by the percentage fat in the sample and multiplying by 100 gives the percentage of *trans*-unsaturated fatty acids in fat. Scholfield et al. (38) reported ca. 20% *trans* unsaturation in 5 shortenings and Kummerow (39) reported 20-30% *trans* in U.S. shortenings. Except for 2 vegetable shortenings, the values in Table IV were considerably less than 20%.

The analysis of fatty acids on a 100-m SP2340 column took ca. 100 min. In order to shorten this time, we tried the analysis on 60-, 20- and 10-m SP2340 columns. The 20-m and 10-m columns did not separate individual *trans*-octadecenoates, but gave one *trans*-octadecenoate peak preceding either 1 or 2 *cis* octadecenoate peaks. In an earlier paper (40), we reported fairly good quantitative agreement between determination of *trans* fatty acids on 10-m and 100-m columns for a number of foods which did not contain hydrogenated fats, but the shorter columns were inadequate for separating the individual isomers of 18:1 and of linoleate. A 60-m column was found satisfactory and reduced the analysis time

TABLE IV

trans Fatty Acid Content of Shortenings

Name	Type of fat	*trans* Fatty acids (g/100 g of food)
Crisco	Soybean oil	12.59
A&P Dexo	Vegetable oil	23.17
Giant	Vegetable oil	9.93
Golden Fluffo	Vegetable oil	8.02
Grand Union	Vegetable oil	9.12
Nu-Made	Corn oil	23.91
Swift'ning	Meat fats & vegetable oils	3.68
Velkay	Meat fats & vegetable oils	6.57
White Beauty	Meat fats & vegetable oils	2.77

from 100 min to ca. 60 min and still separated the first 5 *trans* 18:1 isomers and linoleate from its geometric isomers. The *trans* contents of 6 shortening samples determined gy GCGC on a 60-m SP2340 column and by IR are given in Table VI. IR analysis for the *trans* unsaturation was done by Allen's method (13) which measures the ratio of absorbance at 10.6 μm for *trans* unsaturation to absorbance at 8.5-8.6 μm for methyl esters, compared to that of methyl

TABLE V

trans Fatty Acids in Fast Foods

Item[a]	Company[b]	*cis-trans* 9,12-Octadecadienoate and *trans-cis* 9,12-octadecadienoate (g/100 g food)	*trans* Fatty acids[c] (g/100 g food)	Fat (%)
Hamburger	BC	0.01	0.39	13.99
Hamburger	BK	0.01	0.57	14.40
Hamburger	MD	Tr[d]	0.38	11.08
Big Shef	BC	0.06	0.61	16.85
Super Shef	BC	0.05	0.50	16.03
Double beef hamburger	BK	0.09	0.70	17.38
Whopper	BK	0.04	0.47	15.66
Whopper, Jr.	BK	0.03	0.47	15.35
Double Beef Whopper	BK	0.09	0.63	17.67
Quarter Pounder	MD	0.03	0.79	15.45
Cheeseburger	BC	0.06	0.45	15.52
Cheeseburger	BK	0.07	0.60	16.61
Cheeseburger	MD	0.02	0.69	14.92
Double cheeseburger	BC	0.04	0.66	19.90
Double beef cheeseburger	BK	0.05	0.56	18.66
Whopper w/cheese	BK	0.01	0.45	18.47
Double Beef Whopper w/cheese	BK	0.07	0.63	19.46
Big Mac	MD	0.14	0.55	13.51
Quarter Pounder w/cheese	MD	0.03	0.68	18.26
Skippers Treat	BC	0.03	0.35	12.58
Whaler	BK	0.12	0.43	14.00
Whaler w/cheese	BK	0.06	0.42	16.60
Filet-O-Fish	MD	0.06	0.22	18.26
Yumbo	BK	0.02	0.19	13.11
Rancher	BC	0.08	0.69	20.38
Mariner	BC	0.01	0.76	18.57
Scrambled eggs	MD	Tr	0.21	19.90
Hot cakes w/butter	MD	Tr	0.52	6.83
English muffin w/butter	MD	0.02	0.19	10.03
Egg McMuffin	MD	Tr	0.08	11.57
Sausage sandwich	MD	Tr	0.04	16.80
Apple pie	BK	0.20	0.68	13.91
Apple pie	MD	0.06	1.06	18.99
Apple turnover	BC	0.25	1.75	15.70
Lemon turnover	BC	0.17	2.02	17.20
McDonaldland cookies	MD	0.06	0.49	14.70
Vanilla shake	BC	Tr	0.08	3.86
Vanilla shake	BK	Tr	0.06	3.30
Vanilla shake	MD	0.01	0.07	3.13
Chocolate shake	BC	Tr	0.10	3.89
Chocolate shake	BK	Tr	0.06	3.73
Chocolate shake	MD	Tr	0.05	3.10
Strawberry shake	BC	Tr	0.06	3.20
Strawberry shake	BK	Tr	0.05	2.83
Strawberry shake	MD	Tr	0.07	3.44
French fries	BC	Tr	0.52	18.18
French fries	BK	0.05	0.56	16.82
French fries	MD	Tr	0.72	19.22
Onion rings	BK	0.30	1.26	16.68

[a]All foods analyzed as purchased for direct consumption except condiments were left off.

[b]BC = Burger Chef; MD = McDonald's; BK = Burger King.

[c]*trans*-Octadecenoates plus *cis-trans* 9,12-octadecadienoate and *trans-cis* 9,12-octadecadienoate.

[d]Tr, less than 0.01 g/100 g food.

TABLE VI

trans Content of Selected Shortenings Determined by GCGC and IR

Sample	Lot	Gas chromatography: Octadecenoate 18:1ωX*trans* (g/100 g)	Gas chromatography: Octadecadienoate 18:2ω6*trans* (g/100 g)	Gas chromatography: Total *trans* fatty acids (g/100 g)	IR: *trans* unsaturation (%)
Crisco	A	10.72 ± .07	1.41 ± .04	12.84 ± .09	12.34 ± .45
Crisco	B	11.86 ± .05	1.10 ± .01	13.70 ± .05	12.87 ± 1.79
Giant	A	7.65 ± .38	0.86 ± .04	8.93 ± .40	8.02 ± .24
Giant	B	8.78 ± .05	1.37 ± .04	10.84 ± .06	9.68 ± .20
Golden Fluffo	A	9.58 ± .07	1.30 ± .02	11.27 ± .17	9.69 ± .41
Golden Fluffo	B	9.65 ± .05	1.10 ± .12	11.24 ± .11	9.04 ± .36

N = 3 for each lot except Crisco lot A, where N = 2. GCGC 60 m SP2340 column.

elaidate. This procedure, like other IR procedures, does not take into account differences in *trans* absorbance for fatty acids of different chain length; in addition, mono-*trans* dienes have only an 85% as great absorption as elaidate and *trans-trans* dienes ca. 166% (41). Therefore, data from the GCGC and IR methods can be compared only approximately. The percentage of *trans* unsaturation in shortenings found by IR compared to GCGC ranged from 90-80% in our study. Perkins et al. analyzed the *trans* unsaturated fatty acid content of 12 margarines by 20-ft OV-275 packed columns and IR and found a comparison of 144-90%. While neither the 60-m nor 100-m SP2340 columns completely separate all *cis* and *trans* isomers, both columns appear adequate for most quantitative analyses of *trans* unsaturated fatty acids in foods. Although there is some overlap of *trans* unsaturated monoene fatty acids into the *cis* unsaturated region, a more complete separation probably is not possible using only single column gas chromatography.

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Chromatographic Separation of the Stereoisomers of α-Tocopherol

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ABSTRACT

The diastereoisomers of 2-*ambo*-α-tocopherol were completely separated as TMS ethers by gas chromatography on a 115 m × 0.25 mm glass capillary column coated with SP2340, at a column temperature of 195 C. In the same way, *all-rac*-α-tocopherol was separated into four peaks, corresponding to the four racemates present, and having the same retention ratios as the four diastereoisomers of 4′-*ambo*-8′-*ambo*-α-tocopherol (produced by the hydrogenation of natural α-tocotrienol). Retention data and relative peak areas for the diastereoisomers of the synthesized α-tocopherols and several commercial products were determined. Limited data on the isomers of other tocopherols also are reported.

INTRODUCTION

Gas chromatographic separations of the tocopherols have usually been made on nonpolar phases at fairly high temperatures. Stereoisomers are not separated, even on highly efficient capillary columns. We recently found that tocopherol TMS ethers may be successfully chromatographed on glass capillary columns coated with the highly polar phase SP2340, with the separation or partial separation of diastereoisomers. We have used this technique to examine the isomers in a number of synthetic α-tocopherols in an effort to answer some of the questions regarding their stereoisomeric composition, and to distinguish and determine the relative amounts of synthetic and natural tocopherols in mixtures.

The tocopherols, with three chiral carbon atoms at carbons 2, 4′ and 8′, have eight possible stereoisomers. The forms of α-tocopherol that are discussed here are given in Table I with their approved names (1). The native forms of all the tocopherols are optically pure and exist only in the RRR configuration, whereas the synthetic tocopherols are mixtures of stereoisomers. The condensation of trimethylhydroquinone with natural phytol produces 2-*ambo*-α-tocopherol (formerly named dl-α-tocopherol) that is a mixture of RRR and SRR diastereoisomers. The acetate of 2-*ambo*-α-tocopherol was formerly the International Standard for vitamin E, used to define the now discontinued International Unit of vitamin E activity. The ratio of the two diastereoisomers was initially thought to be equal, but more recently, the possibility of departures from equal distribution has been considered, and the current IUPAC-IUB Tentative Rules and Recommendations for the Nomenclature of

TABLE I

α-Tocopherols Chromatographed

Trivial name (1)	Description	Configuration
RRR-α-tocopherol (or[d]-α-Tocopherol)	Natural α-tocopherol	(1) 2R, 4′R, 8′R
2-*ambo*-α-Tocopherol	Mixture of 2 isomers formed by synthesis using natural phytol	(1) 2R, 4′R, 8′R (2) 2S, 4′R, 8′R
all-rac-α-Tocopherol	Mixture of 8 isomers produced by total synthesis using synthetic phytol or synthetic isophytol	(1) 2R, 4′R, 8′R (2) 2S, 4′S, 8′S (3) 2R, 4′R, 8′S (4) 2S, 4′S, 8′R (5) 2R, 4′S, 8′R (6) 2S, 4′R, 8′S (7) 2R, 4′S, 8′S (8) 2S, 4′R, 8′R
4′-*ambo*-8′-*ambo*-α-Tocopherol	Mixture of 4 isomers produced by hydrogenation of natural α-tocotrienol	(1) 2R, 4′R, 8′R (2) 2R, 4′R, 8′S (3) 2R, 4′S, 8′R (4) 2R, 4′S, 8′S

Tocopherols and Related Compounds states "the ratio may vary from one preparation to the next depending on the reaction conditions" (1). This possibility of variable amounts of the two isomers in different preparations is important because the isomers have different vitamin E activities; the activity of SRR-α-tocopherol (2-*epi*-α-tocopherol) acetate has been found to vary from 14 to 26% of that of RRR-α-tocopherol acetate, depending on the assay used (2). Random variations in the ratios of the isomers in this reference standard are clearly undesirable.

The total synthesis of α-tocopherol results in *all-rac*-α-tocopherol (formerly named dl-α-tocopherol), the common form of synthetic vitamin E used for food and feed supplementation. This synthetic tocopherol is now used as a reference standard, and is the form of the USP ALPHA-TOCOPHEROL RS used to define the USP Unit of Vitamin E activity (3). It consists of four racemates whose relative amounts have been thought to vary; the IUPAC Tentative Rules of Nomenclature defines *all-rac*-α-tocopherol as "a mixture of four racemates in unspecified proportions" (1).

The α-tocopherol isomers formed by the hydrogenation of α-tocotrienol consist of four diastereoisomers each with the R-configuration at carbon 2. This form has been designated 4′-*ambo*-8′-*ambo*-α-tocopherol (1).

No analytical separation of the stereoisomers of α-tocopherol has been reported. The only available method for distinguishing RRR-α-tocopherol and *all-rac*-α-tocopherol is based on the difference in the optical rotations of the products formed by oxidation with potassium ferricyanide (4).

MATERIALS AND METHODS

Tocopherols were either bought on the open market, received as gifts (Hoffman-LaRoche, Inc., Nutley, NJ; U.S. Pharmacopoeial Convention, Rockville, MD), or synthesized in our laboratory.

Tocopherol Synthesis

2-*ambo*-α-Tocopherol was synthesized by heating trimethylhydroquinone (Aldrich Chemical Company, Inc., Milwaukee, WI) with natural phytol in formic acid, as described by Karrer and Fritzsche (5). The *all-rac*-α-tocopherols were made by using synthetic phytol or isophytol in the reaction instead of natural phytol. The 5,7-dimethyltocols were made by heating *m*-xylohydroquinone (Fairfield Chemical Co., Blythwood, SC) either with natural phytol to form 2-*ambo*-5,7-dimethyltocol or with synthetic phytol to form *all-rac*-5,7-dimethyltocol (5). A mixture of 2-*ambo*-monomethyltocols was made by condensing natural phytol with methylhydroquinone. The 4′-*ambo*-8′-*ambo*-α-tocopherol used was formed by hydrogenating natural α-tocotrienol (a gift from Hoffman-LaRoche, Inc., Nutley, NJ) in ethanol under ca. 8 psig H_2 with PtO_2 catalyst for 6 hr. No α-tocotrienol remained, as shown by gas chromatography.

Derivatization

The TMS ethers were prepared by adding 0.5 ml of Regisil (Regis Chemical, Morton Grove, IL) to 10 mg or less of tocopherol, allowing it to stand for 30 min, dissolving the mixture in isooctane and washing 6× with distilled water to remove the excess reagent. The isooctane solution was dried over anhyd. Na_2SO_4, then solvent was removed with a stream of dry nitrogen. The TMS ethers were redissolved in isooctane to give solutions containing ca. 2 mg/ml.

Gas Chromatography

Gas liquid chromatography (GLC) separations were made on a 115 m × 0.25 mm glass capillary column coated with SP2340 (Quadrex Corporation, New Haven, CT), with a capacity factor of 13.9 for RRR-α-tocopherol TMS ether at 195 C. The chromatograph was a Hewlett-Packard Model 5840, equipped with a flame ionization detector, an automatic liquid sampler (Model 7671A), and an all-glass J&W splitter (J&W Scientific, Inc., Orangevale, CA). The chromatographic conditions were: attn. 0; split ratio, 1/50 to 1/100; sample size, ca. 1.7 μ of a solution containing ca. 2 mg/ml; injection port temperature, 280 C; column temperature, 195 C; detector temperature, 300 C; carrier gas, H_2 at 20 to 24 psig; avg. linear velocity, 19 cm/sec. The oven was maintained under a positive pressure of nitrogen and the split flow was vented out the window to minimize the danger from the use of hydrogen as the carrier gas. Nitrogen was introduced at the end of the column as the auxilliary gas at a flow rate 1.4 times that of the hydrogen flow to the detector. Peak areas were determined by integrating 0.02-min slices under the curve and summing the relevant portions to obtain peak areas.

RESULTS AND DISCUSSION

In the separation of the tocopherol stereoisomers by capillary gas chromatography, separation efficiency and peak shape were unusually sensitive to sample size. The sample sizes used were a compromise between the

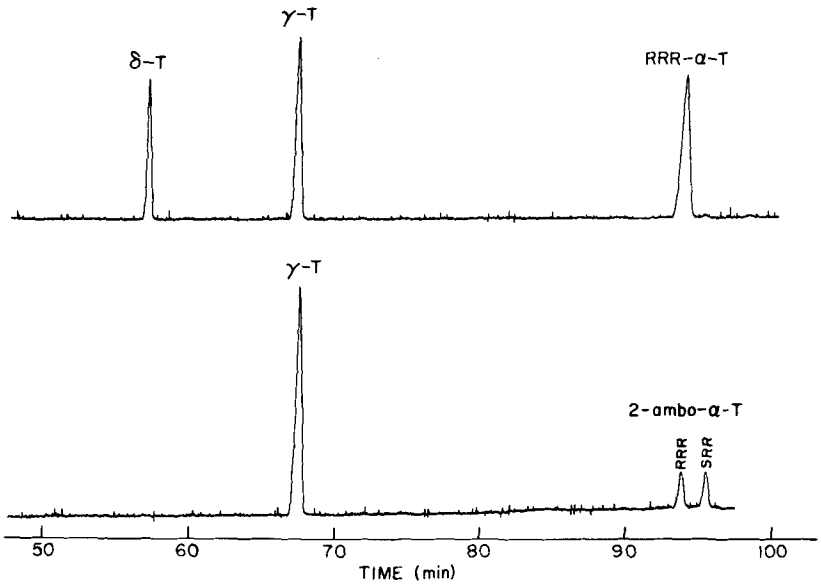

FIG. 1. Separation of 2-*ambo*-α-tocopherol TMS ethers compared with RRR-α-tocopherol on SP2340. For chromatographic conditions, see Materials and Methods.

amounts needed for adequate area measurements and those needed for good separation. A column temperature of 195 C was found optimal for our system and performance deteriorated at higher or lower temperatures.

The separation of the isomers of 2-*ambo*-α-tocopherol is shown in Figure 1, and compared with the single peak for RRR-α-tocopherol. γ-Tocopherol was included in each sample as a reference. The RRR and SRR diastereoisomers were completely separated and gave symmetrical, approximately equal peaks. Repeated injections (N = 13) gave area percent distributions of 49.7 +/-1.28% for the RRR isomer and 50.3+/-1.28% for the SRR isomer. In this one preparation, then, the amounts of the two isomers were equal. It is still possible, of course, that other preparations might contain unequal amounts of the two isomers. Further evidence for an even distribution of isomers in this form was given by Ames (6), who also reported approximately equal amounts of the isomers on the basis of measurements of optical rotations. We tried to obtain samples of the former International Standard for vitamin E to measure its isomer distribution, but none is available.

The 4′-*ambo*-8′-*ambo*-α-tocopherol, prepared by hydrogenation of natural α-tocotrienol [2,5,7,8-tetramethyl-2- (4′, 8′, 12′-trimethyltrideca-3′, 7′, 11′-trienyl) chroman-6-ol], consists of only the four diastereoisomers with the R configuration at the 2-position: RRR, RSR, RRS, and RSS. Figure 2a is a typical chromatogram of this tocopherol. Four peaks were obtained for the four diastereoisomers present. When RRR-α-tocopherol was mixed with this preparation the second peak was increased (Fig. 2b). When 2-*ambo*-α-tocopherol was mixed with the preparation, peaks 2 and 4 were increased (Fig. 2c). Therefore, peak 2 in the chromatogram of 4′-*ambo*-8′-*ambo*-α-tocopherol was the RRR isomer and peak 4 was the RSS isomer. Peaks 1 and 3 were the RSR and RRS isomers, but no evidence is available for assigning a specific identity to either peak.

The *all-rac*-α-tocopherols also gave four peaks, corresponding to the four racemates; Figure 3 is a typical chromatogram. The peaks follow the same pattern and have the same retentions relative to γ-tocopherol as those of 4′-*ambo*-8′-*ambo*-α-tocopherol. The second peak must, therefore, be the racemate consisting of the RRR and SSS enantiomers, and the fourth peak the racemate consisting of the SRR and RSS enantiomers. The other two peaks are the RRS + SSR and RSR + SRS racemates, but specific identities cannot be assigned.

Both peak height and peak width varied among the four peaks for *all-rac*-α-tocopherol and 4′-*ambo*-8′-*ambo*-α-tocopherol (Fig. 2 and 3). Peak 1 was consistently the broadest and peaks 2 and 4 were consistently narrower than the other two. Because this was true not only

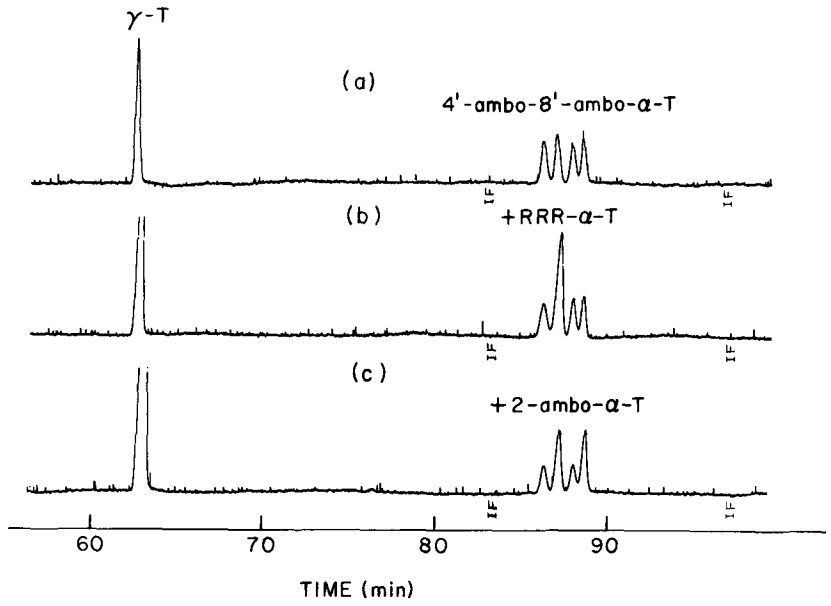

FIG. 2. Separation of 4'-*ambo*-8'-*ambo*-α-tocopherol TMS ethers (a) alone, (b) mixed with RRR-α-tocopherol, and (c) mixed with 2-*ambo*-α-tocopherol. For chromatographic conditions, see Materials and Methods.

for the composite peaks of racemates in the *all-rac*-α-tocopherol, but also for the peaks of the 4'-*ambo*-8'-*ambo*-α-tocopherol, this variation must be due to some intrinsic molecular property and not to the partial separation of enantiomers. The molecular structures of the isomers differ in the configurations at the chiral atoms in the side chain: for peaks 1 and 3, the broader pair, the two chiral atoms have different configurations; for peaks 2 and 4, the pair with the narrower widths, both chiral atoms have the same configuration.

The distribution of isomers in *all-rac*-α-tocopherol and the effect of preparation were evaluated by chromatographing several preparations and measuring the areas of the peaks by the area slice method. The samples examined included two commercial preparations, a sample of the USP ALPHA-TOCOPHEROL RS, a vitamin E capsule, and two *all-rac*-α-tocopherols synthesized in our laboratory. We also measured peak areas for the 4'-*ambo*-8'-*ambo*-α-tocopherol preparation. We have information only on the methods used in our laboratory, but we assume that there are differences in the methods used to make commercial tocopherols,

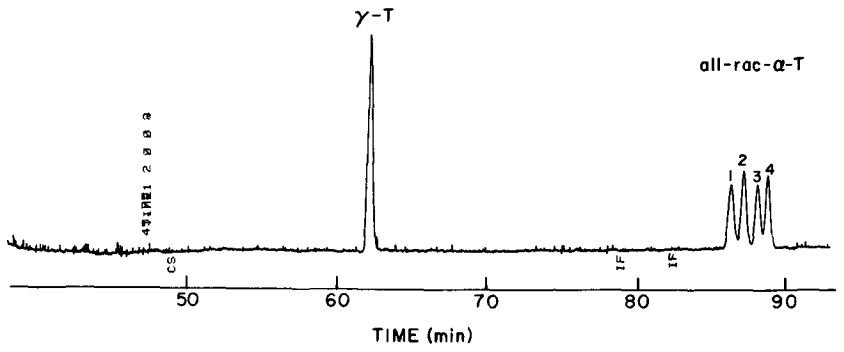

FIG. 3. Separation of *all-rac*-α-tocopherol TMS ethers on SP2340. For chromatographic conditions, see Materials and Methods. 1. RRS + SSR or RSR + SRS; 2. RRR + SSS; 3. RRS + SSR or RSR + SRS; 4. RSS + SRR.

TABLE II

Peak Area Distribution Found for Different α-Tocopherol Preparations (%)

Sample identity	N	Peak number[a] 1 Mean	1 SD	2 Mean	2 SD	3 Mean	3 SD	4 Mean	4 SD
all-rac-α-Tocopherol: commercial sample 1	5	22.9	0.56	25.6	0.36	25.2	0.39	26.3	0.61
all-rac-α-Tocopherol: commercial sample 2	3	22.7	1.08	24.3	1.22	25.9	0.64	27.1	1.55
all-rac-α-Tocopherol: USP reference	4	22.7	0.78	24.4	0.24	26.3	0.31	26.7	0.76
all-rac-α-Tocopherol: vitamin E capsule	4	23.0	0.84	24.6	0.15	26.2	0.15	26.2	0.83
all-rac-α-Tocopherol: made with synthetic phytol	6	22.9	1.43	24.8	0.39	25.7	0.55	27.6	1.16
all-rac-α-Tocopherol: made with synthetic isophytol	8	25.7	2.82	23.9	0.91	25.7	1.21	24.8	1.90
4′-*ambo*-8′-*ambo*-α-Tocopherol: hydrogenated α-tocotrienol	7	25.4	0.44	27.8	0.44	23.0	0.49	23.7	0.22
2-*ambo*-α-Tocopherol: made with natural phytol	13			49.7	1.28			50.3	1.28

[a]Peak identities: 1—RSR + SRS or RRS + SSR; 2—RRR + SSS; 3—RSR + SRS or RRS + SSR; 4—RSS + SRR.

that these differ from the methods we used, and that the preparations examined were representative of α-tocopherols as a whole.

The area distributions obtained are given in Table II. The areas of the four peaks were approximately equal in all preparations, but there were small, consistent differences both among preparations and among peaks. In the two commercial products, the USP sample and the vitamin E capsule, the first peak had the smallest area and the last peak the largest area. If the areas of these peaks are averaged, the peak areas increased in the order of their elution and were, respectively, 22.8, 24.8, 25.8 and 26.5%. The order for the product we synthesized from synthetic phytol was the same, but for the product we prepared from isophytol, peak 2 was the smallest. The peak area distribution was unique for 4′-*ambo*-8′-*ambo*-α-tocopherol; peak 3 had the smallest area and peak 2 the largest. The fact that our peak area measurements for the different preparations were reproducible suggests that the distribution of isomers actually did differ slightly among these tocopherols. But, because the differences were small, the peak areas measured were small and the chromatography was difficult, the results should be confirmed, perhaps in several laboratories, before conclusions are drawn regarding tocopherols in general.

Because the evidence suggested that the differences in isomer distribution are small, we evaluated the possibility that the separation might be used to estimate the amounts of natural and synthetic tocopherols in mixtures. Supplemented foods and feeds commonly

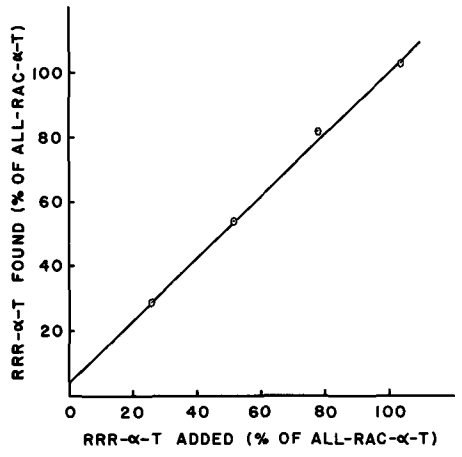

FIG. 4. Correlation of known and found percentages of RRR-α-tocopherol in mixtures with *all-rac*-α-tocopherol.

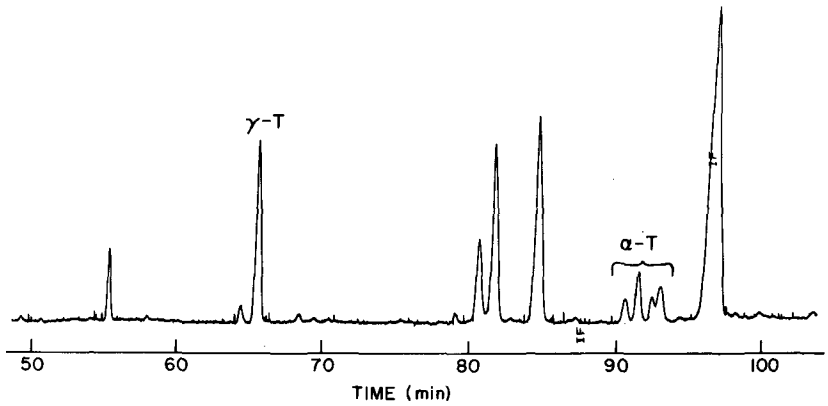

FIG. 5. Separation of the TMS ethers of the unsaponifiable fraction of a baby food formula containing soybean oil and fortified with *all-rac*-α-tocopherol.

contain both tocopherol forms, and their relative contributions to the vitamin E content could be important.

We prepared known mixtures in which natural RRR-α-tocopherol was 25, 50, 75 and 100% of the amount of *all-rac*-α-tocopherol. These were derivatized and chromatographed and the areas of the α-tocopherol peaks measured by the area slice method. We assumed that any increase in peak 2 over that expected from the average of the *all-rac*-α-tocopherols would be due to the added RRR-α-tocopherol. The areas of peaks 3 and 4 were used to estimate the contribution of the *all-rac*-α-tocopherol to the observed area of peak 2. The remaining area of peak 2 was divided by the total area of the *all-rac*-α-tocopherol, multiplied by 100 and plotted against the known weight percentages. Correlation was high between known and calculated compositions (Fig. 4). When an infant formula that contained, according to its label, both supplemented *all-rac*-α-tocopherol and soybean oil (and therefore RRR-α-tocopherol) was extracted, saponified, and the TMS ethers of the total unsaponifiable fraction chromatographed (Fig. 5), the pattern of the peaks in the α-tocopherol region was not that expected, probably because of interference from other peaks. The results suggested that successful analysis would require a preliminary purification of the unsaponifiable fraction.

In addition to the work with α-tocopherol, some mono- and dimethyltocols were prepared and chromatographed. 5,7-Dimethyltocol, which we use as an internal standard in the gas chromatographic estimation of tocopherols, was prepared with both natural and synthetic phytols. The product formed from natural phytol, 2-*ambo*-5,7-dimethyltocol, gave two partially separated peaks; that from synthetic phytol gave three (Fig. 6), although 4 racemates were present. Both 5,7-dimethyltocols overlapped the δ-tocopherol region on the chromatogram. The mixture of monomethyltocols produced by condensing monomethylhydroquinone with natural phytol gave three pairs of diastereoisomers (Fig. 7). The δ-tocopherol pair was well-separated, but those of 7-methyltocol and 5-methyltocol (tentatively identified by analogy to their elution order on nonpolar phases) were only partially separated. The chromatographic behavior of synthetic non-α-tocopherols may become of interest if, as has been suggested, they are used as reference standards to replace the scarce natural products.

The retention ratios of all the tocopherol

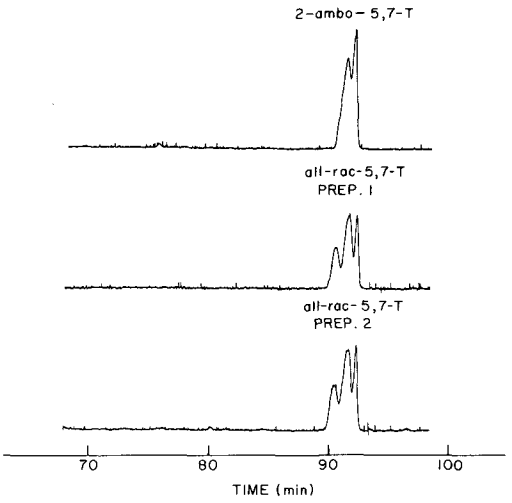

FIG. 6. Separation of 5,7-dimethyltocol TMS ethers on SP2340. For chromatographic conditions, see Materials and Methods.

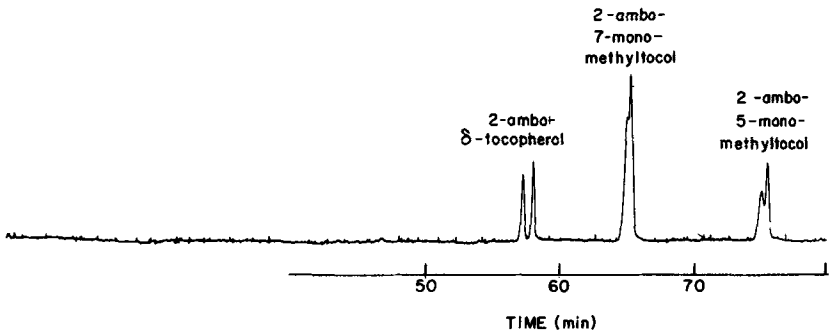

FIG. 7. Separation of monomethyltocol TMS ethers on SP2340. For chromatographic conditions, see Materials and Methods.

forms investigated are summarized in Table III. Those for the α-tocopherol forms are given relative not only to RRR-γ-tocopherol but also to RRR-α-tocopherol. The latter are more consistent, and are helpful in the confirmation of the identities of the 2-*ambo*-α-tocopherol isomers, whose retention ratios relative to γ-tocopherol did not coincide with their proper identities, perhaps because of the distorted peak shape of the γ-tocopherol in these chromatograms.

Separations of the stereoisomers were also achieved on a 100 m x 0.25 mm glass column coated with OV275. However, the results were not quite as good as those on the SP2340 column, and are not reported here.

This capillary gas chromatographic technique has been demonstrated as useful for evaluating the distribution of diastereoisomers of synthetic tocopherols, for distinguishing

TABLE III

Relative Retention Ratios of Tocopherol Isomers on SP2340 at 195 C

Identity	Relative retention ratio	
	γ-T = 1.000	RRR-α-T = 1.000
RRR-α-T	1.469	1.000
2-*ambo*-α-T		
Peak no. 1 (RRR)	1.456	1.000
Peak no. 2 (SRR)	1.485	1.020
4'-*ambo*-8'-*ambo*-α-T		
Peak no. 1	1.450	0.988
2 (RRR)	1.467	1.000
3	1.484	1.012
4 (RSS)	1.497	1.020
all-rac-α-T		
Peak no. 1	1.450	0.990
2 (RRR + SSS)	1.465	1.000
3	1.483	1.012
4 (RSS + SRR)	1.495	1.020
2-*ambo*-5,7-T		
Peak no. 1	1.467	
Peak no. 2	1.478	
all-rac-5,7-T		
Peak no. 1	1.424	
2	1.451	
3	1.463	
Monomethyltocols		
RRR-δ-T	0.818	
SRR-δ-T	0.831	
RRR-7-T[a]	0.955	
SRR-7-T[a]	0.960	
RRR-5-T[a]	1.128	
SRR-5-T[a]	1.136	

[a]Tentative identity.

natural from synthetic tocopherols, and for estimating the relative amounts of each in mixtures. Diastereoisomers of other relatively nonpolar compounds may also be separated in the same way. For example, we have successfully separated sterol diastereoisomers epimeric at C-24 under essentially the same conditions (7).

ACKNOWLEDGMENT

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COMMUNICATIONS

The Relationship between Dietary Phytosterols and the Sterols of Wild and Cultivated Oysters

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ABSTRACT

Wild oysters *(Crassostrea virginica)* contained cholesterol, 24-methyl-cholesta-5, 22-dienol, 24-methylenecholesterol, 22-dehydrocholesterol, 24-methylcholesterol, 24-ethylcholesterol, 24-norcholesta-5, 22-dienol, 24-ethylcholesta-5, 22-dienol and fucosterol. The same species was cultivated on a defined diet of *Thalassiosira pseudonana* and *Isochrysis* sp. The dietary algae were cultured and their sterol compositions were analyzed by gas chromatography and mass spectroscopy. *T. pseudonana* and *Isochrysis* sp. had 24-methylenecholesterol and 24-methyl-cholesta-5, 22-dienol as their major sterols. The sterol composition of the cultivated oysters revealed the predominance of cholesterol (19%), 24-methyl-cholesta-5, 22-dienol (21%) and 24-methylenecholesterol (46%). Therefore, oysters must be able to bioconvert phytosterols to cholesterol, concentrate dietary cholesterol, or synthesize cholesterol de novo.

INTRODUCTION

Anisomyarian bivalves contain complex sterol mixtures. It is likely that many of the sterols observed in these bivalves are dietary. The contribution from de novo sterol synthesis has been investigated, but in the oyster, the results are contradictory. Some authors contend that no sterol synthesis can be detected from radioactive precursors (1, 2). Conversely, several authors have observed some sterol synthesis, but in amounts which are inadequate for fulfilling the sterol demand of the oyster (3).

The ability of bivalves to metabolize sterols has been demonstrated in *Mytilus edulis* and *Ostrea edulis* (4,5). In *M. edulis*, desmosterol was bioconverted to cholesterol, 22-dehydrocholesterol and 24-methylenecholesterol. It was suggested that desmosterol was an intermediate in sterol biosynthesis. The conversion of fucosterol to cholesterol via desmosterol in the oyster supports this hypothesis (5).

This study was designed to investigate the relationship between the sterol content of the oyster and of its diet. The sterols observed in oysters that were reared on a defined diet were compared to those of the dietary algae and of wild oysters grown in natural oyster bars.

METHODS AND MATERIALS

Healthy, mature, wild oysters, *Crassostrea virginica,* were taken in the winter of 1979–80 from natural oyster bars in the following geographical sites: Long Island Sound, Cape Hatteras, Chesapeake Bay, Delaware Bay and St. Augustine. One-year-old cultivated oysters (supplied by Dr. Ellis Bolton of the University of Delaware) were hatchery-reared in filtered sea water and maintained on a defined algal diet of *Isochrysis* sp. *(Isochrysis* aff. *galbana)* Green and *Thalassiosira pseudonana* Hasle et Heimdal. Axenic cultures of these algae were provided by Dean Day of the University of Delaware.

These algae were grown in 19-ℓ carboys fitted with a glass bubbling tube surrounded by a cotton stopper. The cultures were constantly bubbled with 2% CO_2 in air. The carboys contained 10 ℓ of a modified Guillard medium (6). The medium was adjusted to pH 7.6 and a salinity of 20 parts per thousand prior to sterilization. The *Isochrysis* sp. cultures were grown at room temperature and illuminated with fluorescent lighting at 9,000 lux. *T. pseudonana* cultures were grown at 18 C with a light intensity of 5,000 lux. The cells were harvested by continuous centrifugation and freeze-dried.

Shucked oysters were ground with acetone in a Waring blender. The slurry was allowed to set for 36 hr and was then filtered. The acetone was removed from the filtrate by flash evaporation. The crude lipid extract was saponified in 20% KOH in 80% aqueous MeOH for 45 min and then partitioned against diethyl ether and water. The ether was removed under reduced

pressure and sterols were obtained by alumina chromatography.

The algal sterols were obtained in a similar manner except the lyophilized algae were extracted in a Soxhlet apparatus with chloroform/methanol (2:1).

Argentation column chromatography was used to separate complex sterol mixtures as their acetates. The column packing was prepared by the Teshima and Kanazawa method (7), and sterol fractions eluted according to Teshima et al. (3). The separated sterol acetates were then saponified for analysis as free sterols on gas liquid chromatography (GLC) and gas chromatography/mass spectrometry (GC/MS).

The sterols were tentatively identified by GLC on a Varian Model 3700 gas chromatograph coupled with a Varian CDS 111 data system. The glass column was 3% SE-30 on 100/120 mesh Gas-Chrom Q, maintained at 246 C. Mass spectral data were obtained on an LKB 9000 GC/mass spectrometer as described previously (3).

RESULTS AND DISCUSSION

Sterols from Wild Oysters

All algal and oyster sterols were identified on the basis of GLC retention times, affinity for silver nitrate on column chromatography and mass spectral data as compared to knowns. Cholesterol was the dominant sterol. The results of the sterol analyses of these oysters are presented in Table I. The same sterols were observed in all groups. However, the oysters from Long Island Sound and Cape Hatteras contained detectable levels of desmosterol and trace amounts of an unidentified sterol, in addition to the other sterols. Desmosterol eluted with fucosterol and 24-methylenecholesterol in argentation chromatography. Its mass spectrum gave a molecular ion at m/e 384 and other ions at m/e 369, 366, 351, 299, 271, 225, 229 and 213, which was in agreement with the spectrum of the authentic compound.

Sterols of Cultivated Oysters

The major sterols observed in these oysters are listed in Table I. The sterol compositions of the 2 algae used in the diet, *Thalassiosira pseudonana* and *Isochrysis* sp., are presented in Table II. The sterols found in the oyster reflected those of its diet. There were some notable exceptions, the most important of which was the increased concentration of cholesterol in the oyster compared to its diet. This may reflect bioconversion of other dietary sterols, de novo synthesis, or selective uptake of cholesterol by the oyster. Two sterols were present in the oyster which were not detectable in the diet. They were 24-norcholesta-5, 22-dienol and 24-ethylcholesta-5, 22-dienol. One phytosterol, 24-methyl-5, 24(25)-cholestadienol, was not detected in the oyster. This sterol apparently either was not assimilated or was metabolized immediately by the oyster. The results from this study support the earlier work of Tamura et al. (8), who reported an increase in the relative percentage of cholesterol in the total sterol content of oysters which had been held on a sterol-free diet for 5 months. They also noted a depletion of the sterol content of the oyster meat, which suggested a dietary requirement of sterols. Several authors have reported conversion of labeled sterols to cholesterol in bivalves (4,5). Therefore, it is possible that conversion accounts for some of the increased cholesterol content observed in

TABLE I

Sterol Compositions[a] of Wild and Cultivated Oysters

Sterol	RRT[b]	Geographic source of wild oysters: Long Island	Cape Hatteras	Delaware Bay	Chesapeake Bay	St. Augustine	Cultivated
24-Norcholesta-5, 22-dienol	.65	5	5	4	7	3	1
22-Dehydrocholesterol	.91	7	8	7	12	8	trace
Cholesterol	1.00	32	32	33	30	36	20
Desmosterol	1.09	2	2	–	–	–	–
24-Methylchclesta-5, 22-dienol	1.12	10	14	18	17	16	21
24-Methylenecholesterol	1.26	15	14	12	7	10	42
24-Methylcholesterol	1.29	8	7	12	6	6	10
24-Ethylcholesta-5, 22-dienol	1.40	4	2	7	5	5	3
24-Ethylcholesterol	1.60	7	9	6	9	9	1
Fucosterol	1.60	7	6	3	4	4	trace

[a]Percentage of total sterol.

[b]Retention time relative to cholesterol.

TABLE II

Sterol Composition of *Thalassiosira pseudonana* and *Isochrysis* sp.

Sterol	RRT[b]	Composition (%)[a] *Thalassiosira pseudonana*	*Isochrysis* sp.
Cholesterol	1.0	0.5	1.0
24-Methylcholesta-5, 22-dienol	1.12	trace	97.0
24-Methylenecholesterol	1.26	83.0	–
24-Methylcholesterol	1.29	9.0	–
24-Methyl-5, 22(25)-cholestadienol	1.45	3.0	–
24-Ethylcholesterol	1.60	4.0	–
Fucosterol	1.60	trace	–

[a]Percentage of total sterol.

[b]Retention time relative to cholesterol.

these oysters.

The major sterols observed in wild and cultivated oysters were the same, but in different concentrations. However, when one group of cultivated oysters was transferred to a natural oyster bar 3 months prior to harvest, the relative sterol concentrations observed were those of wild oysters.

The relative proportions of specific sterols are rather consistent in wild oysters from different geographic areas. In these oysters, 24-norcholesta-5, 22-dienol, 22-dehydrocholesterol, 24-ethylcholesterol and fucosterol make up 24% of the total oyster sterol, but make up only 2% of the total sterol of the cultivated oyster. These sterols are presumably of dietary origin in wild oysters, but were not provided by the diet of the cultivated oysters. Large quantities of 24-methyl-cholesta-5, 22-dienol and 24-methylenecholesterol in the cultivated oysters can be attributed to the algal diet of these oysters. The fact that cholesterol is 19% of the total sterol of the cultivated oyster whereas its dietary sterol is only 1% cholesterol indicates that the oyster synthesizes cholesterol, dealkylates phytosterols to cholesterol, or concentrates the cholesterol of its diet.

Total sterol composition (% of body weight) of the oyster is known to vary by as much as a factor of 2 from season to season (9). It is interesting to note that the Cape Hatteras oysters, which reputedly have exceedingly rapid growth rates (Tillet and Marshall, personal communication), have 3–10 times the total sterol concentration of the Mid-Atlantic oysters. Work is continuing to determine the significance of this phenomenon.

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: VII. Volatile Thermal Decomposition Products of Pure Hydroperoxides from Autoxidized and Photosensitized Oxidized Methyl Oleate, Linoleate and Linolenate

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ABSTRACT

To clarify the sources of undesirable flavors, pure hydroperoxides from autoxidized and photosensitized oxidized fatty esters were thermally decomposed in the injector port of a gas chromatograph-mass spectrometer system. Major volatile products were identified from the hydroperoxides of methyl oleate, linoleate and linolenate. Although the hydroperoxides from autoxidized esters are isomerically different in position and concentration than those from photosensitized oxidized esters, the same major volatile products were formed but in different relative amounts. Distinguishing volatiles were, however, produced from each type of hydroperoxide. The 9- and 10-hydroperoxides of photosensitized oxidized methyl oleate were thermally isomerized in the injector port into a mixture of 8-, 9-, 10- and 11-hydroperoxides similar to that of autoxidized methyl oleate. Under the same conditions, the hydroperoxides from autoxidized linoleate and linolenate did not undergo significant interconversion with those from the corresponding photosensitized oxidized esters. The compositions of the major volatile decomposition products are explained by the classical scheme involving carbon-carbon scission on either side of alkoxy radical intermediates. Secondary reactions of hydroperoxides are also postulated, and the hydroperoxy cyclic peroxides from methyl linoleate (photosensitized oxidized) and methyl linolenate (both autoxidized and photosensitized oxidized) are suggested as important precursors of volatiles.

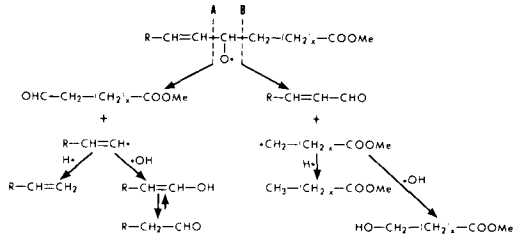
INTRODUCTION

The problems of oxidation of unsaturated fats continue to attract the attention of lipid chemists. Although considerable information is available on lipid oxidation (1-6), the mechansim of flavor deterioration (7-9) and the biological effects (10,11) are not well understood.

The isomeric structures of hydroperoxides formed as primary products of autoxiation and photosensitized oxidation are now well established (12-15), but the mechanism for their decomposition into volatile cleavage products is not clear. A natural fat like soybean oil containing mixtures of oleate, linoleate and linolenate can produce 10 positionally isomeric hydroperoxides by free radical autoxidation and 12 by singlet oxygen from photosensitized oxidation (5,15). In addition to these known hydroperoxides, speculations have been made in the literature to explain the origin of volatiles from certain hydroperoxides for which there is no evidence for their formation (16-18). It is not surprising, therefore, that studies on the source of volatile products from oxidized fats are still controversial and difficult to interpret (9).

To clarify the sources of undesirable flavors, it is necessary to study simple systems, and pure hydroperoxides would be ideal. Evans et al. (19) were the first to decompose a single hydroperoxide prepared by lipoxygenase action. They thermally decomposed the 13-hydroperoxide of linoleic acid by direct injection at 260 C onto a gas chromatograph, and identified pentane as the principal hydrocarbon, a product previously predicted by indirect evidence (20). The same approach was used in recent studies with the pure isomeric hydroperoxides of methyl linoleate (9) and of methyl oleate (21).

In a preliminary communication (21), oleate hydroperoxides were shown to be important precursors of volatiles produced from triolein heated at 192 C. A major part of the volatiles (91%) identified by gas chromatography-mass spectrometry (GC-MS) could be accounted for by the well-recognized mechanism of carbon-carbon scission on either side of the alkoxy radical intermediate produced from hydroperoxides (20) (Scheme I). However, the data were insufficient to prove that this mechanism is the only route by which such products form. The yields of A and B cleavage products from the 8-, 9-, 10- and 11-hydroperoxides of methyl



SCHEME I

oleate were in good agreement. This paper compares the thermal decomposition products of pure hydroperoxides from autoxidation with those from photosensitized oxidation of methyl oleate, linoleate and linolenate.

EXPERIMENTAL METHODS

The same methyl oleate (98%), linoleate (99%) and linolenate (99.9%) were used as described previously (12). Procedures for autoxidation and photosensitized oxidation were the same as before (12,15).

Two methods were used to purify hydroperoxides. Silicic acid column chromatography with methanolic benzene (22) was used to isolate the hydroperoxides of autoxidized oleate. Reverse-phase high pressure liquid chromatography was used with a 1:1(w/w) H_2O/acetonitrile solvent system (23) to isolate all other hydroperoxides. The hydroperoxides were checked for purity by thin layer chromatography (TLC, silica gel, solvent mixture of ether/petroleum ether, 60:40). Isomeric compositions were determined by GC-MS (12) after hydrogenation and silylation of the hydroperoxide samples. Isomeric analyses on purified hydroperoxides (Table I) were in agreement with previous analyses on oxidized fatty esters (12,15).

Hydroperoxides were decomposed in the injector port of a gas chromatograph (on column injection Model 7400, Packard Instrument Co., Inc., Downers Grove, IL) at 210 C. The He carrier flow was decreased to ca. 5 ml/min just prior to and 1 min after injection of a neat sample (4 to 10 μl). The flow was then increased (ca. 36 ml/min) and temperature programming started from 25 to 275 C at 2 C/min. The GC column (glass 14 ft x 4 mm id) was packed with 10% OV-101 on Chromosorb G. The same computerized GC-MS system was used as previously (21,24) to identify volatile compounds by matching mass spectra with those of reference compounds and confirming by GC-retention data.

To study thermal isomerization of hydroperoxides during CG pyrolysis, a glass liner was inserted into the injector of another gas chromatograph (Model 5711A, Hewlett-Packard, Palo Alto, CA). The liner was filled with the same column OV-101 packing and loosely plugged with siliconized glass wool. After

TABLE I

GC-MS Analysis (12) of Isomeric Hydroxystearates from Hydroperoxides before and after Partial GC Pyrolysis[a]

Hydroperoxide esters	Oxidation (conditions)[b]	Pyrolysis (200 C)	Relative percent					
			8-OH	9-OH	10-OH	11-OH		
Oleate	Auto (40 C, PV 1051)	Before	27	23	23	27		
Oleate	Photo (0 C, PV 1727)	Before		50	50			
		After	18	26	31	25		
			9-OH	10-OH	12-OH	13-OH		
Linoleate	Auto (27 C, PV 2970)	Before	50			50		
		After	47	2	4	47		
Linoleate	Photo (0 C, PV 1124)	Before	32	17	17	34		
		After	28	19	21	32		
			9-OH	10-OH	12-OH	13-OH	15-OH	16-OH
Linolenate	Auto • (27 C, PV 790)	Before	32		11	11		46
		After	31		10	14		45
Linolenate	Photo (0 C, PV 1566)	Before	23	13	12	14	13	25
		After	22	14	8	13	15	28

[a]Conditions given in Experimental Methods.

[b]Auto: free radical autoxidation in air (12); PV = peroxide value. Photo: photosensitized oxidation in O_2, methanol solution in presence of methylene blue (15).

injecting the sample of hydroperoxide, the carrier gas flow was decreased again for 1 min, the injector port was disassembled and the glass liner was cooled immediately in Dry Ice. The liner was then rewarmed to room temperature and purged with absolute ethanol. The organic material dissolved in ethanol was hydrogenated (platinum oxide catalyst, atmospheric H_2), and the hydroxystearate products were then silylated and analyzed for isomeric composition by GC-MS (12) (Table I).

RESULTS

Direct injection of different purified hydroperoxides onto the GC system affords immediate anaerobic pyrolysis necessary for efficient separation of volatile products. This approach also eliminates or minimizes further reactions and interactions of secondary cleavage products with unoxidized esters that would complicate the interpretation of results. Table II compares the GC-MS analysis of volatiles from pure hydroperoxides of autoxidized oleate (8- + 9- + 10- + 11-OOH) with those from photosensitized oxidized oleate (9- + 10-OOH). Although the two starting hydroperoxide mixtures are isomerically different, both samples formed the same volatile products. Major peaks identified in both samples correspond to those products expected by carbon-carbon scissions A and B on the isomeric 8-, 9-, 10- and 11-hydroperoxides of oleate (Scheme I). Photooxidized oleate hydroperoxides produced not only all the volatiles expected from the 9- and 10-isomers but also those expected from the 8- and 11-isomers. The photosensitized oxidation-derived hydroperoxides produced much more octane, 2-decenal, 2-undecenal, 1-octanol, methyl heptanoate and octanoate, and much less octanal and methyl 10-oxodecanoate than the autoxidation-derived hydroperoxides. The high relative values for 2-undecenal and methyl heptanoate are unexpected because they come from the 8-oleate hydroperoxide that was not present in the sample from photosensitized oxidation. The other comparative values are those expected from a sample containing initially more 9- and 10-hydroperoxides than 8- and 11-hydroperoxides.

Chan et al. (9) mentioned unpublished data that oleate hydroperoxides undergo isomerization, but did not indicate what isomers were involved. To check whether or not thermal isomerization occurs under our decomposition conditions, the hydroperoxides from photosensitized oxidation were injected in the gas chromatograph, and the nonvolatile organic materials adsorbed on the glass insert in the injector port were recovered and hydrogenated (see Experimental Methods). GC-MS analyses

TABLE II

GC-MS Analysis of Volatiles from Thermally Decomposed Methyl Oleate Hydroperoxides

Compound	Elution temp (C)	Autoxidation[a] (rel %)	Photosensitized oxidation (rel %)	Origin[b]
Heptane	106	4.4	4.6	11-OOH
Octane	121	2.7	10	10-OOH
Heptanal	151	0.5	0.5	?
1-Heptanol	161	0.4	0.4	11-OOH
Octanal	169	11	3.8	11-OOH
Me heptanoate	170	1.5	4.9	8-OOH
1-Octanol	181	0.4	1.0	10-OOH
Nonanal	186	15	10	9-/10-OOH
Me octanoate	189	5.0	9.7	9-OOH
2-Nonenal	197	0.5	0.7	?
Decanal	201	3.9	2.0	8-OOH
Me nonanoate	203	1.5	0.8	?
2-Decenal	211	5.4	12	9-OOH
2-Undecenal	225	1.7	7.1	8-OOH
Me 8-Oxooctanoate	230	3.5	3.0	8-OOH
Me 9-Oxononanoate	245	15	11	9-/10-OOH
Me 10-Oxodecanoate	256	12	1.7	11-OOH
Me 10-Oxo-8-decenoate[c]	265	3.4	5.0	10-OOH
Me 11-Oxo-9-undecenoate[c]	275	5.8	4.6	11-OOH
Unidentified peaks		6.4	6.7	

[a]Data from ref. 21.

[b]Based on Scheme I (5,21).

[c]Tentative identification.

(12) of the hydroxystearate trimethylsilyl ether derivatives showed that the mixture of 9- and 10-hydroperoxides from photosensitized oxidation of oleate isomerized into a mixture of 8-, 9-, 10- and 11-hydroperoxides (Table I). The volatile composition from these hydroperoxides can be explained, therefore, by the significant isomerization observed under our conditions of thermal decomposition in the GC injector port.

The GC-MS analysis of volatiles from respective hydroperoxides of autoxidized and photosensitized oxidized linoleate is given in Table III. Both sets of hydroperoxides produced the same volatiles, but significant differences in composition are evident. The autoxidized linoleate hydroperoxides produced much more pentane, 2-pentylfuran, 2,4-decadienal, and methyl octanoate and much less methyl 10-oxo-8-decenoate and 2-heptenal than the photooxidized linoleate hydroperoxides.

Chan et al. demonstrated that the 9- and 13-hydroperoxides of methyl linoleate are readily interconverted (25,26), and found that both of these isomers gave the same volatile cleavage compounds but in different amounts (9). Under our thermal decomposition conditions, very little interconversion of linoleate hydroperoxide mixtures occurred. The 50:50 mixture of 9- and 13-hydroperoxides in the autoxidation sample, after heating in the injector port, produced a mixture containing also 2% 10- and 4% 12-hydroperoxides. After the same treatment, the hydroperoxides from photosensitized oxidized linoleate showed very little change in isomer composition (Table I). Although not all the cleavage products expected from linoleate hydroperoxides were identified, more volatiles expected from the 9- and 13-hydroperoxides were formed from the autoxidized sample. Similarly, more volatiles expected from the 10- and 12-hydroperoxides were formed from the photosensitized oxidized sample. 2-Pentylfuran is a unique product of autoxidation hydroperoxide, but its origin is not well established. 2-Heptenal is a unique product of photosensitized oxidation hydroperoxides and would originate from the 12-hydroperoxide by cleavage A according to Scheme I.

The composition of volatiles from linolenate hydroperoxides is most complicated because we deal with 4 isomers (9- + 12- + 13- + 16-OOH) in the autoxidation sample and 6 isomers (9- + 10- + 12- + 13- + 15- + 16-OOH) in the photosensitized oxidation sample (Table IV). However, as with oleate and linoleate hydroperoxides, common volatiles were produced from both types of linolenate hydroperoxides. The autoxidation linolenate hydroperoxides pro-

TABLE III

GC-MS Analysis of Volatiles from Thermally Decomposed Methyl Linoleate Hydroperoxides

Compound	Elution temp (C)	Autoxidation (rel %)	Photosensitized oxidation (rel %)	Origin[a]
Acetaldehyde	70	0.3	0.4	?
Pentane	88	9.9	4.3	13-OOH
Pentanal	117	0.8	0.3	13-OOH
1-Pentanol	129	1.3	0.3	13-OOH
Hexanal	136	15	17	12-/13-OOH
2-Heptenal[b]	165	Tr	9.9	12-OOH
1-Octen-3-ol[b]	165	Tr	1.9	10-OOH
2-Pentylfuran[b]	165	2.4	0.6	?
Me heptanoate	170	1.0	0.3	?
2-Octenal	182	2.7	1.5	?
Me octanoate	189	15	7.6	9-OOH
2-Nonenal	195, 197[c]	1.4	1.6	9-/10-OOH*
2,4-Nonadienal	208	0.3	0.3	?
2,4-Decadienal	219, 223[c]	14	4.3	9-OOH
Me 8-Oxooctanoate	230	1.3	0.9	?
Me 9-Oxononanoate	245	19	22	9-/10-OOH
Me 10-Oxodecanoate	256	0.7	0.7	?
Me 10-Oxo-8-decenoate[d]	265	4.9	14	10-OOH
Unidentified peaks		9.9	12	

[a]Based on Scheme I (5,21); *isomerized.

[b]Not separated by GC, estimated by MS.

[c]Different peaks due to geometric isomers.

[d]Tentative identification.

TABLE IV

GC-MS Analysis of Volatiles from Thermally Decomposed Methyl Linolenate Hydroperoxides

Compound	Elution temp (C)	Autoxidation (rel %)	Photosensitized oxidation (rel %)	Origin[a]
Ethane/ethene	65	10	3.2	16-OOH
Acetaldehyde	70	0.8	0.6	?
Propanal/acrolein	80	7.7	9.0	15-/16-OOH
Butanal	97	0.1	0.8	?
2-Butenal	109	0.5	11	15-OOH
2-Pentenal	131	1.6	1.2	13-OOH
2-/3-Hexenal	137	1.4	3.4	12-/13-OOH
2-Butylfuran	158	0.5	0.3	?
Me heptanoate	170	1.8	1.0	?
2,4-Heptadienal	174, 178[b]	9.3	8.8	12-OOH
Me octanoate	189	22	15	9-OOH
4,5-Epoxyhepta-2-enal	194	0.2	0.2	?
3,6-Nonadienal[c]	196, 198[b]	0.5	1.1	9-/10-OOH
Me Nonanoate	203	0.7	0.3	?
Decatrienal	219, 226[b]	14	4.8	9-OOH
Me 8-Oxooctanoate	230	0.6	0.4	?
Me 9-Oxononanoate	245	13	12	9-/10-OOH
Me 10-Oxodecanoate	256	1.0	1.5	?
Me 10-Oxo-8-decenoate[d]	267	4.2	13	10-OOH
Unidentified peaks		11	12	

[a]Based on Scheme I (5,21).

[b]Different peaks due to geometric isomers.

[c]Identified as 2,6-nonadienal.

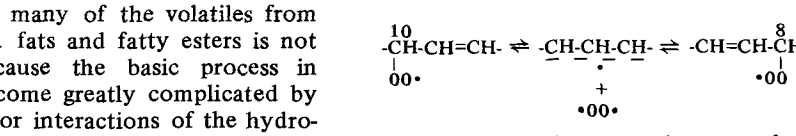
[d]Tentative identification.

duced more decatrienal, methyl octanoate and ethane and less methyl 10-oxo-8-decenoate and 2-butenal than the photosensitized oxidation hydroperoxides. The most distinguishing volatiles include ethane for autoxidation hydroperoxides and 2-butenal for the photooxidation hydroperoxides. As with linoleate, each of the hydroperoxide mixture from either autoxidized or photosensitized oxidized linoleate showed little or no change in isomeric composition after pyrolysis in the GC injector port (Table I).

DISCUSSION

The source of many of the volatiles from different oxidized fats and fatty esters is not clear (5,7-9) because the basic process in Scheme I can become greatly complicated by further reactions or interactions of the hydroperoxides, the aldehydes and the unsaturated substrates. To elucidate this complex problem, pure hydroperoxides are useful precursors of volatile oxidation products. However, this approach is further complicated by the thermal interconversion of different isomeric hydroperoxides and the formation of secondary products that may also produce volatiles.

This work showed that the interconversion previously demonstrated between the 9- and 13-hydroperoxides of linoleate (25,26) is also significant between the 9- and 10-hydroperoxides of oleate formed by photosensitized oxidation and the mixture of 8-, 9-, 10- and 11-hydroperoxides formed by autoxidation (Table I). Chan et al. (26) showed that the rearrangement of linoleate hydroperoxides involves complete exchange of peroxy oxygen with atmospheric oxygen. Therefore, thermal rearrangement of oleate hydroperoxides by the same process suggested by Chan et al. for linoleate would involve the same allylic 3-carbon intermediates as those recognized for autoxidation (4,5).



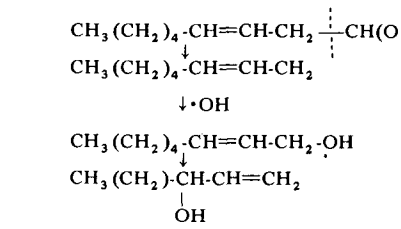
As shown in Table II, both types of hydroperoxides produce the same major volatiles. However, more of the volatiles derived from the 9- and 10-hydroperoxides (octane, 1-octanol, 2-decenal and methyl octanoate) were identified in the photosensitized oxidation hydroperoxides. Therefore, some decomposition of the hydroperoxides may occur before rearrangement, and these two processes may be competitive and controlled kinetically. Never-

theless, the volatile compounds analyzed are only those best amenable to our GC-MS detection technique, and their relative concentration is probably also affected by their thermal stability.

With linoleate hydroperoxides, the list of volatile decomposition products from the 9- and 13-isomers in Table III is more complete and comprehensive than that reported by Chan et al. (9), which includes only four products. All of the products expected from the autoxidation hydroperoxides by Scheme I were detected except the 12- and 13-carbon unsaturated aldehyde esters, for which no authentic references were available. In contrast to Chan et al. (9), we identified 2-enals and 2-pentylfuran among the volatiles from the autoxidation hydroperoxides. Although their origin is not clear, 2-octenal is speculated to come from the nonconjugated 11-hydroperoxide (16-18) and 2-pentylfuran from a 10-hydroperoxide intermediate (27). 2-Nonenal may result by isomerization of 3-nonenal, a product expected from tautomerization of the vinyl alcohol formed by reacting the diene fragment of 9-linoleate hydroperoxide with a hydroxy radical (Scheme I).

Although the autoxidation and photosensitized oxidation hydroperoxides formed most of the same volatile products (Table III), very little interconversion was observed when the two samples were heated in the GC injector inlet. 2-Heptenal derived from the 12-hydroperoxide in the photosensitized oxidation sample is a distinguishing volatile. Methyl 10-oxo-8-decenoate and 1-octen-3-ol are unique products expected from the 10-hydroperoxide. 2-Heptenal is an important product reported in autoxidized fats containing linoleate (17,18,28, 29) and may well originate from the 12-hydroperoxide that we identified in significant amounts in many vegetable oil esters at low levels of oxidation (15,30).

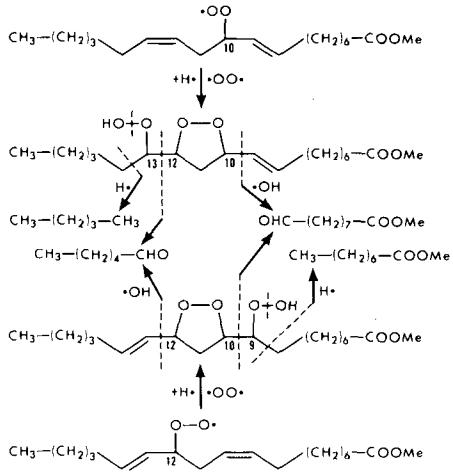
1-Octen-3-ol is a rearrangement product from 2-octen-1-ol expected from the reaction of 2-octen radical with a hydroxy radical.



Other products from the photosensitized oxidation hydroperoxides, such as pentane, hexanal and methyl 9-oxononanoate, may originate from cyclic peroxides that we (31) and others (32) identified as secondary oxidation products of photosensitized oxidized methyl linoleate. According to Scheme II, the 13-hydroperoxy-10,12-cyclic peroxide formed from the 10-hydroperoxide of linoleate would produce pentane, hexanal and methyl 9-oxononanoate by carbon-carbon scission on either side of the alkoxy radical and of the cyclic peroxide. Similarly, the 9-hydroperoxy-10,12-cyclic peroxide formed from the 12-hydroperoxide of linoleate would produce hexanal, methyl octanoate and methyl 9-oxononanoate. If the 10 and 12-hydroperoxides of photosensitized oxidized linoleate isomerized like oleate, conversion to the corresponding 8- and 14-hydroperoxides would be expected. The absence of these isomers (Table I) supports cyclization of the 10- and 12-hydroperoxides as shown in Scheme II. Malonaldehyde is another expected product of cleavage on either side of the cyclic peroxide, which was previously postulated by Pryor et al. (33) from the endoperoxides and prostaglandin-like compounds of linolenate hydroperoxides. However, this dialdehyde was not identified under our GC-MS conditions.

Unpublished evidence was cited by Chan and Levett (34) that linolenate hydroperoxides do not undergo the same interconversions as observed for linoleate hydroperoxides, and that the 9- and 13-linolenate hydroperoxides (prepared by lipoxygenase action) decompose at the same rate. As shown in Table IV, autoxidation and photosensitized oxidation hydroperoxides of linolenate produce many of the same volatiles, most of which can be accounted for by cleavage according to Scheme I. Expected products that were not identified include the methyl C-12, C-13, C-14, C-15 and C-16 unsaturated aldehyde esters, for which no reference compounds were available, as well as the unsaturated hydrocarbons (2-pentene and 2,5-octadiene). Distinguishing products found in significant amounts in the photosensitized oxidation hydroperoxides of linolenate include 2-butenal expected from the 15-hydroperoxide and methyl 10-oxo-8-decenoate expected from the 10-isomer.

Hydroperoxy cyclic peroxides are expected by cyclization and further oxidation of the internal 12- and 13-hydroperoxides from autoxidized linolenate (12,35). A hydroperoxy cyclic peroxide has now been identified from the autoxidation of the 13-linolenate hydroperoxide prepared by lipoxygenase action (35), and several isomeric hydroperoxy cyclic peroxides derived from the 12- and 13-hydroperoxides have been identified in autoxidized methyl linolenate (31). The internal 10- and



SCHEME II

15-hydroperoxides from photosensitized oxidized linolenate would also be expected to cyclize the same way as the 10- and 12-hydroperoxides of photosensitized oxidized linoleate. On the basis of Scheme II, the hydroperoxy cyclic peroxides from linolenate would contribute the same type of aldehyde cleavage products as the hydroperoxides before cyclization. These volatile products derived from secondary oxidation of hydroperoxides would account for many of the common products, such as 3-hexenal, propional and methyl 9-oxononanoate, observed from both types of hydroperoxides (autoxidation and photosensitized oxidation).

Under the anaerobic pyrolytic conditions in this and previous studies (9,19,21), any interactions between hydroperoxides and unsaturated substrate have been eliminated. However, secondary products may occur between free radical intermediates and the peroxy radicals produced thermally. Therefore, contrary to the view of Chan et al. (9), these secondary products may make an important contribution even under anaerobic pyrolysis of pure hydroperoxides. Further study is necessary to establish a mechanism of decomposition involving the secondary products of hydroperoxides.

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Composition and Variability of the Branched-Chain Fatty Acid Fraction in the Milk of Goats and Cows

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ABSTRACT

Branched-chain fatty acids of the milk fat of goats were analyzed by high resolution gas chromatography-mass spectrometry. Iso- and anteiso-acids predominated, but a range of other monomethyl-branched components, mostly with methyl-substitution on carbons 4 and 6, was present. Analysis of the milk fat of cows revealed the presence of iso- and anteiso-fatty acid; other monomethyl-substituted fatty acids, as found in the milk fat of the goat, were virtually absent. Only a trace amount of 6-methylhexadecanoate was detected. The difference between goats and cows in the effectiveness with which these animals metabolize propionyl-CoA and methylmalonyl-CoA is discussed.

INTRODUCTION

Studies related to the occurrence of branched-chain fatty acids in milk fat have been conducted on the human and cow (1). Multi-branched fatty acids (phytanic acid and catabolites) have been shown to be present in butterfat (2-4) and human milk fat (5).

Monomethyl-branched fatty acids with methyl-substitution on even- and odd-numbered carbon atoms are reported in milk fat of these species (5,6). Exclusively even carbon methyl-substitutions were found by Ackman et al. (7) in the C_{15} and C_{17} fraction of butterfat. In all these studies, the separation of the methyl esters was achieved on high resolution capillary gas chromatography (GC) columns with (5,7) or without (6) enrichment of the branched-chain fatty acid fraction. Strocchi and Holman (8) confirmed the presence of iso- and anteiso-fatty acids in butterfat with other monomethyl-branched fatty acids being absent. In this work (8), conventional GC was used without enrichment of the branched-chain fatty acid fraction.

Hitherto, no studies have been related to the occurrence of branched-chain fatty acids in the milk fat of the goat. In this study, the composition of the branched-chain fatty acids of goat milk fat was examined and compared to cow's milk fat. The composition of the branched-chain fatty acids was determined by capillary GC and their identity was established by gas chromatography-mass spectrometry (GC/MS) after urea fractionation. The variation in fatty acid composition in the milk of different goats and the variability in the milk taken from a single animal at different times also were investigated.

MATERIALS AND METHODS

Source of Fat

Milk was obtained from 10 goats of mixed breeds, nonpregnant and giving a good milk yield. They were kept indoors under controlled conditions of feeding and management and received 1.3 kg of concentrates per goat daily and hay ad libitum. The concentrate mixture consisted of 16.5% crude protein, 15% digestible protein, 3% fat, 36% sugar + starch, 9% minerals and 8.5% crude fiber. The goats were milked daily in the morning and in the evening and, once a week, a composite milk sample of one day was obtained from each. This was done 3 times, so that the complete sampling program took 3 weeks. The mean milk yield was 1,117 ml ± 45 ml/day (range 620-1720 ml) and the milk fat percentage amounted to 5.5 g% ± 0.2 g% (range 3.5-7.5 g%).

Milk also was obtained from 6 lactating cows. The food of 2 of them was qualitatively similar to the goats' diet, hay ad libitum and concentrates amounting to 8 kg/day, whereas the others were fed on diets based on concentrate, pulp and hay.

Extraction and Analysis of Triacylglycerols

Triacylglycerols (TG) were obtained from crude milk fat after adsorption chromatography (9) and were transmethylated (10). The methyl esters were extracted with petroleum ether. After evaporation of the solvent, the esters were hydrogenated and analyzed by capillary GC.

Gas Chromatographic Analysis

The esters were subjected to high resolution

capillary GC on a wall-coated, open-tubular column, 50 m in length and 0.5 mm id, coated with polymerized butanediolsuccinate (BDS). The column was operated isothermally at 170 C with the carrier gas flow at 7 ml/min in a Hewlett-Packard 7620A gas chromatograph fitted with a flame ionization detector. The equivalent chain lengths (ECL) of the eluting methyl esters were calculated. Peak areas were integrated using a chromatographic data system model 111C (Varian Assoc., Inc. Palo Alto, CA), and the proportions of the eluting compounds were determined.

The methyl esters were identified on the basis of the accordance of their ECL values with those of the methyl esters of enriched fractions, which were identified by GC/MS under similar chromatographic conditions as described next.

Identification by Mass Spectrometric Analysis

To permit identification of the minor compounds by GC/MS, an enrichment by urea fractionation was necessary, followed in some cases by preparative GC. Several samples of milk fat of goats (0.5 g) were treated with 3 ml methanol saturated with urea as described by Ackman and Hooper (11), yielding a non-adducting and an adducting fraction. The nonadducting fraction was further treated 3 or 4 times. Before each step of fractionation, one part of the nonadducting fraction was taken for GC/MS analysis. For the branched-chain fatty acids C_{17} and C_{18}, further purification was achieved by preparative GC on a Varian Autoprep Model A700. A column of 3 m x 4 mm od packed with 5% EGSS-X on Gaschrom Q 80-100 mesh was operated at 170 C with a carrier gas flow of 80 ml/min.

The enriched branched-chain fatty acid esters were analyzed by GC/MS using a Varian Model 2700 coupled to a Varian Mat 112. A BDS column (50 m in length, 0.5 mm id) was operated isothermally (170 C) as well as temperature-programmed from 100 to 220 C at 1-2 C/min. The column was coupled to the mass spectrometer by a direct inlet system consisting of a glass capillary (50 cm, 0.113 mm id) heated to 230 C under a vacuum of 7.10^{-6} torr. The spectrometer was operated at an acceleration voltage of 820 V, with an ionization energy of 70 eV and an ion source temperature of 250 C. Identification of the branched-chain fatty acids was done by comparison with published mass spectra (5,12-15).

Analysis of the Milk Fat of Cows

The methyl esters were prepared as already outlined, and subjected to capillary GC under similar chromatographic conditions. The branched-chain fatty acids were identified on the basis of the accordance of their ECL values with those of the methyl esters of goat milk fat.

Statistical Analysis

The results obtained from the analysis of the goat milk fat were subjected to an analysis of variance using a complete design (10 [goats] x 3 [weeks] x 2 [replicate samples]) (16). Interactions were not taken into account in the testing of significance of the factors.

RESULTS

Milk Fat of Goats

The conclusions obtained from the GC/MS results and the ECL values for the branched-chain fatty acids are summarized in Table I.

The mass spectra of the peak with ECL-value 10.40 (C=11) from different runs show, by the varying intensities of the ion-peaks, that a mixture is present and this is evaluated as methyl 4-methyl- and methyl 6-methyldecanoate. The substance with ECL 11.20 (C=12) was found to be a mixture of 4,8-dimethyl-C10:0 and 6,8-dimethyl-C10:0. The eluting component with ECL 11.85 (C=13) has a base peak at m/e 88 and is defined as methyl 2-methyldodecanoate.

For 6-methyl- and 4-methyltetradecanoate, ECL values of 14.31 and 14.40, respectively, were found. Three distinct peaks were observed with ECL 16.31, 16.39 and 16.42, and were identified as 6-methylhexadecanoate, 4-methylhexadecanoate and 12-methylhexadecanoate, respectively. Traces of 8-methyl- and 10-methylhexadecanoate were found in the mass spectra of the compounds with ECL 16.31, 16.39, but were not separated or visible on the chromatogram. The identity of most of the branched-chain fatty acids as summarized in Table I can be compared with those reported by Smith et al. (17).

A range of monomethyl-substituted fatty acids is present in goats' milk fat in detectable quantities amounting to 2.02% of the total milk fatty acids (Table I). These monomethyl-branched fatty acids vary in chain length from 9 to 19 carbon atoms. Iso- and anteiso-acids representing ca. 81% of the total branched-chain fatty acid fraction and acids with methyl-substitution in the mid-chain are present. It was found that methyl-substitution occurs only on even-numbered carbon atoms and mostly on carbons 2, 4 or 6. Fatty acids with 4 and 6 methyl-substitutions have not been identified in the C_9 and C_{10} fatty acids of milk fat until now. Because the identification is based only

TABLE I

Equivalent Chain Length (ECL), Identity (As Derived from GC/MS + Urea Fractionation in Some Cases) and Composition (wt %) of Branched-Chain Fatty Acids in Milk Fat of Goats

C[a]	ECL[b]	Identity	(%)	C	ECL	Identity	(%)
9	ND	Methyl 4-methyloctanoate[d]	ND[c]	15	14.55	Methyl 13-methyltetradecanoate (iso)	0.17 ± 0.01
9	ND	Methyl 6-methyloctanoate[d]	ND	15	14.71	Methyl 12-methyltetradecanoate (anteiso)	0.35 ± 0.01
10	ND	Methyl 4-methylnonanoate[d]	ND	17	15.07	Methyl 5,9,13-trimethyltetradecanoate	Trace
11	10.40	Methyl 4-methyldecanoate	0.12 ± 0.01	16	15.30	Methyl 6-methylpentadecanoate	Trace
		Methyl 6-methyldecanoate		16	15.37	Methyl 4-methylpentadecanoate	Trace
12	11.20	Methyl 4,8-dimethyldecanoate	0.03 ± 0.003	16	15.55	Methyl 14-methylpentadecanoate (iso)	0.24 ± 0.01
		Methyl 6,8-dimethyldecanoate		16	15.70	Methyl 13-methylpentadecanoate (anteiso)	Trace
12	11.55	Methyl 10-methylundecanoate (iso)	Trace	17	15.82	Methyl 2-methylhexadecanoate	Trace
13	11.85	Methyl 2-methyldodecanoate	Trace	17	16.31	Methyl 6-methylhexadecanoate	0.01 ± 0.003
13	12.35	Methyl 6-methyldodecanoate	Trace			+ trace 8-methylhexadecanoate	
13	12.40	Methyl 4-methyldodecanoate	0.05 ± 0.003	17	16.39	Methyl 4-methylhexadecanoate	0.05 ± 0.003
13	12.55	Methyl 11-methyldodecanoate (iso)	0.01 ± 0.001			+ trace 10-methylhexadecanoate	
13	12.70	Methyl 10-methyldodecanoate (anteiso)	0.01 ± 0.001	17	16.42	Methyl 12-methylhexadecanoate	
14	13.55	Methyl 12-methyltridecanoate (iso)	0.07 ± 0.002	17	16.55	Methyl 15-methylhexadecanoate (iso)	0.35 ± 0.01
14	13.71	Methyl 11-methyltridecanoate (anteiso)	Trace	17	16.70	Methyl 14-methylhexadecanoate (anteiso)	0.40 ± 0.01
15	13.85	Methyl 2-methyltetradecanoate	Trace	20	17.02	Methyl 3,7,11,15-tetramethylhexadecanoate	Trace
16	14.17	Methyl 4,8,12-trimethyltridecanoate	Trace	18	17.52	Methyl 16-methylheptadecanoate (iso)	0.03 ± 0.01
15	14.31	Methyl 6-methyltetradecanoate	0.05 ± 0.003	18	17.67	Methyl 15-methylheptadecanoate (anteiso)	Trace
15	14.40	Methyl 4-methyltetradecanoate	0.06 ± 0.003	19	18.61	Methyl 16-methyloctadecanoate (anteiso)	Trace

The values given are means for 10 goats ± standard error of mean (SEM).

[a]C: total number of carbon atoms in the molecule.

[b]ECL: equivalent chain length.

[c]ND: not determined.

[d]Tentative identification (see Results).

TABLE II

Equivalent Chain Length (ECL) and composition (wt %) of the Branched-Chain Fatty Acids in Milk Fat of the Cow

C[a]	ECL[b]	Identity	(%)	C	ECL	Identity	(%)
11	10.40	Methyl 4-methyldecanoate	–[c]	16	15.55	Methyl 14-methylpentadecanoate (iso)	0.48 ± 0.05
		Methyl 6-methyldecanoate		16	15.70	Methyl 13-methylpentadecanoate (anteiso)	Trace
13	12.40	Methyl 4-methyldodecanoate	–	17	16.31	Methyl 6-methylhexadecanoate	0.01 ± 0.00
13	12.55	Methyl 11-methyldodecanoate (iso)	0.05 ± 0.01	17	16.39	Methyl 4-methylhexadecanoate	–
13	12.70	Methyl 10-methyldodecanoate (anteiso)	0.02 ± 0.01	17		Methyl 10-methylhexadecanoate	
14	13.55	Methyl 12-methyltridecanoate (iso)	0.19 ± 0.02	17	16.42	Methyl 12-methylhexadecanoate	–
14	13.71	Methyl 11-methyltridecanoate (anteiso)	Trace	17	16.55	Methyl 15-methylhexadecanoate (iso)	0.47 ± 0.03
15	14.31	Methyl 6-methyltetradecanoate	–	18	16.70	Methyl 14-methylhexadecanoate (anteiso)	0.67 ± 0.05
15	14.40	Methyl 4-methyltetradecanoate	–	18	17.52	Methyl 16-methylheptadecanoate (iso)	0.11 ± 0.01
15	14.55	Methyl 13-methyltetradecanoate (iso)	0.36 ± 0.05	19	17.67	Methyl 15-methylheptadecanoate (anteiso)	Trace
15	14.71	Methyl 12-methyltetradecanoate (anteiso)	0.71 ± 0.08		18.61	Methyl 16-methyloctadecanoate (anteiso)	Trace

The values are means for 6 cows ± standard error of mean (SEM).

[a]C: total number of carbon atoms in the molecule.

[b]ECL: equivalent chain length.

[c]Below detection limit of GC analysis.

on the mass spectrum (the ECL was not determined), the identification should be considered as tentative only.

Mass spectrometric analysis shows the presence of trace amounts of multi-branched fatty acids (3,7,11,15-tetramethylhexadecanoic acid) and their catabolites (5,9,13-trimethyltetradecanoate and 4,8,12-trimethyltridecanoate) in the milk fat of the goat. The primary catabolic product of 3,7,11,15-tetramethylhexadecanoic acid, i.e., 2,6,10,14-tetramethylpentadecanoate, also may be present. However, its ECL value is very close to 16:0 and, because no very stringent preliminary fractionation step was done, the quantity of 16:0 is still too high to allow its detection. These acids have already been found in butterfat (2-4) and human milk fat (5). The mass spectrometric analysis also revealed trace amounts of 11-cyclohexylundecanoate. This acid was already isolated in cow's butterfat (18), in bovine rumen bacteria (19) and in sheep perinephric fat (7).

Milk Fat of Cows

The milk fat of cows contains 3.07 ± 0.30% branched-chain fatty acids and this fraction consists almost entirely of acids of the iso- and anteiso-series (Table II). As to the fatty acids with methyl-substitution in the chain, traces of 6-methylhexadecanoate were detected. Strocchi and Holman have found iso- and anteiso-acids. Other branched-chain fatty acids with mid-chain substitution could only be detected in butterfat after thorough enrichment of the branched-chain fatty acids as shown by Ackman et al. (7).

A survey of what is known about the identity of the branched fatty acids in milk fat from this and other studies (5-8) is given in Table III. The results of this investigation confirm and complete the data existing in the literature.

DISCUSSION

In the milk fat both of goats and cows, no large differences exist between the amounts of iso-acids with even-numbered and odd-numbered C-atoms. However, acids with an odd number of C-atoms account for nearly the total anteiso-acid content. Similar results were found in adipose tissue of ruminants (20), and it is assumed that these branched-chain fatty acids may have a common metabolic origin. Indeed, odd- and even-numbered iso-acids are synthesized by rumen bacteria from the products of oxidative deamination of the branched-chain amino acids valine and leucine, and the odd-numbered anteiso-acids are similarly synthe-

sized from isoleucine (21,22). Keeney et al. (23) suggested that significant quantities of the branched-chain fatty acids of milk fat may originate from rumen microbial synthesis.

This investigation clearly shows the occurrence of branched-chain fatty acids other than the iso- and anteiso-acids in milk fat of the goat: 4-methyldodecanoate, 6-methyltetradecanoate, 4-methyltetradecanoate, 6-methylhexadecanoate and 4-methylhexadecanoate. They probably are formed by another metabolic route, i.e., the utilization of methylmalonyl-CoA instead of malonyl-CoA in the chain-lengthening process of fatty acids synthesized with acetyl-CoA as the primer unit. The synthesis of branched acids of intermediate chain length from [^{14}C]methylmalonyl-CoA incubated with acetyl-CoA, malonyl-CoA and NADPH was demonstrated in chicken liver and subcutaneous adipose tissue of barley-fed lambs (24,25).

Analysis of cow's milk fat demonstrates that branched fatty acids other than iso- and anteiso-series are practically absent (Table II) and agrees with the results of Strocchi and Holman (8). The present investigation shows a marked difference between the milk fat of goats and cows in the proportions of the branched-chain fatty acids with ECL values 10.40, 12.40, 14.31, 14.40, 16.31 and 16.39. This observation is in accordance with the fact that no significant incorporation of methylmalonic acid was shown in fatty acids by incubation of bovine mammary tissue slices (26). When feeding a barley-rich diet to sheep, goats and cattle, Duncan and Garton (20) found relatively high proportions of branched fatty acids with methyl-substitution different from iso- and anteiso-acids in adipose tissue of the goats and sheep, but not in that of the cattle. Nevertheless, the incorporation of methylmalonyl-CoA in adipose tissue preparations from cattle was demonstrated (27). From all these previous results and our own findings, it might be concluded that an interspecies difference exists between goats and cattle in the effectiveness with which these animals metabolize propionate and its carboxylation product, methylmalonyl-CoA. This conclusion also was reached by others (28,29).

The variability of the composition of the even- and odd-numbered straight- and branched-chain fatty acids was studied in the milk fat of 10 goats, with samples taken weekly over a 3-week period. Earlier studies (30-32) concerned only with the main component fatty acids, demonstrated that the fatty acid composition of milk fat is subjected to a high degree of genetic control. In our investigation, about

TABLE III

Survey of the Methyl Branch Position of the Branched Fatty Acids in Milk Fat of the Goat and Cow, in Butterfat and in Human Milk Fat

Total number of carbon atoms in the molecule	9	10	11	12	13	14	15	16	17	18	19
	Carbon number with methyl branch position										
Goat's milk fat[a] (partially enriched by urea fractionation)	4 6	4	4 6	(4,8) (6,8) 10	2 4 6 10 11	11 12	2 4 6 12 13	4 6 13 14	2 4 6 8 10 12 14 15	15 16	16
Cow's milk fat[a] (not enriched)	ND[f]	ND	ND	ND	10 11	11 12	12 13	13 14	6 14 15	15 16	16
Butterfat[b] (not enriched)				9	10 11	12	7 8 9 10 11 12 13	14	8 9 10 11 12 14 15		16
Butterfat[c] (enriched)	ND	ND	ND	ND	ND	ND	4 6 8 10	ND	4 6 8 10 12	ND	ND
Butterfat[d] (not enriched)					10 11	11 12	12 13	13 14	14 15	15 16	16
Human milk fat[e] (enriched)	3 4 5 7 8 9			3 4 5 10	2 3 4 5 6 7 8 10 11	4 5 11 12	2 4 5 6 7 8 12 13	2 4 13 14	4 7 8 12 14 15	5 9 10 11 12 16 10 11	

[a]This study.
[b]Adapted from reference (6) Ryhage (1967).
[c]Adapted from reference (7) Ackman et al. (1972).
[d]Adapted from reference (8) Strocchi and Holman (1971).
[e]Adapted from reference (5) Egge et al. (1972).
[f]ND: not determined.

TABLE IV

Significance at the 1% ($p<0.01$) and 5% ($p<0.05$) Levels of the Variability of Normal and Branched-Chain Fatty Acids of Goat's Milk Fat, Expressed As Percentage of the Total Amount of Fatty Acids between Weeks and Between Goats

ECL	Significance (between weeks)	Significance (between goats)	ECL	Significance (between weeks)	Significance (between goats)
10.00	–[a]	$p<0.01$	14.72	$p<0.01$	$p<0.01$
10.40	–	$p<0.01$	15.00	$p<0.01$	$p<0.01$
11.00	$p<0.01$	$p<0.01$	15.55	$p<0.01$	$p<0.01$
11.20	$p<0.01$	$p<0.01$	16.00	$p<0.01$	$p<0.01$
12.00	–	$p<0.01$	16.31	–	$p<0.01$
12.40	$p<0.05$	$p<0.01$	16.39	–	$p<0.01$
12.55	$p<0.05$	$p<0.01$	16.55	$p<0.05$	$p<0.01$
12.70	–	–	16.70	–	$p<0.01$
13.00	$p<0.01$	$p<0.01$	17.00	–	$p<0.01$
13.55	–	$p<0.01$	17.53	–	$p<0.01$
14.00	–	$p<0.01$	18.00	–	$p<0.01$
14.31	–	$p<0.01$	19.00	–	$p<0.01$
14.40	$p<0.05$	$p<0.01$	20.00	–	$p<0.01$
14.55	$p<0.01$	$p<0.01$			

[a]Not significantly different.

TABLE V

Significance at the 1% ($p<0.01$) and 5% ($p<0.05$) Levels of the Variability of Branched-Chain Fatty Acids of Goat's Milk Fat, Expressed As Percentage of the Total Amount of Branched-Chain Fatty Acids between Weeks and between Goats

ECL	Significance (between weeks)	Significance (between goats)	ECL	Significance (between weeks)	Significance (between goats)
10.40	$p<0.05$	$p<0.01$	14.55	–	–
11.20	$p<0.01$	$p<0.01$	14.72	–	$p<0.01$
12.40	–[a]	$p<0.01$	15.55	–	–
12.55	$p<0.01$	$p<0.01$	16.39	–	$p<0.01$
13.55	–	$p<0.01$	16.55	–	$p<0.01$
14.31	–	$p<0.01$	16.70	$p<0.01$	$p<0.01$
14.40	–	$p<0.01$	17.52	$p<0.05$	$p<0.05$

[a]Not significantly different.

30 fatty acids were studied and the composition of all component fatty acids significantly differed among animals ($p<0.01$) (Table IV). The week-to-week variation of the individual animals was much smaller than that observed among the different animals.

When compared to the total amount of branched-chain fatty acids, the week-to-week variability of the individual branched fatty acids becomes still less significant (Table V). We conclude that the cause of .the observed original week variation is due mostly to the variability of the total amount of branched fatty acids in milk fat and less to the variability of the proportions of the individual fatty acids. Variance analysis of the total amount of the branched fatty acids in the different milk fat samples shows that a highly significant week-to-week variation ($p<0.01$) occurs.

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Phospholipid Acyl Group Composition in Normal and Tumoral Nerve Cells in Culture

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ABSTRACT

We have studied the fatty acid composition of total phosphoglycerides from various types of nerve cells in culture. Primary cell cultures were compared with tumoral cell strains. Glial cells exhibited no characteristic pattern when compared to neurons. Tumoral cell phosphoglycerides contained much higher levels of octadecenoic acid and lower levels of C-20 to C-22 polyunsaturated fatty acids than normal cell phosphoglycerides. This observation seems to be a general feature in tumoral cell membranes. It could be of interest in respect to the membrane fluidity of cancer cells.

INTRODUCTION

Numerous membrane functions are dependent on the lipid composition of the membrane. For instance, numerous enzyme activities have been shown to be regulated in part by their lipid environment (for review, see ref. 1). Transport of metabolites across the plasma membrane can also be modulated by the lipid composition of membrane (2). In this respect, the phospholipid fatty acid pattern of cell membranes is considered to play a particularly important role in the microviscosity of membranes (3,4).

Variations in the lipid composition of cultured cell membranes have been reported according to the level of differentiation of the cells or to their tumoral transformation. At present, significant results have been obtained for glycolipids (see review in ref. 5), particularly gangliosides; differentiated cells always exhibit a much more complex pattern of glycosidic moieties than undifferentiated or tumoral cells. On the other side, numerous authors have demonstrated significant differences in membrane lipid fatty acid profiles between normal tissues and tumors. The phospholipids of hepatomas (6-11) or brain tumors (12-14) contain lower levels of C-20 and C-22 polyunsaturated fatty acids than the corresponding normal tissues. However, we have little information until now about the phospholipid acyl chains of similar cultured cell types differing by various transformation processes (15-17). Such information should be valuable in view of the strikingly different membrane properties between normal differentiated cells and transformed cells.

We have already observed that neuroblastoma or glioblastoma cell phosphoglycerides contained no more than 15-20% polyunsaturated fatty acids (18,19) whereas brain phospholipids are particularly rich in these acyl groups, of both n-3 and n-6 series. In order to know whether this fact was related to cell culture or to the transformation process, we studied the acyl chain distribution in phospholipids from various types of nerve cells in culture, of glial or neuronal origin, either grown in established continuous lines ("tumoral cells") or grown in primary cultures ("normal cells").

MATERIALS AND METHODS

Cell Culture

Primary cultures of nerve cells were obtained from rat or mouse cerebral hemispheres, either from 15-day-old embryos or from newborn animals (20,21). Neuron-rich cultures were prepared from 15-day-old embryos; after disruption of the tissue, the cells were seeded on polylysine-coated plastic Petri dishes and were harvested 5-7 days later. Astrocyte-rich cultures were prepared from newborn animals or embryos; the cells were seeded directly onto the plastic Petri dishes and were harvested at least 14 days later, after neurons and oligodendrocytes have progressively disappeared.

Cultures in continuous lines originated from various clonal strains. A clonal line of the C1300 mouse neuroblastoma was studied—the NIE 115 clone (22). Various SV40-transformed cells were also studied and were obtained by Louis from primary mouse embryo hemisphere cultures (23); two of them (MT9 and MT16) presented glial characteristics, whereas two others presented neuronal aspects (MT4 and MT17). The morphological and biochemical characterization of these cells has been presented elsewhere (23). Various other cell lines (glioblastoma, neuroblastoma) had been already studied (18,19).

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The cells were cultivated either in Petri dishes (primary cultures) or in 75 cm^2 flasks (continuous lines) with Eagle-Dulbecco medium (GIBCO) supplemented with fetal calf serum (20% for primary cultures and 10% for continuous lines) (GIBCO). They were maintained at 37 C in a wet atmosphere containing 5% CO_2. The medium was changed each 2-3 day for continuous lines, which were usually replicated every 5-7 days. The MT lines were studied at the 8th-10th replication (40-70 days) after the transformation, and the NIE 115 strain between the 30th and the 35th passage (150-240 days). The cells were harvested after confluency had been reached, generally 5 or 6 days after the last replication. For primary cultures, medium was changed every 5-7 days and no replication occurred. The cells were harvested at various times after seeding; glial cells could be maintained for long periods in culture, whereas it was necessary to harvest the cultures enriched in neuronal cells much earlier.

Fatty Acid Analysis

The cells were recovered by scraping the culture dish, and were then washed in isotonic saline and pelleted. An aliquot of the pellet was taken out for protein measurement (24). Lipids were extracted according to Folch et al. (25) and the chloroform phase was taken to dryness under a stream of nitrogen and chromatographed on a small column of silicic acid suspended in hexane. Neutral lipids were eliminated with chloroform and phospholipids were eluted with methanol and collected. Phospholipids were assayed according to Macheboeuf and Delsal (26). An aliquot of this eluate was subjected to mild alkaline methanolysis which provides fatty acid methyl esters from phosphoglyceride acyl groups (27). This methanolysis was performed with 0.1 N KOH in methanol at 37 C for 30 min; fatty acid methyl esters were recovered in the chloroform layer obtained after addition of 2 vol of chloroform and 0.6 vol of water, and evaporated under nitrogen. The fatty acid methyl esters were then separated on a 402 B Hewlett-Packard gas chromatograph equipped with a flame ionization detector. The separation was achieved on a 1.5 m long glass column filled with 10% EGSS-X in Chromosorb W-HP. Analyses were made isothermally at 185 C. Peaks areas were measured with and ICAP 10 integrator. Peaks were identified as already described (18).

RESULTS

The phospholipid concentrations ranged between 0.125 and 0.170 μmol/mg protein in tumoral cells and between 0.215 and 0.390 μmol/mg protein in normal cells. Individual values are listed at the tops of Tables I and II.

The phosphoglyceride acyl group composition of the various nerve cell strains grown in continuous lines is presented in Table I and that

TABLE I

Phosphoglyceride Acyl Group Distribution in Tumoral Nerve Cells in Culture

	C1300 Neuroblastoma	SV40-transformed cells			
		Neuronal		Glial	
Phospholipids (μmol/mg protein)	NIE 115	MT4	MT17	MT9	MT16
	0.170	0.168	0.165	0.141	0.125
14:0	2.0	1.1	0.8	1.3	1.4
16:0	19.0	16.9	15.1	19.6	18.3
16:1	8.8	10.9	7.2	11.2	9.2
18:0	9.8	16.4	17.0	12.9	12.7
18:1	38.8	35.8	37.8	32.1	35.3
18:2	2.0	2.4	3.1	1.9	2.2
18:3+20:1	3.5	1.2	1.4	1.2	1.4
20:2	1.3	0.8	0.5	0.2	0.6
20:3 (n-9)	1.8	1.0	0.6	1.3	1.4
20:3 (n-6)	1.0	1.0	1.1	0.8	0.7
20:4 (n-6)	4.6	5.5	5.9	7.7	7.1
20:5 (n-3)	1.5	1.0	1.2	1.8	1.5
22:4 (n-6)	0.4	0.6	0.5	0.7	0.7
22:5 (n-6)	0.2	tr	0.1	tr	tr
22:5 (n-3)	2.5	2.7	3.9	3.4	3.7
22:6 (n-3)	2.5	2.8	3.6	3.8	3.8

Fatty acids are abbreviated in the usual manner, i.e., a number indicating the number of carbon atoms followed by number of double bonds of the molecule; the "n" refers to the number of carbon atoms between the methyl end of the molecule and the first double bond as determined by comparison of retention times with standards. Results are expressed as weight percentages. Tr: not measurable amounts.

TABLE II

Phosphoglyceride Acyl Group Distribution in Normal Nerve Cells in Culture

			Glial cells of mouse			Neuron-enriched cultures	
	Glial cells of newborn rats		From embryo	From newborn animals		Rat embryo	Mouse embryo
Days in culture	14	28	14	15	70	7	5
Phospholipids (μmol/mg protein)	0.215	0.228	ND	0.263	ND	0.390	0.335
14:0	0.8	0.8	1.2	0.3	tr	0.1	1.7
16:0	22.0	24.1	30.7	23.4	34.3	13.3	27.0
16:1	4.1	5.1	9.2	7.0	3.0	1.4	7.4
18:0	13.6	13.7	12.0	14.6	12.7	21.7	17.5
18:1	29.8	22.6	26.0	29.2	24.2	21.8	22.1
18:2	0.8	0.9	tr	tr	2.0	1.0	0.2
18:3+20:1	0.9	0.6	0.3	0.7	1.0	1.5	0.2
20:2	tr	tr	0.2	0.3	tr	0.4	tr
20:3 (n-9)	1.8	0.8	2.3	1.9	0.2	0.5	0.5
20:3 (n-6)	0.8	0.3	tr	0.5	0.2	1.2	0.5
20:4 (n-6)	14.2	18.1	9.4	12.0	11.6	17.3	9.2
20:5 (n-3)	tr	tr	tr	tr	0.5	0.6	tr
22:4 (n-6)	1.9	1.5	1.6	1.6	1.5	1.8	2.2
22:5 (n-6)	0.6	0.7	0.3	0.3	tr	0.3	0.6
22:5 (n-3)	2.1	1.9	1.9	2.6	2.1	5.3	2.5
22:6 (n-3)	6.5	8.6	5.1	4.9	5.9	11.8	8.3

Same abbreviations as in Table I; nd = not determined.

of cells in primary cultures in Table II. The embryologic origin of the cells (glial or neuronal) has no influence on this distribution. The characteristic of cell growth, however, is of particular importance, and striking differences can be observed between primary cell cultures ("normal cells") and cell strains grown in continuous lines ("tumoral cells"). The tumoral cells contain higher levels of monounsaturated fatty acids and of linoleic acid than the normal cells do. These differences are compensated in normal cells by an increase in C-20 to C-22 polyunsaturated fatty acids and in some cases by an increase in saturated fatty acids. Considering individual acyl groups, the main differences concern octadecenoic, linoleic, arachidonic, docosatetraenoic and docosahexaenoicacids. During aging of primary cells in culture from 15 to 70 days, several changes occurred (increase in palmitic acid, decrease in palmitoleic acid). None of these changes was similar to the changes observed after transformation.

DISCUSSION

Very similar results with those reported here already had been observed in other tumoral nerve cell strains: NN and C6 glioblastomas (18) and the M1 clone of mouse C1300 neuroblastoma (19).

Two hypotheses can explain such a difference between tumoral and normal cells. Nutritional fatty acids are provided to the cells by the fetal calf serum with which they grow. The fatty acid composition of two different batches of this serum has been published (18) and we have already demonstrated, as numerous authors did (e.g., see 28), that the fatty acids present in serum are incorporated into cultured cells (18,19). Normal and tumoral cells were cultivated with the same serum, but the continuous established lines were in contact with serum for many generations, whereas most primary cultures were in contact with serum for a few weeks when harvested. This difference could explain the changes observed in fatty acid distribution between normal and tumoral cells, the tumoral cells resembling more closely the fatty acid distribution of serum lipids. However, the primary cultures showed no "adaptation" of their acyl group pattern to their nutritional environment after 2.5 months of culture, whereas some SV40-transformed cells were studied less than two months after transformation. It seems, therefore, that no nutritional artifactual difference can explain our observation.

The other hypothesis we can postulate is that the differences in fatty acid distributions are intrinsic to the nature of the cells, normal or tumoral. The special fatty acid pattern exhibited by tumoral cells may result from the existence of constraints (or from the suppression of constraints) exerted on the cell membranes. Numerous observations are in agreement with this hypothesis. From our data, it is evident that whatever the origin of the tumor cells (virus transformation, chemical induction, spontaneous cancerization), the same modifications in their acyl group distribution occurred. It has been shown that hepatomas (6-11) or brain tumors (12-14) contained also higher levels of octadecenoic acid and lower levels of C-20 or C-22 polyunsaturated fatty acids than the corresponding normal tissues. Cultured cells have not been studied in detail until now. Perkins and Scott (15) and Ruggieri et al. (16) have pointed out similar differences between the plasma membranes of 3T3 cells and the same cells after transformation by SV40 (SV3T3 cells). Burns et al. (17) observed in L1210 leukemia cells a 2.5-fold reduction of the proportion of arachidonate compared to normal lymphocytes. Simon (29), working on myeloid leukemia cells, compared the membrane fatty acids of an easily differentiable strain (D^+) to a nondifferentiable strain (D^-). D^+ cells contained much less oleic acid and more arachidonic and palmitic acids than D^- cells. In contrast, we found only one observation of a 5-fold increase of arachidonic acid proportion in cancerous white blood cells (30). We can therefore postulate that the differences we observed between normal and tumoral cells could be attributed to a fundamental change in structure and function of the membranes in the tumors. Since the same phenomenon is observed in several types of transformed cultured cells, as well as in several types of solid tumors and leukemias, it can be considered a general characteristic of cancer cell membranes, the significance of which remains to be elucidated.

Such differences in fatty acid compositions may be related to differences in desaturase activities. Indeed, a reduction has been observed of these enzyme activities in various tumoral cells (18,19,31-34). However, since all the fatty acids identified in the cell phosphoglycerides are found in the nutritive serum, changes in the uptake of serum lipids by the cells could also explain the differences observed in membrane acyl groups. No direct evidence permits a choice between these two mechanisms.

It is difficult to know if the changes we observed are really related to the growth char-

acteristics of the cells or if it is only a side effect of the transformation without any important physiological signification. Wood, considering the acyl group distribution in phospholipids from embryonic chick (35), suggested that the decreased levels of polyunsaturated fatty acids and the increased levels of octadecenoic acid in cancer cells may be related to the neoplastic process and not to the rapid cell proliferation. Moreover, the analysis of reverted cells from SV3T3 cells (16) does not show a reversal of the acyl group profile to that of normal cells. However, a continuous line of glial cells could be differentiated by 5′-bromodeoxyuridine (36); the acyl group composition of such differentiated cells, the growth of which is stopped, is very similar to that of primary cell cultures, the only difference being the level of docosapentaenoic acid (n-3) compared to that of docosahexaenoic acid (36).

It is also difficult to speculate about the actual effect of the acyl group differences observed between normal and tumoral cells on the physical properties of cell membranes. The low level of polyunsaturated fatty acids in tumoral cells is compensated by both an increase in the level of monounsaturated fatty acids and by a slight decrease in the level of saturated fatty acids. No conclusion, therefore, can be drawn from our data to predict changes in the microviscosity of cell membranes during transformation; such a physical study will be undertaken soon. Some authors have studied the microviscosity of hepatoma cell membranes (37) and leukemia cells (38) compared to that of normal corresponding tissues. A significant decrease of this parameter, evaluated by fluorescence polarization or by electron spin resonance, was observed in both cases. However, an increase in membrane lipid microviscosity was observed after virus transformation of 3T3 cells (39). The modification of membrane fluidity in tumor cells may be of particular importance in the understanding of several aspects of malignancy.

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Acyl Exchange between Oleoyl-CoA and Phosphatidylcholine in Microsomes of Developing Soya Bean Cotyledons and Its Role in Fatty Acid Desaturation

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ABSTRACT

Microsomes of developing soya bean cotyledons transfer oleate from oleoyl-CoA to phosphatidylcholine (PC) by two different mechanisms: one in which oleate transfer is accompanied by the release of free CoA and another which results in the exchange of oleate from oleoyl-CoA for unsaturated 18-carbon fatty acids of PC. The acyl exchange can be demonstrated only when bovine serum albumin is present in the incubation medium. ATP-dependent acyl-CoA synthetase is not involved in the exchange process, which apparently does not require any cofactors. In light of this exchange process, the oleate desaturase system was reinvestigated in order to determine what the actual substrate for this system is. Upon incubation of microsomes with high concentrations of [^{14}C] oleoyl-CoA, bovine serum albumin and NADH, it could be conclusively demonstrated that most oleic acid is desaturated while part of the PC molecule. The amounts of [^{14}C] linoleoyl-CoA formed could be explained entirely by the acyl exchange. The physiological significance of the acyl exchange system is discussed. A new method for separation of acyl-CoA from other lipids and free CoA using reversed phase column chromatography also is described.

INTRODUCTION

The true substrate for the oleate desaturase system in plants has been a matter of controversy. Oleoyl-CoA (1), oleoyl-phosphatidylcholine (2-5), or both (6,7) have been suggested as being the actual substrate. In an attempt to resolve this question, we examined the interaction of acyl-CoA with phosphatidylcholine (PC) in microsomes from developing soya bean cotyledons.

These microsomes have previously been shown to convert oleate into linoleate (5). Although oleoyl-CoA is an excellent substrate for desaturation, the kinetics of linoleate appearance indicate that oleate is first transferred from oleoyl-CoA to PC and subsequently desaturated (5). In this paper, an investigation of the mechanisms of acyl transfer between PC and acyl-CoA in microsomes from developing soya bean cotyledons is presented.

MATERIALS AND METHODS

Soya plants (*Glycine max* var. Fiskeby V) were grown outdoors in a controlled environment using a 16-hr light period at 20 C and 8-hr night at 10 C. Bovine serum albumin (fraction V, fatty-acid-free), oleoyl-CoA, CoASH, NADH and ATP were purchased from Sigma Chemical Company, St. Louis, MO. [1-^{14}C] Oleic acid (1.96 MBq/mol) and [1-^{14}C] linoleic acid (1.92 MBq/mol) were obtained from Radiochemical Centre, Amersham, England, and [^{3}H] CoASH (44.4 MBq/mol) from New England Nuclear, Boston, MA. SEP-PAK^{TM} C_{18} cartridges were procured from Waters Associates Inc., Farmingham, MA, and precoated silica gel 60 thin-layer chromatography plates from Merck, Darmstadt, GFR. PCS^{TM} scintillation liquid was purchased from Amersham Searle. [1-^{14}C] Oleoyl-CoA, [1-^{14}C] oleoyl-[^{3}H] CoA and [1-^{14}C] linoleoyl-CoA were prepared enzymatically with avocado acetone powder according to Galliard and Stumpf (8), except that gluthathione was replaced by 1 mM mercaptoethanol in the incubation media. Purification of the radioactive acyl-CoA was performed with SEP-PAK^{TM} cartridges as described next.

Preparation of Microsomes

All procedures were done at 1-4 C. Pods of soya beans were harvested 15-20 days after flowering. The cotyledons were picked from the pods and, after removal of the seedcoat, were ground in a mortar with 2 parts (w/v) of 0.1 M sodium phosphate, pH 7.2, containing 0.25 M sucrose. The extract was passed through a double layer of Miracloth®, then diluted 5 times with the grinding medium and centrifuged at 18,000 × g for 20 min. The resulting supernatant was filtered through one layer of Miracloth® and centrifuged at 105,000 × g for 60 min. The 105,000 × g pellet, the microsomes, was resuspended in 0.1 M phosphate buffer, pH 7.2, with a small Potter-Elvehjelm homogenizer. This preparation was used either

fresh or after being stored frozen at -70 C.

Enzyme Assays

Assays were done at 30 C in a water bath with constant shaking. Each incubation contained bovine serum albumin (BSA), substrates, cofactors and microsomes (at the concentrations stated in the tables) dissolved in 0.1 M potassium phosphate buffer, pH 7.2, in a final volume of 1 ml. Incubations were started by addition of the microsomes.

Separation of CoASH, Acyl-CoA and Lipids Soluble in Chloroform

Reactions were terminated by the addition of 3 ml ice-cold 0.15 M acetic acid. When BSA was not present during the incubation, 10 mg BSA was added just before termination. After transfer of the mixture to a 50-ml conical flask which could be fitted into a vacuum evaporator, 15 ml methanol/chloroform (2:1) were added and the solution was shaken. An additional 5 ml chloroform and 5 ml water were added to obtain a biphasic system according to Bligh and Dyer (9). The lower layer, called the chloroform layer, which contained ca. 97% of the complex lipids and the free fatty acids, was removed with a Pasteur pipet. One ml H_2O was added to the upper phase, which contained essentially all the acyl-CoA. After shaking, the remaining chloroform was removed in a rotary evaporator at room temperature. The pH of the mixture was then adjusted to 7.4 by dropwise addition of 0.5 M NaOH with stirring and the flask was placed in an ultrasonic bath until aggregates of proteins were dispersed. The solution was thereafter passed through a SEP-PAK^{TM} C_{18} column. The eluate, designated FI, contained all the free CoA and soluble proteins and ca. 5% of the acyl-CoA present in the mixture. The column was rinsed with 15 ml water to remove remaining soluble proteins and water-soluble substances. Acyl-CoA was thereafter eluted from the column with 15 ml 0.4 M NH_4OH in methanol/water (4:1). The eluate, designated FII, was immediately mixed with 6.4 ml 1.1 M acetic acid and thereafter 11.25 ml chloroform was added and the mixture shaken. Most of the acyl-CoA was now recovered in the upper phase, whereas remaining complex lipids and free fatty acids partitioned into the lower phase. After removal of the lower layer, the upper phase was saponified with 1 g NaOH overnight, acidified with 2 ml 30% H_2SO_4, and extracted with 10 ml hexane. After methylation of the hexane phase with diazomethane, the fatty acid composition of the acyl-CoA was determined by gas chromatography (GLC) as described in the next section.

In those cases where intact acyl-CoA was required for analysis on thin layer chromatography (TLC) or for use as substrate, the FII fraction was instead immediately evaporated to dryness under vacuum at room temperature. The residue was then dissolved in water and extracted with chloroform/methanol according to Bligh and Dyer (9) to remove any traces of chloroform-soluble lipids. However, acyl-CoA was hydrolyzed in the alkaline NH_4OH solution to an extent of 10-20%, depending on the efficiency of the evaporator. Thus, the radioactive acyl-CoA substrates used contained corresponding percentages of free CoA.

Recovery of the [^{3}H]CoASH in the FI fraction was essentially quantitative, but ca. 5% of the acyl-CoA was eluted in the FI fraction. In the subsequent water rinse, an additional 4% of the acyl-CoA was lost. Elution of the remaining acyl-CoA by methanolic NH_4OH was quantitative. The method was tested using 0.1-500 nmol oleoyl-CoA, 3 mg microsomal protein, and 10 mg BSA. With amounts of oleoyl-CoA above 50 nmol, the recovery in the FII fraction was lowered and cross contamination of the FI fraction was elevated. The acyl-CoA fraction obtained after the final methanol/chloroform extraction was free from common microsomal lipids, as judged by GLC of the methyl esters of the fatty acids of the fraction. No oleic, linoleic or linolenic acids were detected in that fraction in incubations of microsomes in the absence of acyl-CoA. Small amounts of stearic, palmitic and shorter fatty acids of unknown origin were, however, detected. The purity of acyl-CoA was also confirmed by TLC after incubation with [^{14}C]acyl-CoA and various ^{14}C-labeled complex lipids. Over 99.5% of the radioactivity cochromatographed with authentic acyl-CoA in the two different TLC systems described in the next section.

The method has its obvious limitations, since it does not allow quantitation of the acyl-CoA and since the CoASH fraction is contaminated with some acyl-CoA.

Chromatographic Procedures

PC and lyso-PC were separated from the total lipids of the chloroform phase by TLC on silica gel plates in chloroform/methanol/acetic acid/water (170:30:20:7) and in chloroform/methanol/acetic acid/water (150:36:24:7), respectively. Free fatty acids were separated on silica gel plates in hexane/diethylether/acetic acid (70:30:1). The acyl-CoA was identified by TLC on silica plates using two systems: chloroform/methanol/acetic acid/water (170:30:20:7), in which acyl-Coa remains at the origin, and

n-butanol/acetic acid/water (5:2:3). Lipids were stained lightly with iodine and removed from the plates according to Stymne and Appelqvist (3) for scintillation counting or for methylation with sodium methoxide (10).

Fatty acids were analyzed as their methyl esters by GLC with methylated heptadecanoic acid as an internal standard. A glass column (2 m × 2 mm) containing 6% EGA on Anachrom ABS 80-90 mesh was used in a Varian Model 2100 gas chromatograph connected to a Hewlett Packard Model 3380 A integrator. PC was quantitated on the basis of its fatty acid content. The distribution of radioactivity among the fatty acids was determined by radio-GLC of the methyl esters, as previously described (3).

Liquid Scintillation Procedures

All samples were counted by liquid scintillation in 10 ml PCS™/xylene (2:1) using a Beckman LS-230 liquid scintillation system with an efficiency of 90% for ^{14}C and 32% for ^{3}H. The amount of [^{3}H]CoASH in the incubations was determined on the basis of the counts in the ^{3}H window of aliquots (1/50) of the FI fraction. Because the FI fraction contained some [^{14}C]acyl-[^{3}H]CoA, the counts were corrected based on the $^{14}C/^{3}H$ ratio of this fraction and the $^{14}C/^{3}H$ ratio of the FII fraction. The channel ratio of the acyl-CoA was determined by the ratio of ^{14}C Iso-Set window to ^{3}H-window of an aliquot (1/50) of the FII fraction. About 30% of the ^{14}C counts were counted in the ^{3}H window whereas all the ^{3}H counts were counted in the ^{14}C Iso-Set window.

RESULTS AND DISCUSSION

Metabolism of Oleoyl-CoA at High Concentrations

Microsomes from developing soya beans were tested in their capacity to incorporate [^{14}C]oleate in the presence of high concentrations of [^{14}C]oleoyl-CoA. Incubations in the absence of BSA demonstrated that the most active enzyme acting on the oleoyl-CoA was the thioester hydrolase, which yields free oleic acid (Table I). Only a small portion of the [^{14}C]-oleate was transferred to other lipids such as PC, phosphatidylethanolamine (PE) and triacylglycerols.

In the presence of 1% BSA, the thioester hydrolase was inhibited by 90%, as evident by the amount of [^{14}C]oleic acid remaining after 80 min of incubation (Table I). On the other hand, the incorporation of [^{14}C]oleate into PC was stimulated over 10-fold. The amount of [^{14}C]oleate found in other chloroform-soluble lipids was somewhat lowered (Table I). After 180 min of incubation in the presence of BSA, ca. 11% of the total fatty acids in PC consisted of [^{14}C]oleate (Table I).

Two mechanisms exist by which acyl groups can be transferred from acyl-CoA to PC. The first is the de novo synthesis of PC from its precursors via the Kennedy pathway. We can exclude any significant contribution by this pathway in our microsomal system, because no CDP-choline or other cofactors were added. The second mechanism is the acylation of lyso-PC. However, the amount of lyso-PC required to explain our findings is more than 20% of the total PC (Table I). It could be shown that the amount of lyso-PC found in the microsomes constituted maximally 4.5% of the total PC. If, though it seems unlikely, a small pool of lyso-PC is regenerated during the acylation of lyso-PC, lyso-PC could still be the acceptor for [^{14}C]oleate. The results in this case would be an accumulation of free fatty acids derived from PC.

TABLE I

Metabolism of [^{14}C]Oleoyl-CoA by Microsomes from Soya Bean Cotyledons[a]

	Incubation time (min)	[^{14}C] Fatty acids (nmol) in:			
		Acyl-CoA[b]	PC	FFA	Other lipids in $CHCl_3$ phase
BSA omitted	0	27	0.0	0.0	0.0
↓	20	23	0.2	2.9	1.1
↓	80	18	0.4	6.7	2.1
BSA added	0	27	0.0	0.0	0.0
↓	20	25	0.8	0.2	0.6
↓	80	20	5.0	0.6	1.2
↓	180	18	6.7	0.9	1.8

[a]Each incubation contained 27 nmol [^{14}C]oleoyl-CoA (400,000 cpm) and microsomes containing 31 nmol phosphatidylcholine.

[b]Calculated on the basis of the ^{14}C activity in the aqueous phase of the Bligh and Dyer extraction (see Materials and Methods).

A third possible mechanism for the incorporation of acyl groups into PC, suggested by Shine et al. (11), is a free acyl exchange. To test this hypothesis, incubations with high levels of [^{14}C] oleoyl-[^{3}H] CoA with microsomes in the presence of BSA were performed.

Incubations with [^{14}C] Oleoyl-[^{3}H] CoA

Possible microsomal reactions involving [^{14}C] oleoyl-[^{3}H] CoA are shown in Figure 1. Reactions A and D are catalyzed by thioester hydrolase, reaction B represents a nonexchange incorporation into glycerolipids, and reaction C an acyl exchange between acyl-CoA and glycerolipids. Reactions A and B will yield an amount of free [^{3}H] CoASH equal to the amount of [^{14}C] oleate transferred from acyl-CoA and an unchanged ^{14}C/^{3}H ratio in the remaining acyl-CoA. Reaction C will result in a nonstoichiometric release of CoASH and a lowered ^{14}C/^{3}H ratio in the remaining acyl-CoA. It should be noted that the combined action of reactions C and D is impossible to discriminate from reaction B using this approach.

In Table II, data from three incubations using high levels of [^{14}C] oleoyl-[^{3}H] CoA in the presence of BSA are presented. In all three cases, the amount of [^{3}H] CoASH released was ca. 25% of the amount of [^{14}C] oleate transferred from oleoyl-CoA to lipid and released as free fatty acids. Most of the [^{14}C]-oleate was incorporated into PC, and after 180 min of incubation, 11% of the total fatty acids in PC consisted of [^{14}C] oleate. The ^{14}C/^{3}H-channel ratio of the acyl-CoA was markedly

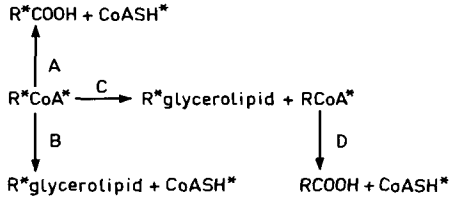

FIG. 1. Possible microsomal reactions involving oleoyl-CoA. R = acyl chain; * = radioactive label.

lowered in all incubations.

The fatty acid compositions of PC and of the acyl-CoA at zero and 180 min in two of the incubations are presented in Table III. The oleate content of the PC was more than doubled at the expense of linoleate and linolenate. The content of saturated fatty acids was essentially unchanged. Upon incubation of microsomes for 180 min in the absence of oleoyl-CoA, the fatty acid composition of PC was unchanged (data not shown). Analysis of the acyl-CoA showed that no linoleic or linolenic acid was present at zero time, whereas after 180 min, those acids represented over half of all unsaturated fatty acids in the remaining acyl-CoA (Table III).

Data from incubations using low concentrations of [^{14}C] oleoyl-[^{3}H] CoA relative to PC with and without BSA are presented in Table IV. In experiment 1, when BSA was added, the ratio of nmol [^{3}H] CoASH released to nmol [^{14}C] oleate transferred from [^{14}C] oleoyl-CoA was 0.5 after 15 min of incubation. The ^{3}H/^{14}C ratio of the remaining acyl-CoA also was markedly changed. In a corresponding incuba-

TABLE II

Metabolism of [^{14}C] Oleoyl-[^{3}H] CoA at High Concentrations and in the Presence of BSA by Microsomes from Soya Bean Cotyledons[a]

	Incubation time (min)	[^{14}C] Oleate (nmol) in: PC	FFA	Other lipids in $CHCl_3$ phase	Acyl-CoA[b]	[^{3}H] CoASH[c]	^{14}C/^{3}H Channel ratio[d] in acyl-CoA fraction
Experiment 1	0[e]	0.0	0.9	0.0	44	3.8	2.01
↓	180	16	5.7	3.7	18	10	1.82
Experiment 2	0[e]	0.0	1.7	0.0	44	3.8	1.98
↓	180	23	6.5	5.3	9.3	13	1.69
Experiment 3	0[e]	0.0	0.2	0.0	85	15	1.74
↓	180	42	7.4	15	20	32	1.48

[a]Experiments 1 and 2 contained 44 nmol [^{14}C] oleoyl-[^{3}H] CoA (^{14}C=246,000 cpm; ^{3}H=62,000 cpm) and experiment 3 contained 85 nmol [^{14}C] oleoyl-[^{3}H] CoA (^{14}C=291,000 cpm; ^{3}H=106,000 cpm). Experiments 1, 2 and 3 contained microsomes equivalent to 77, 108 and 194 nmol phosphatidylcholine, respectively.

[b]Calculated as nmol [^{14}C] oleate added minus nmol [^{14}C] oleate found in $CHCl_3$ phase.

[c]Calculated on the basis of the counts in ^{3}H window of fraction FI (see Materials and Methods).

[d]Determined in an aliquot of the FII fraction (see Materials and Methods).

[e]About 5 sec.

tion in the absence of BSA (Table IV, experiment 1), more [^{3}H]CoASH was released relative to the amount of [^{14}C]oleate transferred from [^{14}C]oleoyl-CoA (ratio 0.8), but the small amount of acyl-CoA remaining at the end of the incubation had a lowered ^{3}H/^{14}C ratio. When the ratio of oleoyl-CoA to PC was increased, incubation in the absence of BSA yielded a release of [^{3}H]CoASH that was equal to the [^{14}C]oleate transferred from oleoyl-CoA (Table IV, experiment 2). Furthermore, no change in the ^{3}H/^{14}C ratio of the remaining acyl-CoA was seen at the end of the incubation. Negligible acyl-CoA thioester hydrolase activity was observed in all incubations with low concentrations of oleoyl-CoA (Table IV).

These results demonstrate an acyl exchange between PC and acyl-CoA, as evident by the nonstoichiometric release of [^{3}H]CoASH, a change in the ^{14}C/^{3}H ratio of the remaining acyl-CoA, and a change in the fatty acid composition of PC and acyl-CoA. The oleate of oleoyl-CoA was principally exchanged for linoleate and linolenate of PC. If a minor exchange between oleoyl-CoA and oleoyl-PC exists, it was too small to detect in these experiments.

In the experiments with BSA added and high ratios of oleoyl-CoA to PC, the dominating mechanism of oleate transfer to PC appears to be via an acyl exchange. In incubations with low concentrations of oleoyl-CoA relative to PC in the absence of BSA, the major mechanism seemed to be incorporation without acyl exchange (Table IV). This conclusion can be drawn because of the very low thioester hydrolase activity under these conditions, which means that reactions A and D in Figure 1 can be neglected. The increase in [^{3}H]CoASH must therefore be due to a nonexchange incorporation of [^{14}C]oleate into the glycerolipids, i.e., reaction B in Figure 1. In fact, no acyl exchange seems to occur at all in experiment 2 in Table IV.

An acyl exchange between acyl-CoA and PC might be explained as follows: phospholipase A continuously regenerates a pool of lyso-PC, which serves as an acceptor for oleate from oleoyl-CoA. The free fatty acids released by the action of the phospholipase A would then be activated by an acyl-CoA synthetase. Such a process would require energy in the form of cofactors such as ATP.

In experiment 3 of Tables II and III, the amount of fatty acids exchanged between PC and acyl-CoA is at least 20 nmol. To test the capacity of the microsomes to activate free fatty acids, incubations with the same microsomal preparation used in that experiment were done with NH_4-[^{14}C]oleate, NH_4-[^{14}C]-linoleate, BSA, and CoASH in the absence and in the presence of ATP and $MgCl_2$. The results show that the microsomes do contain an active acyl-CoA synthetase which, in the presence of ATP and $MgCl_2$, is able to activate free fatty acids to their CoA esters and subsequent transfer to PC can occur (data not shown). However, no transfer of ^{14}C-fatty acids to either PC or to acyl-CoA was seen in the absence of ATP and $MgCl_2$, although the amount of microsomes used in these incubations was 5 times greater than that used in experiment 3 in Tables II and III. Thus, in the absence of ATP, the microsomes had no capacity to activate free fatty acids and, consequently, the acyl exchange does not seem to involve the ATP-dependent acyl-CoA synthetase.

The two different mechanisms reported here

TABLE III

Change in the Fatty Acid Composition of Phosphatidylcholine and of Acyl-CoA after Incubating High Concentrations of [^{14}C]Oleoyl-[^{3}H]CoA with Microsomes from Soya Bean Cotyledons[a]

		Fatty acids (nmol) in PC[b]					Fatty acids in acyl-CoA (%)		
	Incubation time (min)	16:0	18:0	18:1	18:2	18:3	18:1	18:2	18:3
Experiment 2	0[c]	50	15	20	73	58	100	0.0	0.0
↓	180	54	14	41	60	47	46	34	20
	Difference	+4	-1	+21	-13	-11	-54	+34	+20
Experiment 3	0[c]	85	16	32	141	114	100	0.0	0.0
↓	180	80	14	79	119	96	49	31	20
	Difference	-5	-2	+47	-22	-18	-51	+31	+20

[a]Experiments 2 and 3 are identical with those in Table II.

[b]Calculated on the amount of PC at the zero time of incubation.

[c]About 5 sec.

TABLE IV

Metabolism of Low Concentrations of [^{14}C]Oleoyl-[^{3}H]CoA by Microsomes from Soya Bean Cotyledons[a]

	Incubation time (min)	[^{14}C]Oleate (nmol) in:				[^{3}H]CoASH[c]	^{14}C/^{3}H Channel[d] ratio in acyl-CoA fraction
		PC	FFA	Other lipids in $CHCl_3$ phase	Acyl-CoA[b]		
Experiment 1	0[e]	0	0.0	0	1.9	0.2	2.01
↓ BSA added	15	1.5	0.0	0.2	0.1	0.9	1.48
BSA omitted	15	1.5	0.0	0.3	0.1	1.4	1.67
Experiment 2	0[e]	0	0.0	0	3.8	0.3	2.08
↓ BSA omitted	15	2.6	0.1	0.7	0.5	3.8	2.05

[a]Experiment 1 contained 1.9 nmol of [^{14}C]oleoyl-[^{3}H]CoA (^{14}C=220,000 cpm; ^{3}H=55,000 cpm) and microsomes equivalent to 216 nmol of phosphatidylcholine. Experiment 2 contained 3.8 nmol of [^{14}C]oleoyl-[^{3}H]CoA (^{14}C=440,000 cpm; ^{3}H=110,000 cpm) and microsomes equivalent to 77 nmol of phosphatidylcholine. When indicated, 10 mg of BSA was added.

[b]Calculated as nmol [^{14}C]oleate added minus nmol [^{14}C]oleate found in $CHCl_3$ phase.

[c]Calculated on the basis of the counts in the ^{3}H window of fraction I (see Materials and Methods).

[d]Determined in an aliquot of the FII fraction (see Materials and Methods).

[e]About 5 sec.

by which oleate can be transferred from oleoyl-CoA to PC, i.e., the acyl exchange and the acyl transfer, can be explained by the following hypothesis. The small amount of lyso-PC present in the microsomes (ca. 4% of the total PC) is rapidly esterified by the action of an acyl-CoA:acyl-glycero phosphocholine O-acyl transferase (EC .2.3.1.23). This reaction will yield free CoA. When all the lyso-PC is esterified, further incorporation of oleate from oleoyl-CoA will be accomplished by an acyl exchange. However, while the esterification of lyso-PC can take place with the acyl-CoA substrate in both micellar and monomer form, the exchange reaction occurs only when this compound is reversibly bound to a protein such as BSA. That would explain the large increase in the capacity of the microsomes to incorporate oleate from oleoyl-CoA upon addition of BSA to the incubation medium (Table I).

In view of the acyl exchange discovered here, as well as of questions as to whether oleoyl-CoA or oleoyl-PC is the true substrate for the oleate desaturase (1-7), a reinvestigation of oleate desaturation in microsomes from developing soya bean cotyledons was performed. Microsomes were incubated 30, 60 and 120 min with high levels of [^{14}C] oleoyl-CoA in the presence of BSA and NADH, an essential cofactor (5). At each incubation time, the distribution of radioactivity among the fatty acids in PC and acyl-CoA and the fatty acid composition of these lipids were investigated. At 30 min, no [^{14}C] linoleate was seen in acyl-CoA, whereas 12.3% of the radioactivity in PC was in linoleate (Table V). At 120 min, 2.8% of the radioactivity of the acyl-CoA was present in linoleate. Compared to the changes observed in the unlabeled fatty acids of PC and acyl-CoA (Table V), this finding can be completely accounted for by the acyl exchange.

The data must be interpreted as meaning that the desaturation occurs at the level of PC, unless linoleate from acyl-CoA is preferentially incorporated into PC before the oleate. Incubation of a mixture of [^{14}C] oleoyl-CoA and [^{14}C] linoleoyl-CoA with microsomes revealed that this was not the case (Table VI). Oleate and linoleate were transferred from their CoA esters to PC at about the same rate during a 5-min incubation.

Pugh and Kates (12,13) have shown that, in rat liver microsomes, eicosatrienoate contained in a phospholipid molecule can be desaturated to arachidonate. Eicosatrienoyl-CoA was also an efficient substrate for the reaction. A study of the time course revealed that a portion of the eicosatrienoate was transferred from acyl-CoA to phospholipids and during the first

TABLE V

Microsomal Metabolism of High Concentrations of [^{14}C]Oleoyl-CoA and the Concomitant Change in the Fatty Acid Composition of Phosphatidylcholine and of Acyl-CoA

Incubation time (min)	^{14}C Activity (%)[b] in:				Fatty acid composition (%) of:							
	PC		Acyl-CoA		PC					Acyl-CoA		
	18:1	18:2	18:1	18:2	16:0	18:0	18:1	18:2	18:3	18:1	18:2	18:3
0	0.0	0.0	100	0.0	22	4.1	8.1	36	30	100	0.0	0.0
30	17	2.4	72	0.0	20	3.6	11	36	29	89	6.7	4.1
60	39	4.0	43	0.5	22	3.8	15	33	26	64	22	13
120	58	3.6	14	0.4	20	3.8	19	31	26	52	30	18

[a]Each incubation contained 85 nmol of [^{14}C]oleoyl-CoA (850,000 cpm), microsomes equivalent to 194 nmol of phosphatidylcholine, 10 mg of BSA and 3 μmol of NADH.

[b]Expressed as a percentage of total recovered activity.

30 min of incubation the phospholipids were labeled with [^{14}C]arachidonate at a faster rate than the acyl-CoA fraction (14). The acyl exchange reported here raises the question of whether there really are two separate desaturases in rat liver microsomes, one having acyl-CoA as the substrate and the other utilizing a phospholipid, or whether there is only a phospholipid desaturase, which appears to be the case for the oleate desaturase studied here in microsomes from soya bean cotyledons. The arachidonic acid in the acyl-CoA would then be derived from acyl exchange with phospholipids.

Physiological Significance of the Acyl Exchange

It has been well established from in vivo experiments that oleate is incorporated into PC and that linoleate is transferred from PC to other lipids in various plant tissues (15-20). Some studies suggest that PC is also a donor of linolenate to other lipids (15,18,20,21). As previously mentioned, there is strong evidence for oleoyl-PC being the substrate for the oleate desaturase (2-5), a conclusion which has been confirmed in this study. The kinetics of linoleate desaturation in homogenates of developing soya bean cotyledons (21) suggests that the substrate for this desaturase is linoleoyl-PC or linoleoyl-CoA. On the basis of studies in which [^{14}C]acetate and [^{3}H]glycerol were fed to developing linseed and soya bean cotyledons, Slack et al. (18) have proposed that polyunsaturated fatty acids are transferred from PC to triacylglycerols via the diacylglycerol backbone of PC. This diacylglycerol transfer was later integrated into a scheme for the synthesis of various polar lipids (22). The discovery of the process whereby oleate in oleoyl-CoA is exchanged with linoleate and linolenate of PC with apparently no requirement for cofactors provides an alternative model for the transfer of unsaturated fatty acids between lipids. The oleate synthesized in the plastids (23-25) and ligated to CoA in the plastid envelope (26) would thus be transferred to microsomal PC by acyl exchange with the linoleate and linolenate synthesized from oleate in PC. The linoleoyl-CoA and linolenoyl-CoA formed could then be utilized in synthesis of various other lipids. However, there is also strong evidence that isolated chloroplasts are capable of desaturating both oleate and linoleate with monogalactosyldiacylglycerol as substrate (27), which indicates that the plastids, at least in part, are independent of the microsomes in the supply of polyunsaturated fatty acids.

On the basis of our present findings, one can only speculate as to whether lipids other than PC also undergo acyl exchange with acyl-CoA. It is of relevance in this connection that the fatty acid composition of acyl-CoA after an exchange reaction is not what would be ex-

TABLE VI

Metabolism of [^{14}C]Oleoyl-CoA and [^{14}C]Linoleoyl-CoA by Microsomes from Soya Bean Cotyledons[a]

Incubation time (min)	^{14}C activity (%) in PC[b]		^{14}C activity (%) in Acyl-CoA[b]	
	18:1	18:2	18:1	18:2
0	–	–	39	61
5	44	56	40	60

[a]The incubation contained 4.6 nmol of [^{14}C]-oleoyl-CoA (497,000 cpm) and 7.0 nmol of [^{14}C]-linoleoyl-CoA (742,000 cpm), 10 mg BSA, and microsomes equivalent to 113 nmol of phosphatidylcholine.

[b]Expressed as a percentage of the ^{14}C activity of each lipid. PC contained 4.9% and acyl-CoA 93.4% of the total activity after 5 min of incubation.

pected if exchange had occurred with PC only. In experiments 2 and 3 in Tables II and III, about the same amount of linoleate and linolenate is lost from PC, but the amount of linoleate in acyl-CoA is 1.6 times higher than the amount of linolenate. That must be due to selective acyl thioester hydrolases or to enzymes selectively transferring linolenate from acyl-CoA to glycerolipids other than PC or to an acyl exchange between acyl-CoA and various other lipids.

The possibility of changing the fatty acid composition of the PC in microsomes by incubation with large amounts of oleoyl-CoA in the presence of BSA might be of great methodological interest in studies of the effect of membrane composition and fluidity on the activity of membrane-bound enzymes.

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4-Demethyl-, 4-Monomethyl- and 4,4-Dimethylsterols in Some Vegetable Oils

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ABSTRACT

The content of 4-demethyl-, 4-monomethyl- and 4,4-dimethylsterols in 13 vegetable oils was found to vary between 0.10-1.4%, 0.01-0.08% and 0.02-0.29%, respectively. The largest amount of demethylsterols was found in maize and wheat germ oils, whereas the largest amounts of the dimethylsterols were found in olive and linseed oils. The predominating demethylsterols were sitosterol, campesterol, stigmasterol and Δ^5-avenasterol. Among the 4-monomethylsterols, obtusifoliol, gramisterol, cycloeucalenol and citrostadienol predominated, but usually more than 10 components were found in this fraction. The composition of the 4,4-dimethylsterol fraction was also rather complex, with the 9,19-cyclopropanesterols together with α- and β-amyrin predominating. In most of the oils, characteristically high or low percentages of some sterols were found, and a few specific sterols were also noted. A scheme useful for characterization is presented.

INTRODUCTION

Many papers have been published showing the content and composition of 4-demethylsterols in vegetable oils (1-5). The composition of the intermediates in sterol biosynthesis, 4-monomethyl- and 4,4-dimethylsterols (triterpene alcohols), is more complicated (5-11). The predominating 4-monomethylsterols in edible oils are obtusifoliol, gramisterol, cycloeucalenol and citrostadienol. These are Δ^8- and Δ^7-sterols except cycloeucalenol, which has a 9,19-cyclopropane ring in the steroid skeleton. Among the 4,4-dimethylsterol compounds with a cyclopropane ring, cycloartenol and 24-methylenecycloartanol predominate, together with the pentacyclic triterpenes, α- and β-amyrin. The structures, as well as other biochemical aspects of plant sterols, have been extensively studied (12 and loc. cit.). It has been proposed that sterols could be used as a tool for characterizing edible oils and to detect adulterations (13).

The aim of this study was to determine the sterol content and composition and find out whether characteristic components could be found to identify the oils. The capillary gas liquid chromatography (GLC) technique revealed very complex patterns with many components present in small amounts. The identification of all of these compounds was beyond the scope of this study.

MATERIALS AND METHODS

Commercially prepared crude oils of coconut, cottonseed, maize, peanut, rapeseed (1.5% erucic acid), soybean and sunflower were obtained from AB Karlshamns Oljefabriker, Karlshamn, Sweden. Samples of grape seed (Italian), linseed (Belgian cold-pressed), olive (Spanish), sesame (U.S. cold-pressed) and wheat germ oil (German cold-pressed) were bought at retail level.

The unsaponifiables were extracted from 1.0-2.5 g oil sample according to the AOAC procedure (14). Cholestanol was added as internal standard prior to saponification. The dry residue was dissolved in a few ml chloroform/methanol (2:1), diluted with hexane and stored at -25 C. A portion of the unsaponifiables equivalent to ca. 200 μg sterol was separated on silica gel thin layer chromatography (TLC). Small amounts of the extract were applied as references on both sides of the sample. After developing twice in hexane/diethyl ether/acetic acid (70:30:1), the reference bands were exposed to iodine vapor while the sample was protected with a glass plate. Demethyl-(R_f 0.29-0.43), monomethyl-(R_f 0.44-0.50), and dimethylsterols (R_f 0.51-0.57) were located, the silica gel was scraped off and extracted 3 times with diethyl ether. 5α-Cholestane was added as internal standard to the sterol fractions.

The sterols were converted to TMS ethers (15), extracted with hexane, dried with anhydrous Na_2SO_4 and stored at -25 C. The TMS derivatives were separated at 270 C on a 50 m WCOT glass capillary column coated with OV-1. The column efficiency was ca. N_{eff} = 65,000 (sitosterol, k = 2.5). Sitosterol was eluted after 22 min. A Varian GC 3700 with a solventless (falling needle) injector and a CDS 111 C computing integrator were used. The mass spectra were recorded on an LKB 2091 GC-MS system (70 eV, 200 C ion source temp.) equipped with an LKB Model 2130 data system.

RESULTS

Methodology

The GLC separation of the 3 sterol fractions is illustrated in Figure 1. Δ^5-Avenasterol is separated from sitosterol, which is impossible on packed OV-1 or SE-30 columns. OV-17, often used for GLC of sterols, does not separate Δ^5-sterols from the corresponding saturated stanol, not even on capillary columns. However, WCOT OV-1 columns separate cholestanol and cholesterol. Cycloeucalenol was overlapped in

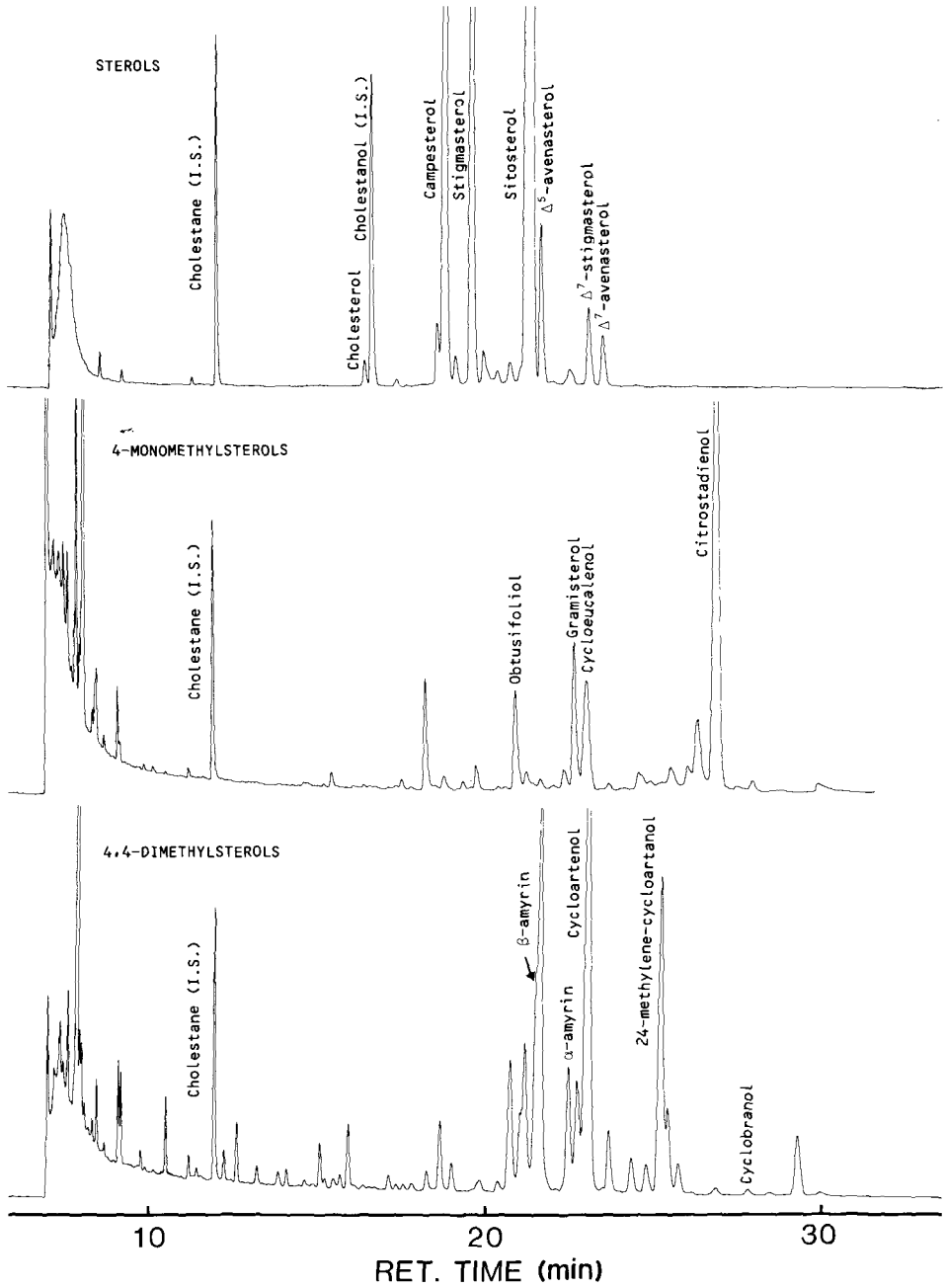

FIG. 1. GLC separation of 4-demethyl-, 4-monomethyl- and 4,4-dimethylsterols from a soybean oil on 50 m WCOT glass capillary column coated with OV-1.

most of the oils (see following), and several minor peaks consisted of 2 or 3 sterols. In olive and soybean oils, the separation of β-amyrin and component IV was very poor because of relatively large amounts of component IV in these particular oils. These incomplete separations are not improved on OV-17 capillary columns. Identification of the sterols was based on a comparison of mass spectra with literature data (7,11,16-23).

The reproducibility of the determination of the sterol contents was calculated after 6 consecutive analyses of a soybean oil. The coefficient of variation ($SD/\bar{X} \times 100$) was found to be 1.0, 2.2 and 1.6% for the 4-demethyl-, 4-monomethyl- and 4,4-dimethylsterol fractions, respectively. By comparing the detector response for pure campesterol, stigmasterol and sitosterol, losses due to column adsorption were observed. If this is accounted for, the 4-demethylsterol contents listed in Table I should be about 10% higher. The differences in sterol composition in Table II were +2% (abs) or less for sitosterol and stigmasterol, and not more than -1% (abs) for campesterol. The contents of 4-demethyl-, 4-monomethyl- and 4,4-dimethylsterols in the various oils are given in Table I.

4-Demethylsterols

The composition of the 4-demethylsterols is given in Table II. Four minor peaks (1-2%) accounted as "others" were irregularly registered between stigmasterol and sitosterol in the chromatograms. 24-Methylenecholesterol (main ions, [rel. abund.]; m/e 470 [90%], m/e 455 [32%], m/e 386 [98%], m/e 365 [23%], m/e 343 [36%], m/e 341 [30%], m/e 296 [38%], m/e 281 [54%]) and Δ^7-campesterol (main ions, [rel. abund.]; m/e 472 [100%], m/e 457 [15%], m/e 382 [–], m/e 367 [–]) were identified. Other 4-demethylsterols, which were registered after Δ^7-avenasterol, are also marked as "others."

4-Monomethylsterols

The gas chromatography-mass spectrometry (GC/MS) analyses of the 4-monomethylsterol fraction showed the predominance of 4 components, obtusifoliol, gramisterol, cycloeucalenol and citrostadienol, though their percentage distribution varied considerably (Table III). Cycloeucalenol was overlapped by another component (M^+: m/e 486 [100%]) in all oils, except coconut, linseed, olive and rapeseed oils. Thus the figures for cycloeucalenol are overestimated in the other oils. A remarkably high percentage (10%) of 31-norlanosterol was found in linseed oil. In grape seed oil, as much as 45% consisted of a sterol, which was tentatively identified as a Δ18-oleanene (fridelane-3-ol). From data obtained by MS, small amounts of this sterol also were found in the oils of cottonseed and olive. Fragmentation patterns and possible structures of the different components are presented in Table III.

4,4-Dimethylsterols

Cycloartenol and 24-methylenecycloartanol were the predominating 4,4-dimethylsterols in

TABLE I

Contents of 4-Demethyl-, 4-Monomethyl- and 4,4-Dimethylsterols in Some Vegetable Oils

Oil	4-Demethylsterols[a] (mg/100 g)	4-Monomethylsterols[b] (mg/100 g)	4,4-Dimethylsterols[b] (mg/100 g)
Coconut	102	7	20
Cottonseed	510	12	17
Grape seed	534	64	90
Linseed	471	39	246
Maize	1441	62	54
Olive	150	68	292
Palm kernel	140	3	22
Peanut	321	18	17
Rapeseed	954	7	18
Sesame	331	47	20
Soybean	394	25	40
Sunflower	494	78	33
Wheat germ	1425	59	59

[a]Cholestanol is used as internal standard.

[b]Cholestane is used as internal standard and the figures are corrected for losses in the preparation.

TABLE II

Composition of 4-Demethylsterols in Some Vegetable Oils

Oil	4-Demethylsterols (%)								
	Cholesterol	Brassicasterol	Campesterol	Stigmasterol	Sitosterol	Δ5-Avenasterol	Δ7-Stigmasterol	Δ7-Avenasterol	Others
Coconut	1	–	8	13	47	26	1	2	2
Cottonseed	tr[a]	–	7	1	86	3	tr	tr	3
Grape seed	tr	–	10	7	69	5	2	1	6
Linseed	1	1	27	8	42	13	–	1	6
Maize	tr	–	17	6	60	10	tr	1	6
Olive	tr	–	3	3	82	4	tr	tr	8
Palm kernel	1	–	10	13	69	7	–	–	–
Peanut	tr	–	18	8	63	7	1	1	2
Rapeseed	tr	10	33	tr	48	3	–	–	6
Sesame	tr	–	19	6	57	6	1	1	10
Soybean	tr	–	20	18	51	4	2	1	3
Sunflower	tr	–	7	7	59	8	6	5	7
Wheat germ	tr	–	19	4	60	7	2	2	5
RRT[b] of individual components on OV-1	1.88	2.07	2.35	2.52	2.87	2.92	3.21	3.29	

[a]Less than 0.5%.

[b]Cholestane is given the relative retention time of 1.00.

all the investigated oils except cottonseed, grape seed and soybean. In linseed and palm kernel oil, cycloartenol and 24-methylenecycloartanol accounted for 93% of the fraction, whereas the corresponding figure for cottonseed and grape seed was 33% and for soybean 39% (Table IV). Cottonseed was distinguished from the other oils by component VII (37%), grape seed by a high percentage (20%) of β-amyrin and soybean by component IV (13%). Cyclobranol, another 9,19-cyclopropanesterol, appeared in most of the oils, but only in significant amounts in the oils of grape seed (7%), olive (10%) and sesame (5%).

The pentacyclic triterpenes α- and β-amyrin were present in most of the oils. However, β-amyrin was not found in the oils of linseed or rapeseed. The mass spectra of the TMS derivatives of α- and β-amyrin were similar and characterized by a base peak at m/e 218, whereas other fragments showed very low intensities. These compounds were therefore very easy to recognize by GC/MS. Soybean oil was distinguished from the other oils, as the 4,4-dimethylsterol fraction of this oil contained 5 components with m/e 218 as base peak. Besides α- and β-amyrin, 3 such components eluted after cycloartenol and accounted as "others" in Table IV were found. Fragmentation patterns and possible structures of the different components are presented in Table IV.

TABLE III

Fragmentation Pattern of 4-Monomethylsterols and Their Distribution in Some Vegetable Oils

Peak no.	RRT[a]	Main ions, m/e (rel.inten.)	Possible structure
I	2.38	500(30%), 485(16%), 457(16%), 287(34%), 274(18%), 69(100%)	Unidentified
II	2.48	484(39%), 469(50%), 394(3%), 379(43%)	31-Norlanosterol (4α, 14α-Dimethyl-5α-cholesta-8, 24-dien-3β-ol)
III	2.79	498(86%), 483(100%), 469(12%), 399(7%), 393(70%), 455(4%)	Obtusifoliol (4α,14α-Dimethyl-24-methylene-5α-cholest-8-en-3β-ol)
IV	2.90	482(63%), 467(9%), 445(9%), 392(–), 377(–)	Δ^7-Sterol
V	2.93	484(100%), 469(35%), 394(30%), 379(14%)	Δ^7-Sterol
VI	3.09	498(65%), 483(60%), 408(14%), 393(50%)[c]	Unidentified
VII	3.14	484(44%), 469(22%), 400(40%), 394(12%), 379(13%), 357(46%)	Gramisterol (4α-Methyl-24-methylene-5α-cholest-7-en-3β-ol)
VIII	3.20	498(39%), 483(38%), 408(100%), 393(90%), 365(11%), 339(10%), 286(8%)	Cycloeucalenol (4α,14α-Dimethyl-24-methylene-9β, 19-cyclo-5α-cholestan-3β-ol)
IX	3.54	1) 498(70%), 483(10%), 408(12%), 393(12%) 2) 512(22%), 497(29%), 407(14%)	Δ^7-Sterol Δ^8-Sterol
X	3.58	484(55%), 469(26%), 400(88%), 394(15%), 379(14%), 457(100%)	4α, 24-Dimethyl-5α-cholesta-7,24-dien-3β-ol
XI	3.70	498(97%), 483(28%), 408(13%), 393(9%), 387(17%), 297(30%)	$\Delta^{7,22}$-Sterol
XII	3.86	500(75%), 485(26%), 410(13%), 400(100%), 395(10%), 359(18%), 357(42%), 269(38%)[e]	24-Ethyllophenol (4α-Methyl-24-ethyl-5α-cholest-7-en-3β-ol)
XIII	3.96	498(11%), 483(9%), 408(6%), 400(75%), 393(5%), 357(74%)	Citrostadienol (4α-Methyl-24-ethylidene-5α-cholest-7-en-3β-ol)
XIV	4.19	498(38%), 483(17%), 408(3%), 400(100%), 393(9%), 357(97%)	Δ^7-Sterol
XV	4.39	512(6%), 279(14%), 232(65%), 203(100%), 190(61%)	Fridelan-3-ol
Others			

[a]Cholestane is given the relative retention time of 1.00.

[b]Less than 0.5%.

[c]Other fragments, which indicated a sterol with a molecular ion at m/e 512, were also present.

[d]The following fragments: 486 (100%), 471 (15%), 396 (15%), 381 (10%), 269 (33%), which can refer to 24-methyllophenol (4α-methyl-24-methyl-5α-cholest-7-en-3β-ol), were also present.

[e]Other fragments, which were similar to those of citrostadienol, were also registered in some oils. The possible structure of this sterol is 4α-methyl-24-ethyl-cholesta-7,24-dien-3β-ol.

DISCUSSION

Previous investigations have shown that the 4-methylsterols display a much more complex composition than the 4-demethylsterols and therefore would be useful for characterizing vegetable oils (5-10). The present study confirmed these observations. Characteristically high or low percentages of some of the methylsterols were found and a few specific components were noted. These observations, together with the characteristics observed for the demethylsterols, are compiled in Table V.

The content of demethyl-, monomethyl- and dimethylsterols in the oils varied significantly. Comparatively large amounts of demethylsterols have previously been found in maize, rapeseed and wheat germ oil (1,3,24), which is in agreement with the present investigation. The data presented in Table I were based on an analysis with an internal standard and showed slightly higher values than literature data based on the digitonin method. Literature data also confirm that olive and linseed oils are rich in dimethylsterols (1,25,26). However, our sample of sunflower oil did not contain notably large amounts of dimethylsterols, as others have reported (25). Generally, higher contents of dimethylsterols than of monomethylsterols were found, with the exception of sesame and sunflower oils. In these oils, twice as much monomethyl- as dimethylsterols were found.

Vegetable oil (%)												
Coconut	Cottonseed	Grape seed	Linseed	Maize	Olive	Palm kernel	Peanut	Rapeseed	Sesame	Soybean	Sunflower	Wheat germ
–	2	tr[b]	1	–	tr	–	1	–	–	1	3	tr
tr	–	–	10	–	–	–	–	–	–	–	–	–
9	8	5	35	21	7	9	19	26	17	6	26	14
tr	3	2	1	tr	3	5	4	2	tr	tr	1	tr
2	1	1	4	2	tr	–	3	2	2	1	2	2
4	2	1	1	3	3	2	5	2	4	2	2	3
4	11	4	16	26	3	2	16	21	15	9	15	25
36	5[d]	4[d]	10	6[d]	14	33[d]	11[d]	17	12[d]	10[d]	3[d]	6[d]
2	4	1	2	2	5	4	–	–	1	–	1	–
–	–	3	–	–	2	5	–	–	8	–	1	tr
3	3	6	2	2	12	2	2	tr	8	3	2	3
2	8	4	2	4	6	6	4	2	5	7	2	5
33	42	11	11	29	22	19	23	16	15	44	38	30
2	1	2	–	–	5	1	–	–	4	1	–	1
–	1	45	–	–	3	–	–	–	–	–	–	–
3	9	11	5	5	15	12	12	12	9	15	4	11

TABLE IV

Fragmentation Pattern of 4,4-Dimethylsterols and Their Distribution in Some Vegetable Oils

Peak no.	RRT[a]	Main ions, m/e (rel.inten.)	Possible structure
I	2.78	498(5%), 483(5%), 393(6%), 204(100%), 189(22%)	Pentacyclic sterol
II	2.85	498(58%), 483(66%), 469(4%), 408(8%), 393(100%)	Δ^8-Sterol
III	2.90	498(8%), 483(2%), 393(2%), 218(100%)	β-Amyrin (5α-olean-12-en-3β-ol)
IV	2.96	498(50%), 483(60%), 469(3%), 408(3%), 393(100%)	Δ^8-Sterol
V	3.01	484(16%), 204(11%), 191(10%), 190(30%), 189(10%), 163(100%)	28-Nor-5α-olean-17-en-3β-ol
VI	3.09	498(12%), 483(4%), 393(6%), 218(100%)	α-Amyrin (5α-urs-12-en-3β-ol)
VII	3.14	1) 512(48%), 497(48%), 407(98%) 2) 498(81%), 483(42%), 393(94%), 385(35%)	24-Methylene-5α-lanost-8-en-3β-ol Δ^8-Sterol
VIII	3.20	498(7%), 483(10%), 408(100%), 393(42%), 365(34%), 339(38%), 286(18%)	Cycloartenol (9β,19-Cyclo-5α-lanost-24-en-3β-ol)
IX	3.39	512(58%), 497(46%), 469(35%), 428(12%), 407(78%), 385(70%)	Δ^7-Sterol
X	3.47	512(69%), 497(58%), 469(28%), 422(12%), 407(67%), 385(62%)	Δ^7-Sterol
XI	3.63	512(9%), 497(10%), 422(100%), 407(38%), 379(33%), 353(21%), 300(14%)	24-Methylene cycloartanol (24-Methylene-9β,19-cyclo-5α-lanostan-3β-ol)
XII	3.96	512(58%), 497(47%), 469(49%), 428(66%), 422(9%), 407(97%), 385(19%), 383(21%)	Δ^7-Sterol
XIII	4.15	512(8%), 497(9%), 422(100%), 407(36%), 379(35%), 353(36%), 300(11%)	Cyclobranol (24-Methyl-9β,19-cyclo-5α-lanost-24-en-3β-ol)
XIV	4.51		Unidentified
Others			

[a]Cholestane is given the relative retention time of 1.00.

[b]Less than 0.5%.

[c]A component, which amounted to 22% if accounted for, was registered. The mass spectra was different from those obtained from the sterols and this component has not been included in the dimethyl sterol composition.

[d]Approximations because of poor separation.

[e]The mass spectra showed the main ions at 498 (100%), 483 (15%), 408 (17%), 393 (23%), 369 (14%), which might refer to a Δ^7-sterol.

The methylsterol patterns in the present investigation showed several dissimilarities with the data presented by others (5-10). Part of this is due to the improved separation achieved by capillary columns; however, it is also interesting to compare data obtained from oils after different degrees of processing. Differences are quite obvious for sterols possessing a double bond at C 24(28), e.g., Δ^5-avenasterol, citrostadienol and 24-methylenecycloartanol. In a study by Itoh et al. (1,7) of 2 olive oils, one French and one Italian, Δ^5-avenasterol represented 2 and 12%, citrostadienol 36 and 59% and 24-methylenecycloartanol 33 and 59% of the appropriate sterol fractions, respectively, in the 2 oils. The corresponding values from the present study, 4, 22 and 31%, resemble those of the French olive oil. The degree of processing of the oils presented by Itoh et al. was not reported, nor was that of the olive oil in the present investigation known. Recently, we analyzed a crude olive oil obtained direct from a Greek plantation. It contained 17% Δ^5-avenasterol, 64% citrostadienol and 58% 24-methylenecycloartanol in the appropriate fractions. These results show that the sterol composition can be used to estimate the degree of processing of an oil. Spencer (27) has shown that the degree of processing and extraction could be evaluated by comparing the contents of 24-methylenecycloartanol and cyclobranol. The virgin oil lacks cyclobranol, which is formed from 24-methylenecycloartanol under acidic conditions by double bond rearrangements from the 24(28)-position in 24-methylenecycloartanol to the 24(25)-position in cyclobranol.

Vegetable oil (%)												
Coconut	Cottonseed	Grape seed	Linseed	Maize	Olive	Palm kernel	Peanut	Rapeseed	Sesame	Soybean	Sunflower	Wheat germ
1	–	6	tr[b]	–	2	1	–	2	2	5	tr	2
2	7	5	4	2	1	–	5	–[c]	3	8	2	3
5	7	20	–	1	4[d]	2	7	–	3	9[d]	5	12
1	3	tr	–	1	5[d]	–	1	–	–	13[d]	–	1
–	–	5	tr	3	2	–	–	–	2	–	–	–
7	2	3	1	2	3	1	7	3	6	5	11	7
4	37	4	1	2	2	1	6	6	5	5	8	7[e]
55	12	21	66	43	18	80	30	49	51	26	19	25
1	3	2	1	4	3	–	5	–	–	–	–	–
1	1	2	tr	1	6	1	1	2	2	1	1	5
22	21	12	27	40	31	13	35	37	16	13	48	33
–	–	2	–	–	8	–	tr	–	1	tr	–	1
1	1	7	–	–	10	tr	tr	–	5	tr	tr	2
–	2	2	–	1	–	–	1	–	1	3	2	–
–	4	9	–	–	5	1	2	1	3	12	4	2

In the present investigation, significant amounts of cyclobranol were found in 3 of the oils (grape seed, olive and sesame) bought at retail level, whereas no cyclobranol was detected in linseed oil (Table IV).

A component marked XII among the dimethylsterols (Table IV) and a component XI among the monomethylsterols (Table III) also seemed to be present preferentially in the retailed oils. Furthermore, it is notable that sterols with an ethylidene group at C-24 minimized deterioration in heated oil (28,29), and that the percentage of Δ^5-avenasterol in poppy seed decreased during storage (30).

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TABLE V

4-Demethyl-, 4-Monomethyl- and 4,4-Dimethylsterols Useful for Characterization of Some Vegetable Oils[a]

Oil	4-Demethylsterols	4-Monomethylsterols	4,4-Dimethylsterols
Coconut	26% Δ5-Avenasterol	36% Cycloeucalenol	
Cottonseed	86% Sitosterol, 1% stigmasterol	42% Citrostadienol	37% Component VII
Grape seed[b]		45% Component XV, 4% gramisterol, 4% cycloeucalenol	20% β-Amyrin, 7% cyclobranol
Linseed[b]	27% Campesterol, 13% Δ5-avenasterol	35% Obtusifoliol, 10% component II	250 mg/100 g oil, 93% cycloartenol + 24-methylenecycloartanol, 66% cycloartenol, <0.03% β-amyrin
Maize	1,450 mg/100 g oil	26% Gramisterol	
Olive[b]	82% Sitosterol, 3% campesterol	12% Component XI, 5% component XIV, 3% gramisterol	300 mg/100 g oil, 10% cyclobranol
Palm kernel	Δ7-Sterols absent	2% Gramisterol	80% cycloartenol
Peanut	No characteristics were found		
Rapeseed	33% Campesterol, 10% brassicasterol		
Sesame[b]		8% Component X, 8% component XI	5% Cyclobranol
Soybean[c]	18% Stigmasterol	44% Citrostadienol	13% Component IV
Sunflower	11% Δ7-Sterols		48% 24-Methylenecycloartanol
Wheat germ[b]	1,400 mg/100 g oil	25% Gramisterol	12% β-Amyrin

[a]Data from Tables I, II, III and IV.

[b]The oil was bought at retail level and the degree of processing was uncertain.

[c]Five components with a base peak at m/e 218 were found among the dimethylsterols.

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Effect of 2-Hexadecynoic Acid on Cultured 7288C Hepatoma Cells

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ABSTRACT

The effects of 2-hexadecynoic acid on the growth and lipid metabolism of cultured 7288 (HTC) cells have been evaluated. Growth was inhibited by the acetylenic acid: the LD_{50} was 35-85 μM as determined by two methods at low and high cell densities. Reduced growth did not result from damaged plasma membranes as determined by α-amino isobutyrate leakage. DNA synthesis was unaffected by the acetylenic acid and the effect on RNA and protein synthesis appeared to be secondary to the effects on lipid metabolism. The 2-hexadecynoic acid inhibited lipid metabolism of the HTC cells at least at two levels. Data from both mass studies and radioactive acetate distributions in cellular and media lipids indicated that fatty acid elongation and acylation, especially triglyceride synthesis, were inhibited.

A number of fatty acid analogs have been used in our laboratory which affect lipid metabolism in hepatomas and host animals (1-4). Recently, Wood et al. (4) reported a detailed study of the effect of 2-hexadecynoate on hepatic fatty acid metabolism. Low levels of 2-hexadecynoate fed to normal rats caused a decrease in body weight and in the appearance of essential fatty acid deficiency symptoms, whereas hepatoma 7288CTC-bearing host animals (5) were somewhat protected (4). This protective effect may have been due to the ability of the hepatoma to metabolize 2-hexadecynoate at an increased rate, but other factors may have contributed to the reduced toxicity of 2-hexadecynoate. The effects of potassium salt of 2-hexadecynoic acid (acetylenic acid; 16=1) on cultured hepatoma 7288C cells (HTC) are described in the present studies.

MATERIALS AND METHODS

Materials

The preparation of 2-hexadecynoic acid has been described (4). Thymidine-methyl-^{3}H (specific activity [sp act] 6.7 Ci/mmol), uridine-5-^{3}H (sp act 28.5 Ci/mmol) and α-amino isobutyrate (AIB) 1-^{14}C (sp act 51.6 mCi/mmol) were obtained from New England Nuclear, Boston, MA. [^{14}C] Amino acid mixture (sp act 50 mCi/mmol) was obtained from ICN Pharmaceuticals, Irvine, CA. [2-^{14}C] Acetate (sp act 54.7 mCi/mM) was obtained from Nuclear Chicago, Chicago, IL. Cell culture media and serums were obtained from Grand Island Biological Co., New York.

Cell Culture

The minimal deviation hepatoma cell line 7288C (HTC) was initially developed by G.M. Tompkins (6,7) and maintained in this laboratory in Swimm's 77 medium supplemented with 5% fetal calf serum and 5% calf serum (5/5) media in a 5% carbon dioxide atmosphere at 37 C using previously described techniques (8). The cell growth was estimated by one of the following methods: counting colonies resulting from single cells; counting the increase in cell numbers in a defined area; counting the trypsinized cells in a hemocytometer or by estimating cell protein by Lowry's method (9). Appropriate amount of acetylenic acid in 10% ethanol was sonicated with 5/5 media for 1 min prior to use.

Precursor Incorporation Studies

Deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis in HTC cells were measured by the incorporation of radioactive precursors in trichloroacetic acid (TCA) precipitable material by previously described methods (10,11). At indicated times, the acetylenic acid (buffer, in case of control) containing 5/5 media was replaced by 2 ml of 5/5 media containing either one μCi[^{3}H]-thymidine for 15 min, 2 μCi[^{3}H]uridine for 30 min or 2 μCi of [^{14}C] L-amino acid mixture for 15 min. The radioactive macromolecules were precipitated from the cells (solubilized in 1 ml of 1% SDS) by 10% ice-cold TCA. The radioactivity was determined by scintillation counting. Separate flasks were used for protein determination (9) that served as a basis for activity calculations.

The uptake and incorporation of [2-^{14}C]-acetate in lipid has been described previously (12). The acetylenic-acid-treated cells (3×10^5 cells/75 cm^2 flask) were exposed to 3 μCi-

[2-^{14}C] acetate for 24 hr. The trypsinized cells from duplicate flasks were pooled and lyophilized. The incorporation of [2-^{14}C] acetate in lipid was determined by counting the Bligh-Dyer extract (13) of the lyophilized sample. The free [2-^{14}C] acetate in the Bligh-Dyer extract was removed by converting it to [2-^{14}C] methyl acetate by diazomethane (14) and evaporated under the stream of nitrogen.

Leakage of α-AIB [1-^{14}C]

HTC cells (10^6 cells/25 cm^2 flask) were prelabeled with ^{14}C-AIB (4 μCi/4 ml of 5/5 media) for 3 days. At the end of 3 days incubation, 7% cpm were internalized by the cells. Washed, prelabeled cells (6×10^5 cells/25 cm^2 flask, which represent 4.6×10^4 cpm/flask) were treated with 68.8 μM acetylenic acid. An aliquot of media was counted at indicated times to determine the amount of ^{14}C-AIB leaked.

Uptake of α-AIB [1-^{14}C]

The uptake of ^{14}C-AIB in acetylenic-acid-treated cells (2×10^5 cells/25 cm^2 flask) was measured in one of Hank's-HEPES buffer (pH 7.4) containing 20 μl of 0.1 mM AIB and one μCi of ^{14}C-AIB at 37 C for 15 min. The uptake was stopped by washing the flask 3 times with 2 ml of ice-cold Hank's HEPES buffer. The radioactivity in the cell was determined after digestion in protosol.

Lipid Analyses

Lipid analyses of the cells and media were done using previously described methods (4,8). The radioactive total lipid samples were resolved by thin layer chromatography (TLC) in hexane/ether/acetic acid (80:20:1) on Silica Gel G. The phospholipids, which were retained at origin, were eluted from the gel by chloroform/methanol/water (60:45:12) mixture. Neutral lipids were separated into classes and were eluted by chloroform and ether. The extraction of radioactive lipid from the cells was optimized by addition of 10 mg carrier lipid to freeze-dried cells prior to Bligh-Dyer extraction (13). In some experiments, the methyl esters of fatty acids were obtained directly from total lipid extracts.

RESULTS

Effect on Cell Growth

The effect of acetylenic acid on cell growth was evaluated at 2 different cell densities: 5×10^2 cells/25 cm^2 flask and 1×10^5 cells/25 cm^2 flask. At lower cell densities, discrete colonies were observed after 7-10 days incubation. Complete inhibition was observed with a concentration of 120 μM of acetylenic acid and 50% inhibition (LD_{50}) of growth was achieved by 40-50 μM acetylenic acid (Fig. 1). An inverse correlation between the colony size and acetylenic acid concentrations was also observed. At higher cell densities, growth was followed by counting the number of cells in a defined area. Estimated LD_{50} by this procedure was 35-85 μM acetylenic acid and complete inhibition was observed >130 μM (Fig. 2). The biochemical effects of acetylenic acid were evaluated at 68.8 μM (20 μg/ml of 5/5 media).

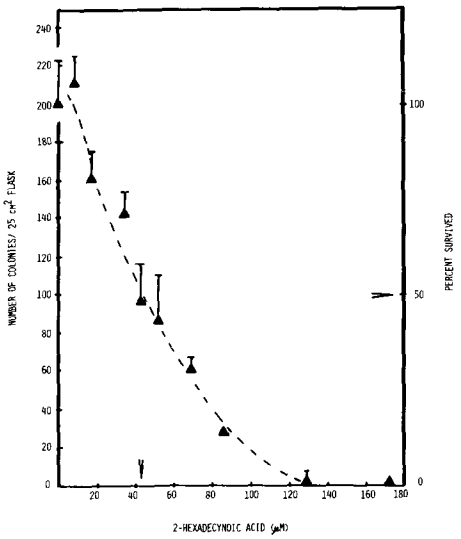

FIG. 1. Effect of 2-hexadecynoic acid on HTC cells colony formation. HTC cells (5×10^2) in a 25-cm^2 flask were treated with increasing concentration of 2-hexadecynoic acid in 5/5 media. The flasks were incubated for 10 days at 37 C 5% CO_2 atmosphere. The colonies were fixed by 10% formaldehyde (ice-cold), followed by staining with 0.25% methylene blue. The data represents the mean ± SD number of colonies in 3 flasks. The arrows correspond to the LD_{50} concentration (ca. 40-50 μM).

A careful examination of Figures 1 and 2 reveal that the inhibition of cell growth was not dependent on cell density and the inhibition showed a lag of ca. 24 hr. The control and treated cells looked similar under the phase contrast microscope for the first 4 days. However, about the fifth day, the treated cells start rounding up and eventually detach. Trypan blue staining indicated that the detached cells were dead.

Effect on Macromolecular Syntheses

Figure 3 shows that acetylenic acid treatment of HTC cell only affected RNA and

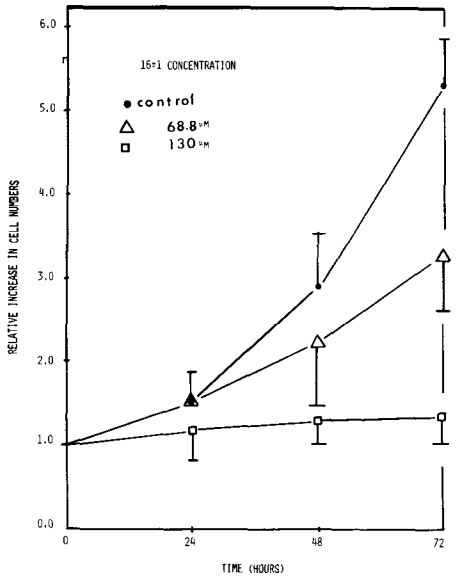

FIG. 2. Effect of 2-hexadecynoic acid on growth of HTC cells. Cells (1×10^5) in a 25 cm^2 flask were treated with increasing concentration of 2-hexadecynoic acid in 5/5 media. The cells were then counted under a microscope in a premarked area (ca. 2 mm square) every 24 hr for 3 days. The data represents the mean ± SD of the cell counts from 2 areas on 2 flasks for each concentration. Concentrations of 2-hexadecynoic acid up to 20 μM gave growth rates similar to control. Concentrations ranging from 35-85 μM showed an approximate 50% inhibition similar to 68.8 μM growth curve shown in the figure. Concentrations above 100 μM inhibited growth curve similar to the 130 μM growth curve shown in the figure.

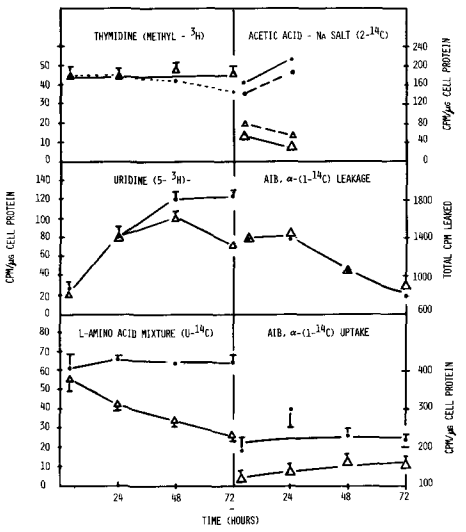

FIG. 3. Effect of 2-hexadecynoic acid on the incorporation of radioactive precursors in DNA, RNA, protein and lipid: on membrane leakiness (leakage of ^{14}C-α=AIB), and on the uptake of ^{14}C-α-AIB. The experimental conditions are described in Materials and Methods. The data in case of DNA, RNA and protein represents mean cpm incorporated ± SD/μg cell protein from duplicate flasks. The data for lipids were obtained from the cells pooled from duplicate flasks and represents cpm incorporated in lipids/μg cell protein. The dotted line shows the data from another set of experiments. The data for ^{14}C-α-AIB leakage represents the average of duplicate flasks. The data for ^{14}C-α-AIB uptake represents the mean CPM internalized ± SD/μg cell protein from duplicate flasks. Symbols 16≡ •—•; control △—△.

protein syntheses after 48 and 24 hr, respectively. In contrast, lipid biosynthesis was affected early. There was no effect on DNA synthesis. These data also suggest that primary effects of acetylenic acid are on lipid metabolism and the delayed effects on protein and RNA syntheses are secondary. Tata (15) has shown that, for proper functioning of ribosomal machinery during growth, a normal level of lipid synthesis is required.

Effects on Membrane and Uptake Process

Two other parameters which are often associated with cell death are the leakiness of plasma membrane and the defects in the uptake of small molecular weight substances. The leakage of 1-^{14}C-AIB, an amino acid analog taken up but not incorporated in proteins from prelabeled cells, was identical in the case of control and acetylenic-acid-treated cells (Fig. 3). These results indicate that acetylenic acid does not affect membrane leakage of the HTC cells.

The amount of AIB uptake/μg cell protein was less in acetylenic-acid-treated cells (60-70% of control). The reduced uptake of AIB probably is not related to cell death as the level of AIB uptake does not decrease with the time of incubation (Fig. 3). The reduced uptake of AIB in acetylenic-acid-treated cells could be attributed to such parameters as response to hormonal signals (16,17), and effect on contact inhibition of growth (18), both of which may be affected in an altered lipid environment. Indeed, Kaduce et al. (19) have shown a decreased AIB uptake upon increasing the fatty acid content of Ehrlich ascites cells.

Effect of Acetylenic Acid on Lipid Metabolism

The effects of acetylenic acid on lipid metabolism were examined by characterizing the lipids from media and HTC cells grown in presence of the acetylenic acid (68.8 μM) for

4 days.

Quantitative analysis of neutral lipid classes from the treated cells revealed that sterol esters are unaffected; cholesterol is slightly increased; and free fatty acids (FFA), monoglyceride, diglyceride and triglyceride levels are decreased (Table I). Although the quantity of sterol esters does not seem to change significantly, the fatty acids esterified to sterols do show some noticeable differences. The percentage of saturated fatty acids was elevated and 18:1 was drastically decreased in acetylenic-acid-treated cells. FFA levels of acetylenic-acid-treated cells show decreased amounts of saturates and monoenes, and a noticeable increase in the amounts of polyunsaturated fatty acids (Table I).

The changes in the phospholipid classes of HTC cells grown in presence of acetylenic acid are presented in Table II. Except for phosphatidylinositol, the level of all phospholipid classes was reduced (50-75% of control). Some general trend in the fatty acid composition of phospholipid classes was also noted. Palmitate percentages were lower in sphingomyelins and phosphatidylcholines whereas stearate percentages were increased in all phospholipid classes from acetylenic-acid-treated cells except two. Octadecenoate was decreased in all phosphoglycerides, whereas hexadecenoate percentages generally increased in the treated cells. All phosphoglycerides of the acetylenic-acid-treated cells showed an increased percentage of polyunsaturated fatty acid.

The 5/5 media contained sterol esters, cholesterol, FFA and triglycerides in amounts of 99.8, 19.9, 16.3 and 0.4 μg/ml of media. When HTC cells are grown in this media, the spent media had increased levels of cholesterol (27.1 μg/ml) and decreased levels of FFA (5.8 μg/ml), whereas the sterol ester content remained essentially unchanged (98.5 μg/ml). After cells were grown in presence of acetylenic acid, the media contained more FFA (31.2 μg/ml), fewer sterol esters (91.2 μg/ml) and the same level of cholesterol (19.1 μg/ml). Among neutral lipid classes examined, only FFA showed significant differences in fatty acid composition (Table III). Accompanying the doubling of the concentration of FFA in the media of the treated cells was an increase in 16:1 and a decrease in polyunsaturated fatty acids. Although excretion of lipids by HTC cells have been previously reported (8), the changes in the concentration and composition of the FFA may also be due to selective uptake, selective hydrolysis and preferential turnover. Phospholipid class composition of media did not change significantly (data not shown) in cells grown on acetylenic acid relative to control cells.

Fate of [2-^{14}C] Acetate in Presence of Acetylenic Acid

The distribution of radioactivity in various lipid classes was similar whether the cells were exposed to the acetylenic acid 4 or 24 hr before the addition of [2-^{14}C] acetate substrate. The data in Table IV represent the earlier time period and control values. The incorporation of

TABLE I

Fatty Acid Composition of Neutral Lipid Classes from HTC Cells Grown in Presence of 2-Hexadecynoic Acid (16≡1)[a]

Neutral lipid class		Quantity (μg lipid/mg dry wt cell)	Fatty acid percentages[b]					
			16:0	16:1	18:0	18:1	18:2	20:4
FFA	Control	41.8	24.4	5.5	17.4	43.7	2.6	–
	16≡1[c]	21.7	12.7	6.3	13.0	30.1	13.5	11.1
Sterol esters	Control	3.5	20.6	3.6	17.7	43.8	0.2	–
	16≡1	2.5	42.7	3.5	27.9	4.5	0.4	–
Triglycerides	Control	10.3	12.6	5.3	10.8	59.0	3.7	0.1
	16≡1[d]							
Others[e]								

[a]HTC cells (3 × 10^5 cells/75 cm^2 flask–initial) were grown in the presence of 68.8 μM potassium salt of 2-hexadecynoic acid for 4 days. The lipid analysis was performed on a composite sample pooled from 10 or more flasks. The variations in quantity of lipids and fatty acid percentages were <2% in duplicate determinations.

[b]The difference between the sum of percentages in any row and 100% represents minor amounts of other fatty acids not given in this table.

[c]Does not include the percentage of an unidentified metabolite of 16≡1.

[d]Quantities too small to permit analysis.

[e]The quantity (μg lipid/mg dry wt cell) of other neutral lipid classes: cholesterol (control, 13.6; 16≡1, 16.8), diglycerides (control, 5.9; 16≡1, 0.4) and monoglycerides (control, 6.7; 16≡1, 2.3).

TABLE II

Fatty Acid Composition of Phospholipid Classes Isolated from HTC Cells Grown in Presence of 2-Hexadecynoic Acid (16≡1)[a]

Phospholipid class		Concentrations (μG P/mg dry wt)	Fatty acid percentages							
			16:0	16:1	18:0	18:1	18:2	20:1	20:4	>20:4
SPH	Control	0.32	40.6	–	7.8	2.7	–	–	–	b
	16≡1	0.20	28.9	4.4	6.9	4.6	–	–	–	c
PC	Control	1.31	23.7	5.8	8.4	41.3	10.2	1.4	2.9	0.8
	16≡1	0.97	19.4	7.1	12.0	29.9	12.4	0.8	9.6	1.3
PI	Control	0.31	8.9	2.3	37.2	32.8	6.3	0.8	5.6	0.9
	16≡1	0.32	7.9	5.8	33.5	27.5	7.1	0.4	8.5	2.6
PS	Control	0.25	4.8	1.6	26.4	38.1	6.2	3.7	4.1	3.5
	16≡1	0.13	6.8	4.5	32.2	31.8	4.8	0.3	5.5	4.8
PE	Control	0.97	5.4	2.4	16.3	49.0	9.2	1.2	8.2	3.0
	16≡1	0.63	8.3	5.3	20.1	31.2	9.6	0.6	14.0	4.8
DPG + SF	Control	0.12	9.6	7.8	5.3	41.2	11.8	2.47	2.1	3.2
	16≡1[d]	0.08	12.4	5.9	8.3	18.2	14.2	–	5.1	5.7

[a]HTC cells (3×10^5 cells/75 cm^2 flask–initial) were grown in the presence of 68.8 μM potassium salt of 2 hexadecynoic acid for 4 days. The lipid analysis was peformed on a composite sample pooled from 10 or more flasks. The variations in quantity of lipids was <10% and in fatty acid percentages was <2% in duplicate determinations.

[b]22:0 = 7.5%, 23:0 = 1.2%, 24:0 = 18.0%, 24:1 = 18.6%, 25:0 = 0.3%, 26:0 = 0.1%.

[c]22:0 = 11.2%, 23:0 = 5.6%, 24:0 = 11.7%, 24:1 = 13.8%, 25:0 = 1.7%, 26:0 = 0.6%.

[d]Does not include the percentage of an unidentified metabolite of 16≡1.

Abbreviations: SPH = sphingomyelins; PC = phosphatidylcholines; PI = phosphatidylinositols; PS = phosphatidylserines; PE = phosphatidylethanolamines; DPG = diphosphatidylglycerols; SF = solvent front.

radioactivity in total lipids was reduced dramatically in the treated cells, but neutral lipids (NL) were affected to a greater extent. Analysis of NL fraction revealed that the treated cells had a lower amount of radioactivity in the triglycerides than control cells, which is in agreement with mass data (Table I). Spent medium lipids from both control and treated cultures contained a higher percentage of radioactivity than the cells, but the medium from the treated culture contained the highest proportion of radioactivity. Analysis of the neutral lipid classes (Table IV) indicate the medium from the treated cultures contained an increased percentage of radioactivity in the FFA, which is consistent with mass values for FFA given in the text earlier. The analysis of total fatty acids from HTC cells showed that most of the [2-^{14}C] acetate is incorporated in monoenes and saturated fatty acids. Detailed analysis (Table V) revealed that acetylenic acid caused accumulation of radioactivity in 16:0 and drastically reduced 18:0 counts. Likewise, 16:1 accumulated radioactivity, whereas 18:1 showed a large loss relative to control values.

DISCUSSION

This is the first study which deals with the effects of acetylenic acid on cultured cells. The growth inhibitory effects of acetylenic acid were not due to its detergent potential, as elevated levels of exogenous fatty acids, palmitate alone (580 μM) or a combination of palmitate, oleate and linoleate in equal amounts (total 420 μM) did not inhibit cell growth.

TABLE III

Composition of Free Fatty Acids (FFA) from Unused Media, Spent Media from Control Cells and Spent Media from Cells Grown in Presence of 2-Hexadecynoic Acid (16≡1)[a]

Media source	Quantity (μg lipid/ml media)	Fatty acid percentages							
		16:0	16:1	18:0	18:1	18:2	20:0	20:4	>20:4
Unused	16.3	11.2	4.4	8.6	36.6	14.6	1.8	5.2	7.5
Control	5.8	6.8	1.6	17.7	51.1	5.8	–	–	4.1
16≡1[c]	31.2	8.7	13.8	10.1	35.5	13.4	1.5	1.5	0.7

[a]HTC cells (10^6 cells/75 cm^2 flask–initial) were grown in the presence of 68.8 μM potassium salt of 2-hexadecynoic acid for 4 days. The lipid analysis was performed on a composite sample (30 ml media) pooled from 3 flasks. The variations in quantity of lipids and fatty acid percentages were <2% in duplicate determinations.

[b]The difference between the sum of percentages in any row and 100% represents minor amounts of other fatty acids.

[c]The percentage of free 16≡1 is not included.

TABLE IV

Distribution of Sodium Acetate [2-^{14}C] in Various Lipid Classes from Cells and Media in Presence of 2-Hexadecynoic Acid (16≡1)[a]

Lipid classes	CPM X 10^{-3}			
	Cells		Media	
	Control	16≡1	Control	16≡1
Phospholipids	13.6	6.9	56.1	42.9
Neutral lipids	15.3	3.7	55.6	34.9
	Percent of neutral lipid[b]			
Cholesterol	42	59	62.0	48.5
Free fatty acids	23	27	21.6	36.3
Triglycerides	27	5	13.3	11.6
Sterol esters	2	2	0.2	0.3

[a][2-^{14}C Acetate was added to the cells after 4 hr of 16≡1 treatment and incubation continued for 24 hr.

[b]The difference between the sum of percentages in any column and 100% represents minor amounts of other neutral lipids not given in this table.

[c]The counts represent the average of duplicate determinations with a variation of <10%.

TABLE V

Distribution of Sodium Acetate [2-^{14}C] in Various Fatty Acids of HTC Cells Grown in Presence of 2-Hexadecynoic Acid (16≡1)[a]

Treatment	Total cpm X 10^3	Chain length-(% of total)[b]				
		<16	16	17	18	>18
				Saturates		
Control	15.2	3.6	53.6	7.5	25.1	10.1
16≡1	11.2	3.0	81.6	6.8	5.4	3.1
				Monoenes		
Control	4.0	–	9.8	1.2	69.6	19.4
16≡1	1.4	0.2	42.8	5.2	34.3	17.5

[a][2-^{14}C]Acetate was added to the cells after 4 hr of 16≡1 treatment and incubation continued for 24 hr.

[b]The difference between the sum of percentages in any row and 100% represent the minor amounts of other fatty acids.

[c]The counts represent the average of duplicate determinations with a variation of <10%.

Growth curves were similar to the control in Figure 2. This is further supported by the inability of the acetylenic acid to induce membrane leakiness in HTC cells (Fig. 3). The results clearly demonstrate that the primary effects of acetylenic acid were on lipid metabolism (excluding cholesterol). The delayed inhibition of RNA and protein syntheses were secondary to the effects on lipid metabolism. The normal levels of cholesterol and DNA syntheses in acetylenic-acid-treated cells support the recent findings that synthesis of cholesterol is a prerequisite for DNA synthesis (20-23).

The acetylenic acid treatment affects lipid metabolism at least at two levels: biosynthesis of glycerides (especially triglycerides), and fatty acid elongation. The effects of acetylenic acid on glyceride biosynthesis appear to be multiple. Quantitative analysis of phosphoglycerides revealed that, except for phosphatidylinositol, the concentration of all phosphoglycerides was lowered in the acetylenic-acid-treated cells (Table II). The less dramatic effects on phosphoglycerides (also sphingolipids) biosynthesis are perhaps due to the slow turnover of phospholipids. The differences in fatty acid profiles of phospholipid classes may also be affected by changes in the level of FFA, monoglycerides, diglycerides and triglycerides of the treated cells. Both mass (Table I) and radioactive (Table IV) data indicate that triglyceride levels were affected most severely. The mass analysis of spent media lipids from treated cells indicated that the reduced triglyceride level in cells was not due to excretion of triglycerides in the media. It is therefore likely that acetylenic acid interferes with esterification of fatty acid to glycerol.

It is clear from the mass data (Tables I-III) that the acetylenic acid treatment affects the level of free and esterified fatty acids. These results are difficult to interpret because of interconversion of various lipid molecules. This is further complicated by the differential excretion of FFA to media in the presence of acetylenic acid. We therefore evaluated the incorporation of [2-^{14}C]acetate in total fatty acids of HTC cells, which were exposed to acetylenic acid for 4 hr (Table V). An elevated level of acetate incorporation in 16:0 and 16:1 and a reduced level of acetate incorporation in 18:0 and 18:1 was observed. These results suggest that acetylenic acid or its metabolite(s) inhibit elongation of saturated and monoene fatty acids. These effects on fatty acid elongation are specific to acetylenic acid or its metabolite, as unlike fibroblasts (24), the fatty acid biosynthesis in hepatoma was not inhibited by exogenous physiological fatty acids (25-27).

The effects of acetylenic acid on cultured cells were quite similar to those on intact normal animals with regard to fatty acid elongation. The inhibition of triglyceride biosynthesis in the cultured cells, however, differs from the increased levels of triglycerides in the liver of normal animals (4). These increased triglyceride levels in intact animals could be accounted for by the mobilization of lipids from other tissues. The lipid mobilization in response to hepatoma has already been reported from this laboratory (28).

This study indicates that the primary effect of 2-hexadecynoic acid on cultured hepatoma cells is on lipid metabolism. Further studies on the metabolism of the acetylenic acid will be

required to determine if the multiple effects are due to the original ynoic acid or a metabolite(s). Studies are currently underway to answer this question.

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Iodination of Docosahexaenoic Acid by Lactoperoxidase and Thyroid Gland in vitro: Formation of an Iodolactone

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ABSTRACT

In the presence of iodide, hydrogen peroxide and lactoperoxidase, docosahexaenoic acid (22:6ω3) was converted into iodinated compounds. The major product was identified as 5-iodo-4-hydroxy-7, 10,13,16,19-docosapentaenoic acid, γ-lactone, on the basis of ^{125}I incorporation, mass spectrometry, chemical modifications and proton nuclear magnetic resonance spectroscopy. Iodolactonization of docosahexaenoic acid occurred in the rat thyroid in vitro and was inhibited by the peroxidase inhibitor methimazole. These data indicate that formation of an idolactone constitutes one pathway of docosahexaenoic acid metabolism which could be expressed in tissues containing an iodide peroxidase.

INTRODUCTION

The lack of information concerning the metabolism of docosahexaenoic acid (22:6ω3) contrasts with the growing knowledge of the multiple metabolic transformations of arachidonic acid (20:4ω6). Docosahexaenoic acid is an important constituent of mammalian phospholipids; it is more abundant than arachidonic acid in the retina of several animal species (1,2), in the human cerebral cortex (3) and in ram spermatozoa (4). In the phospholipids of bovine thyroid, the amounts of arachidonic acid and docosahexaenoic acid are comparable (5). We have recently observed that the rat thyroid transforms arachidonic acid into an iodo-δ-lactone (6). In this report, we show that the major product of lactoperoxidase-catalyzed iodination of docosahexaenoic acid is an iodo-γ-lactone and that the same transformation occurs in the rat thyroid in vitro.

MATERIALS AND METHODS

Materials

Docosahexaenoic acid (purity >99%) was obtained from Nu-Chek-Prep, Elysian, MN. ^{125}I (15.8 mCi/μg) was purchased from Amersham, Arlington Heights, IL. Lactoperoxidase (EC 1.11.1.7) from milk was obtained from Sigma Chemical Co., St. Louis, MO.

Incubation of Docosahexaenoic Acid with Lactoperoxidase

Lactoperoxidase (2.9 μg/ml or 0.17 purpurogallin units/ml), docosahexaenoic acid (0.21 mM), (^{125}I) KI (0.4 mM, 0.5 μCi/ml) and H_2O_2 (0.26 mM) were stirred in phosphate buffer (0.1 M, pH 7.4) for 30 min at room temperature. After addition of sodium thiosulfate, the reaction mixture was extracted with 2 vol of ethyl acetate.

Incubation of Thyroid Lobes

White male Sprague-Dawley rats weighing around 250 g were sacrificed by ether inhalation and the thyroid lobes were incubated for 40-min periods, under air, at 37 C and with constant shaking (80 rpm). Nine lobes (70 mg wet wt) were incubated in 20 ml of Krebs-Ringer phosphate medium containing glucose (8 mM), ^{125}I (0.1 μCi/ml), KI (5 or 25 μM) and docosahexaenoic acid (0, 4, 21 or 107 μM). The incubation medium was then extracted with 2 vol of ethyl acetate.

Liquid Chromatography

Silicic acid chromatography was performed in 1.5-cm-diameter columns packed with a slurry in chloroform of Porasil A (35 to 75-μ particles; Waters Associates, Milford, MA), (column height: 18 cm). Elution was done with chloroform (85 ml) and then methanol (30 ml). Reversed phase-high pressure liquid chromatography (RP-HPLC) was done on a μBondapak C_{18} column (3.9 × 300 mm, 10-μ particles, Waters Associates). The injector (Model U6K) and the pump (Model 6000 A) were from Waters Associates. The samples were injected after dissolving in 100 μl methanol. Elution was done with methanol/water (80:20, v/v) at a flow rate of either 1 or 2 ml/min.

Chemical Modifications and Derivatizations

Alkali treatment: 100 μg of material was dissolved in 3 ml THF to which 2 ml of 0.5 M NaOH in water was added; after 3 hr at room temperature, the reaction mixture was diluted

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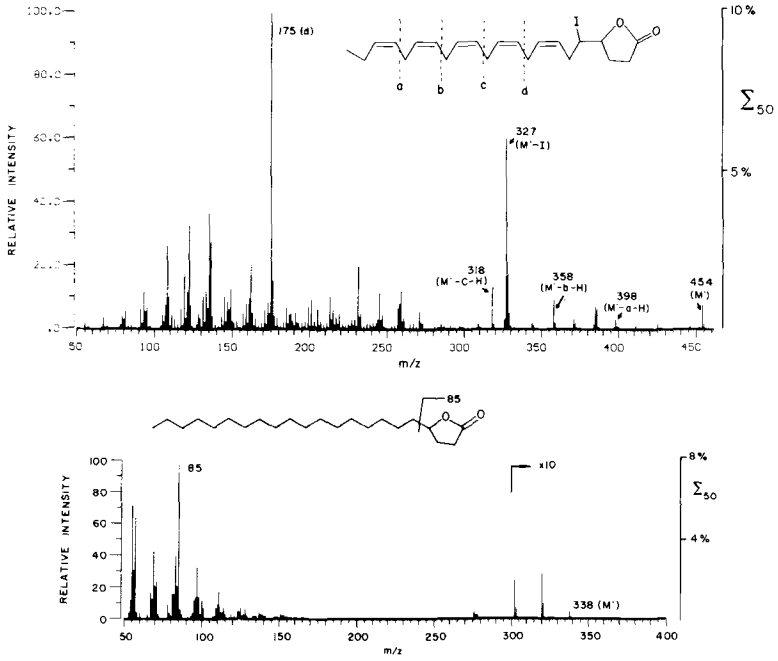

FIG. 1. Top: mass spectrum (12 eV) of the major product of lactoperoxidase-catalyzed iodination of docosahexaenoic acid (peak A of Fig. 2). Bottom: mass spectrum (70 eV) of hydrogenated compound A. The scale on the right (Σ_{50}) represents the intensity of % of the total ion current above m/z 50.

with water and extracted with 2 vol of ethyl acetate. Catalytic hydrogenation: 10 μg of material (ester or lactone) was dissolved in 0.5 ml ethanol to which 1 mg platinum oxide was added; hydrogen gas was bubbled for 2 min, after which the reaction mixture was diluted with water and extracted with diethyl ether. Methyl esters were prepared by reaction with excess ethereal diazomethane; trimethylsilyl ethers were obtained by reaction with excess bis-trimethylsilyl-trifluoro-acetamide (BSTFA) in pyridine.

Mass Spectrometry

Mass spectra were obtained either on a LKB 9000 magnetic instrument operated at 12 eV, using sample introduction by direct inlet probe (Fig. 1, top) or on a Hewlett-Packard combined gas chromatograph-quadrupole mass spectrometer (Model 5982A) operated at 70 eV (Fig. 1, bottom).

RESULTS

Incubation of docosahexaenoic acid with lactoperoxidase in the presence of hydrogen peroxide and iodide resulted in the formation of several iodinated products, which were resolved by silicic acid column chromatography (Fig. 2). One compound (A) largely predominated and was obtained in a 20–40% yield (3 experiments). RP-HPLC of compound A on a μBondapak C_{18} column (solvent: methanol/water, 80:20, v/v) revealed a single peak of ^{125}I

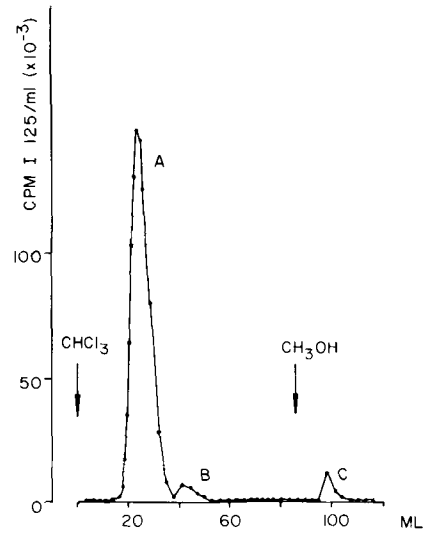

FIG. 2. Silicic acid column chromatography of the products of docosahexaenoic acid iodination catalyzed by lactoperoxidase.

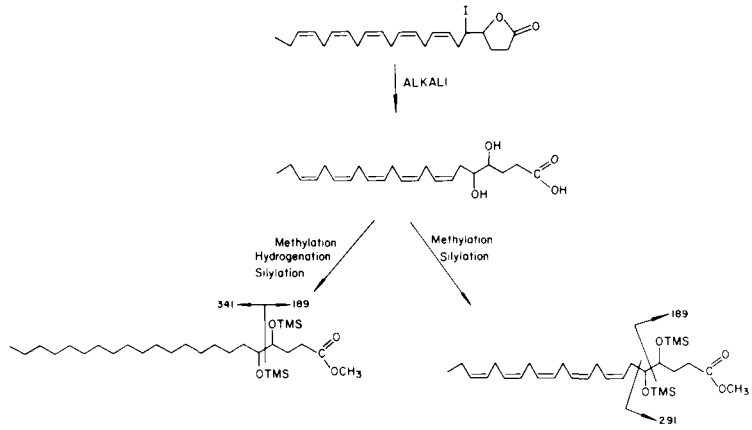

FIG. 3. Degradation of compound A (Fig. 2) at alkaline pH; gas chromatographic-mass spectrometric analysis of the main product. The major fragmentation pattern under electron impact is described.

radioactivity; the retention vol was 26 ml as compared to 23 ml for the iodolactone derived from arachidonic acid (6). Gas chromatographic analysis of compound A (underivatized) showed a single peak with an equivalent chain length C-25.7 (vs fatty acid methyl esters on OV-1). The electron ionization mass spectrum showed a molecular ion at m/z 454, the expected molecular weight of 5-iodo-4-hydroxy-docosapentaenoic acid, γ-lactone, and a prominent peak at m/z 327 (M-127), produced by the loss of iodine which is a typical fragmentation of alkyl iodides (7) (Fig. 1, top). The base peak at m/z 175 corresponds to the fragment represented by d in Fig. 1 (top). Many of the smaller peaks apparently result from the loss of various vinyl moieties together with a distal hydrogen: 390 (M-a-H), 358(M-b-H), 318(M-c-H). After catalytic hydrogenation, a peak with an equivalent chain length of C-23.6 (OV-1) was observed during gas chromatography. The mass spectrum showed a molecular ion at m/z 338 and a base peak at m/z 85, typical of γ-lactones (8) (Fig. 1, bottom). The decrease of the retention time caused by the hydrogenation and the shift from a major fragment ion at m/z 327 to a molecular ion at m/z 338 is consistent with the saturation of 5 double bonds and the substitution of hydrogen for iodine.

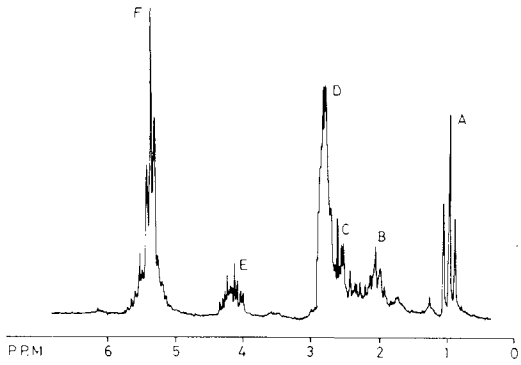

FIG. 4. Proton magnetic resonance spectrum of compound A. The spectrum was recorded on a JEOL FX-90Q Fourier transform spectrometer operated at 90 MHz. The sample was dissolved in deuterochloroform and tetramethylsilane was used as internal reference. The correspondence between the peaks of the spectrum and the protons of the molecule is as follows: A (0.97 ppm), 3H, C_{22}; B (2.07 ppm), 4H, C_3 and C_{21}; C (2.6 ppm), 2H, C_2; D (2.8 ppm), 1OH, C_6, C_9, C_{12}, C_{15} and C_{18}; E (4.2 ppm), 2H, C_4 and C_5; F (5.4 ppm), 10H, C_7, C_8, C_{10}, C_{11}, C_{13}, C_{14}, C_{16}, C_{17}, C_{19} and C_{20}.

Treatment with alkali degraded compound A. The mass spectrometric analysis indicated that the major product is a 4,5 diol (Fig. 3), presumably resulting from iodine displacement by hydroxyl anion and hydrolysis of the lactone ring. Since compound A was identified as a γ-lactone, iodine must be bound to C_5.

The proton magnetic resonance spectrum of compound A was consistent with the proposed structure of 5-iodo-4-hydroxy-docosapentaenoic acid, γ-lactone (Fig. 4). It also indicated that the configuration of remaining double bonds was not changed as compared to the precursor docosahexaenoic acid. The covalent structure of the major product of lactoperoxidase-catalyzed iodination of docosahexaenoic acid is thus 5-iodo-4-hydroxy-7,10,13,16, 19-docosapentaenoic acid, γ-lactone. The absolute configuration of the chiral centers (C_4 and C_5) has not been determined.

The release of this iodolactone by rat thyroid lobes incubated in vitro was measured

by the incorporation of ^{125}I followed by RP-HPLC purification. The recovery of this procedure was high (85%) and the retention volumes highly reproducible: 25.8 ± 1.2 ml (mean ± SD, n = 16). An excellent correlation between the results obtained by this method and those provided by a gas chromatographic-mass spectrometric (GC-MS) assay has been demonstrated in the case of the release of 6-iodo-5-hydroxyeicosatrienoic acid, δ-lactone by the rat thyroid (6). Figure 5 shows a typical RP-HPLC chromatogram of iodinated products released by the rat thyroid in the absence and the presence of exogenous docosahexaenoic acid. The nature of the iodinated components eluted in peaks I and III was not investigated. GC-MS analysis of component II revealed the existence of a peak having a retention time and a mass spectrum identical to those of 5-iodo-4-hydroxy-docosapentaenoic acid, γ-lactone, obtained by the lactoperoxidase reaction. The amounts of iodolactone released depended on the concentration of both iodide and docosahexaenoic acid (2 experiments, Fig. 6). Methimazole (200 μM) completely inhibited the release of the iodolactone.

DISCUSSION

The existence of iodinated lipids in cells has been reported, but their structures have not been fully characterized. Olefin diiodides were detected in thyroid lipids of dogs on a high iodine intake by use of proton nuclear magnetic resonance spectroscopy (9,10). In other studies, it has been shown that lactoperoxidase-catalyzed iodination of intact cells labels not only cell surface proteins, but also several classes of membrane lipids (11–16). The mechanism of these iodinations is presumed to involve either addition of iodine to double bonds or substitution of iodine to hydrogen in unsaturated and saturated fatty acids, respectively. In this report, we have shown that docosahexaenoic acid can be iodinated by intact cells by a third mechanism: iodolactonization.

The formation of 5-iodo-4-hydroxy-7,10,13,16,19-docosapentaenoic acid, γ-lactone, in the rat thyroid is likely to involve the activity of the thyroid peroxidase, since it is blocked by methimazole and mimicked by lactoperoxidase. There is, however, no evidence for a direct interaction between docosahexaenoic acid and the peroxidases. The role of these enzymes might be restricted to the oxidation of I^- into a reactive species, possibly I^+ (17,18), which

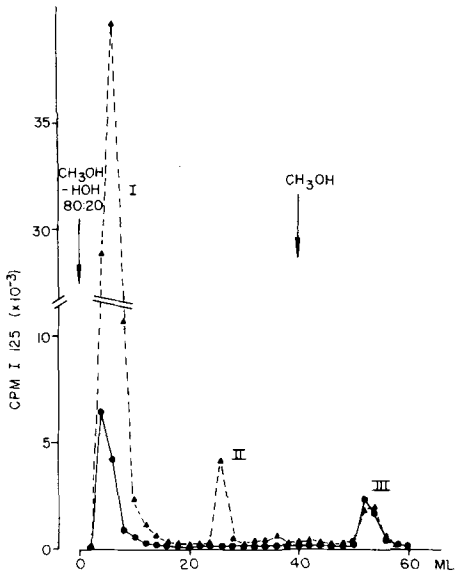

FIG. 5. RP-HPLC chromatogram of iodinated products released by the rat thyroid in vitro. —•—: control; ---▲---: docosahexaenoic acid (107 μM). Incubation and chromatography were performed as described in Methods.

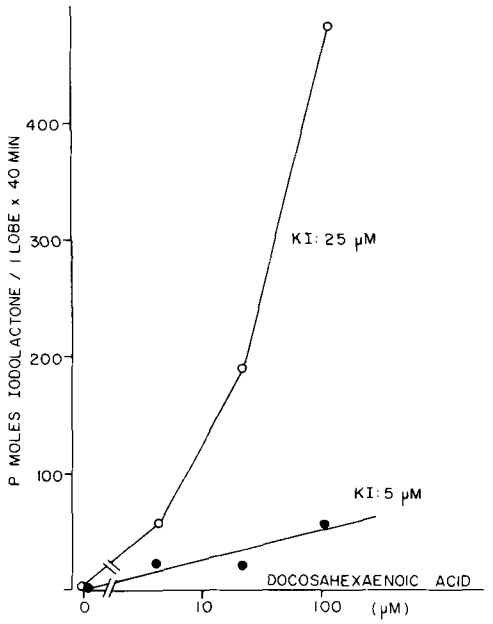

FIG. 6. Dependence of iodolactone formation by the rat thyroid on the concentrations of docosahexaenoic acid and iodide. The incubation of the thyroid lobes and the RP-HPLC of the incubation medium extract were performed as described in Methods. To determine the amounts of iodolactone released, the total molar amount of iodide added was multiplied by the ratio between the ^{125}I radioactivity in peak II (Fig. 5) and the total radioactivity added.

would then react with docosahexaenoic acid by a purely chemical process, analogous to the well known reaction of alkaline iodolactonization of $\beta, \gamma, \gamma, \delta$ and δ, ϵ unsaturated carboxylic acids (19–21). This transformation might be of general biological significance, since docosahexaenoic acid is abundant in phospholipids, e.g., in the thyroid (5), and since, in addition to thyroid and mammary glands, salivary glands (22), polymorphonuclear leukocytes (23) and oocytes (24) contain an iodide peroxidase.

The possible biological activity of 5-iodo-4-hydroxy-7, 10,13,16, 19-docosapentaenoic acid, γ-lactone, is presently unknown. It might be the product of scavenging by docosahexaenoic acid of excess iodine formed by the thyroid peroxidase or play a role as mediator of the inhibitory actions of excess iodide on the thyroid (25, 26).

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Thyroid Control over Biomembranes: VI. Lipids in Liver Mitochondria and Microsomes of Hypothyroid Rats

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ABSTRACT

The lipids of liver mitochondria prepared from normal rats and from rats made hypothyroid by thyroidectomy and injection with ^{131}INa contained similar amounts, per mg protein, of total lipids, phospholipids, neutral lipids and lipid phosphorus. Hypothyroidism caused a doubling of the relative amounts of mitochondrial cardiolipins (CL; to 20.5% of the phospholipid P) and an accompanying trend (although statistically not significant) toward decreased amounts of both phosphatidylcholines (PC) and phosphatidylserines (PS), with phosphatidylethanolamines (PE) remaining unchanged. The pattern of elevated 18:2 fatty acyl content and depleted 20:4 acyl groups of the mitochondrial phospholipids of hypothyroid preparations was reflected to varying degrees in the resolved phospholipids, with PC showing greater degrees of abnormality than PE, and CL showing none. Hypothyroidism produced the same abnormal pattern of fatty acyl distributions in liver microsomal total lipids as was found in the mitochondria. Hypothyroid rats, when killed 6 hr after injection of [1-^{14}C] labeled linoleate, showed the following abnormalities: the liver incorporated less label into lipids, and converted 18:2 not exclusively to 20:4 (as normals do) but instead incorporated the label mainly into saturated fatty acids. These data, together with the known decrease in β-oxidation, suggest that hypothyroidism involves possible defective step(s) in the conversion of 18:2 to 20:4.

INTRODUCTION

The slowed rate of ADP phosphorylation in the liver mitochondria of hypothyroid rats, when measured at 25-30 C, is one aspect of an abnormal velocity-temperature relationship (1-3). Because Arrhenius plot profiles of membrane-dependent catalytic and transport systems alter when membrane unsaturated fatty acyl contents are manipulated (4,5), we measured the phospholipid fatty acyl groups in liver mitochondria (2,3). Hypothyroidism increased the contents of linoleoyl (18:2) and decreased arachidonoyl (20:4) acyl groups and because of the major contribution of 20:4 to the unsaturation index (Σ[% fatty acyl group × number of unsaturated bonds]) decreased overall unsaturation. A similar correlation has been reported between the abnormal Arrhenius profile of State 3 respiration (succinate) and altered unsaturation (6).

The mechanism of these lipid changes is of interest in understanding thyroid hormone actions or effects on energy metabolism. Among the parameters shown to affect biomembrane dependency are phospholipid:protein ratios (7), relative amounts of phospholipid polar groups (8), the degree of fatty acyl unsaturation (4,5) and the cholesterol content (9). We compared the first three of these parameters in mitochondria from hypothyroid and control (euthyroid) rats. Because the pattern of unsaturated fatty acid abnormalities in mitochondria is consistent with a defect in Δ5-desaturation (2,3), and because a decreased rate of overall fatty acyl desaturation has been demonstrated in the livers of hypothyroid rats (10), we also examined the fatty acyl contents of microsomal total lipids. If impaired hepatic desaturase activity accounts for the altered mitochondrial contents of 18:2 and 20:4 acyl groups, we expected the microsomal lipids to show a similar abnormality, such as the one reported in the nuclear envelope (11) that suggested a desaturation defect rather than a lesion specific for mitochondria. To test in vivo conversion of 18:2 to 20:4, which involves a Δ6-desaturation, an acyl chain elongation and the Δ5-desaturation, liver lipids were analyzed 6 hr after injection of labeled linoleate.

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MATERIALS AND METHODS

Male rats weighing ca. 80 g were purchased thyroidectomized. The first series of control litter-mates and hypothyroid animals was obtained from Simonsen Laboratories, Inc., Gilroy, CA, and was maintained on a low-iodine, vitamin-enriched diet (Nutritional Biochemical Corp., Cleveland, OH); the controls received 0.0005% KI solution as drinking water, whereas the hypothyroid animals had 0.01% $CaCl_2$ in their water to prevent parathyroprivic tetany. Although growth retardation was evident in the hypothyroid group after 3 weeks, gas liquid chromatographic (GLC) analyses of mitochondrial phospholipid fatty acyl contents showed smaller abnormalities than those previously reported with hypothyroid rats additionally injected with $Na^{131}I$ (2,3). Further, experiments on the liver microsomal fatty acyl CoA Δ9-desaturase showed that half of a group of rats made hypothyroid by surgery alone were able to induce the enzyme after a fasting-refeeding cycle, whereas none of those additionally pretreated with $^{131}I^-$ could (12). We therefore report here the results comparing controls with thyroidectomized male rats (Spartan Res. Lab., Haslett, MI) treated as described plus an additional preliminary radiothyroidectomy through the ip injection of 0.5 mCi of ^{131}INa a few days after surgery (2,3). Animals were used for experiments at least later than 3 weeks after $^{131}I^-$ injection, and were allowed to feed ad lib up to the time of killing. One control and two hypothyroid animals were killed in each experiment to provide sufficient material for analyses.

After the rats were decapitated, livers were chilled in iced 0.25 M sucrose and homogenized in a 10% suspension using a Teflon-glass tube. The dense pellet that sedimented at 600 × g for 5 min was removed and the mitochondria were sedimented at 10,000 × g for 10 min, resuspended in iced 0.25 M sucrose solution, and resedimented. Liver microsomes were prepared from a 10,000 × g supernatant, as described elsewhere (13). The microsomal pellet was gently resuspended with a Teflon-glass homogenizer in 0.15 M Tris acetate, pH 8.1, leaving behind most of the glycogen (when present) and the microsomes were resedimented at 105,000 × g for 45 min. The pellets were resuspended in cold 0.25 M sucrose and diluted to 20 mg protein/ml. Protein was determined using 0.16% deoxycholate to dissolve membrane-bound proteins, and bovine serum albumin was used as reference standard (14).

Lipids were extracted from freshly prepared mitochondrial suspensions with a 2:1 mixture of chloroform/methanol plus 0.005% butylated hydroxytoluene, using 20 ml/ml suspension. This mixture was homogenized for 1 min with a Tekmar high-speed tissue disintegrator and extracted according to Folch et al. (15). Neutral and polar lipids were separated on a silica gel column by elution with chloroform and methanol, respectively. Aliquots of total lipids and polar lipids were weighed after removal of solvents at 40 C under N_2, and at 23 C under vacuum. Phospholipids were

TABLE I

Lipids in Rat Liver Mitochondria

	Controls (4)	Hypothyroids (6-9)
Total Lipids (μg/mg protein)	0.14 ± .02	0.12 ± .01
Phospholipids (μg/mg protein)	0.08 ± .02	0.07 ± .01
Neutral lipids (μg/mg protein)	0.06 ± .01	0.05 ± .007
Total lipid P (μmol/mg protein)	0.11 ± .01	0.11 ± .02
Phospholipids (% total phospholipid P)		
PC	55.4 ± 3.8	47.7 ± 2.9
PE	22.6 ± 2.7	25.5 ± 2.6
CL	11.9 ± 1.8	20.5[a] ± 2.2
PS	10.2 ± 2.3	6.5 ± 1.2

[a]$p<0.025$.

Mitochondria were prepared, extracted and analyzed as described in Materials and Methods. Aliquots of total lipids, phospholipids and neutral lipids were dried and weighed. The P contents of total lipids and of the phospholipids resolved by TLC (PC = phosphatidylcholine; PE = phosphatidylethanolamine; CL = cardiolipin, PS = phosphatidylserine and phosphatidylinositol) were measured. The numbers in parentheses after Controls and Hypothyroids denote how many separate measurements were done, each on materials from 1 control or 2 hypothyroid animals.

resolved on Silica Gel 60 TLC plates, developed in chloroform/methanol/glacial acetic acid/H_2O (100:60:16:8, v/v) according to Skipski et al. (16); locations were identified by very short exposure of the plates to I_2 vapor, after which the I_2 was removed under vacuum at 23 C. Four phospholipid classes were found: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS; this component reacted with ninhydrin but probably also included phosphatidylinositol) and cardiolipin (CL). The TLC plates were scraped to remove each of the phospholipids, and total phosphorus was measured (17). In separate experiments, each phospholipid was dissolved in benzene, concentrated, and subjected to methanolysis for 45-60 min in methanol-1% H_2SO_4 at 70 C. Fatty acid methyl esters were measured by GLC (Varian Model 3700) using a 10% Silar 10C column with linear temperature programming, 170-200 C at 3 C/min and a hydrogen flame ionization detector at 250 C. Results are given as area percentages.

To measure in vivo conversion of 18:2 to 20:4, rats were injected ip with 0.1 μCi/g body wt of 1-^{14}C-labeled linoleate (57 Ci/mol; New England Nuclear) dissolved in bovine serum albumin, 24 mg/ml. Animals were killed at 6 hr and the livers were extracted as already described. Solvents were removed under N_2 at 40 C. Aliquots of total lipids were: (a) assayed for ^{14}C by liquid scintillation counting; (b) resolved by TLC into neutral and polar lipid classes, which were counted; or (c) converted to fatty acid methyl esters and analyzed by a combined GLC and radio-assay instrument: a Packard Model 824 in combination with a proportional counter #894 that gives simultaneous peaks for mass and radioactivity.

RESULTS

The low-iodine, vitamin-enriched diet fed to both control and hypothyroid rats contained 7.9 g of lipid/100 g dry wt. The fatty acid contents were: 4.5% 14:0, 17.2% 16:0, 0.5% 16:1, 9.1% 18:0, 39.3% 18:1, 28.8% 18:2 and 0.6% 18:3 fatty acids. The only significant source of ω6 fatty acids was thus 18:2; therefore, any 20:4ω6 fatty acyl groups found in liver organelles must have been biosynthesized from linoleate.

The liver mitochondrial contents (on a wt/protein basis) of total extractable lipids, phospholipids and neutral lipids (by difference) were not significantly different from normal levels in hypothyroid rats (Table I). Total lipid phosphorus content/mg protein was identical in both groups, as well. However, the percentage contribution of each of the resolved phospholipid classes, as measured by percentage of total phospholipid P, showed that cardiolipin content was almost twice normal levels ($p<0.025$) in the mitochondria of hypothyroid rats; although the contents of the other phospholipids were not significantly changed, PC and PS decreased slightly.

The fatty acyl contents of the combined and the resolved phospholipids are shown in Table II. The total phospholipids extracted from the liver mitochondria prepared from hypothyroid animals showed the abnormalities previously described (2,3): increased contents of 18:2 and 20:3 acyl groups (evidence in ref. 3 and comparison with standards indicates that this is the 20:3ω6, not the 20:3ω9 isomer), decreased 20:4 acyl groups, decreases in the unsaturation index (-11%) and a decreased ratio of 20:4/18:2. In addition, the current analyses showed a significant 31% increase in the proportion of 18:1 fatty acyl groups. Among the separated phospholipid classes, mitochondria from hypothyroid animals contained PC with even more strikingly elevated 18:2 and 20:3 and depressed 20:4 fatty acyl moieties, and an even lower 20:4/18:2 ratio, than appeared in the total phospholipids. The PE in the hypothyroids similarly contained excess proportions of 18:2 and 20:3 acyl groups, but the depletion of 20:4 acyl groups was less severe, although still significant. Stearic acid content was depressed. The PS(PI) fractions showed no significant fatty acyl group deviations from normal levels, but did reveal a decreased 20:4/18:2 ratio. Cardiolipins from control and hypothyroid preparations were similar in all fatty acyl residue contents.

Comparison of the fatty acyl group compositions of unresolved and resolved mitochondrial phospholipids in Table II indicates that some polyunsaturated acyl moieties were lost during the TLC separation. Using the percentage contribution of each of the phospholipid classes (Table I) together with their fatty acyl compositions (Table II), the calculated recovery of 18:2 acyl residues was 92.0% in control preparations and 104% in hypothyroids. More 20:4 acyl groups were lost, but to a similar degree in controls (78.9% recovery) and hypothyroids (79.2% recovery). Taken together with the similarity of the total phospholipid

TABLE II

Fatty Acyl Contents of Total and Resolved Phospholipids in Liver Mitochondria Obtained from Control (C) and Hypothyroid (H) Rats

		Fatty acyl content (%)								
		16:0	18:0	18:1	18:2	20:3	20:4	22:6	Unsat. index	20:4, 18:2
Total PL	C	12.5 ± 1.1	19.6 ± 1.3	14.3 ± .9	17.0 ± .5	0.9 ± .3	29.0 ± 1.1	4.9 ± .7	201 ± 2.8	1.7 ± .07
	H	12.9 ± 1.1	17.1 ± 1.1	18.7[b] ± 1.1	23.2[d] ± 1.2	2.6[d] ± .3	19.3[e] ± .8	3.4 ± .4	179[d] ± 4.7	0.8[e] ± .07
PC	C	18.2 ± 1.7	22.5 ± 1.2	16.5 ± 1.6	13.4 ± 1.7	.7 ± .2	24.9 ± 1.8	2.1 ± .5	162 ± 4.1	2.1 ± .6
	H	17.2 ± 1.3	19.8 ± 1.4	19.0 ± 2.9	20.7[c] ± .8	3.2[b] ± .8	15.3[d] ± 1.3	1.6 ± .6	151 ± 6.6	0.7[a] ± .07
PE	C	14.9 ± 1.2	30.4 ± 2.5	15.6 ± 2.7	6.6 ± .7	.2 ± .1	25.2 ± .9	4.4 ± 2.4	190 ± 15.9	4.0 ± .5
	H	14.4 ± 1.5	22.5[a] ± .6	18.9 ± .7	11.6[e] ± .3	1.1[b] ± .3	22.0[a] ± .8	3.8 ± .5	176 ± 6.0	1.9[d] ± .08
CL	C	5.7 ± 1.4	4.9 ± .4	17.8 ± 1.4	54.5 ± 3.5	1.0 ± .4	3.5 ± .6		175 ± 2.2	0.1 ± .01
	H	8.0 ± 2.7	6.1 ± 1.9	17.8 ± 1.8	53.6 ± 5.0	1.3 ± .5	3.6 ± .5		164 ± 14.5	0.1 ± .01
PS	C	6.0 ± 1.7	49.4 ± 1.8	9.7 ± .9	2.3 ± .4		29.3 ± 1.9		150 ± 9.2	14.2 ± 2.9
	H	5.9 ± 1.1	40.6 ± 4.3	13.0 ± 2.5	4.2 ± .9		25.3 ± 2.6		180 ± 9.4	6.7[a] ± 1.0

$p<{}^{a}0.05; {}^{b}0.025; {}^{c}0.01; {}^{d}0.005; {}^{e}0.001$.

Liver mitochondria were prepared from control (C; N = 4) and hypothyroid (H; N = 4-6 pairs) rats, and aliquots of extracted phospholipids were analyzed for fatty acyl contents (total PL); aliquots were resolved by TLC into phosphatidylcholine (PC), -ethanolamine (PE), -serine (PS) and cardiolipin (CL) fractions, which were analyzed separately. Results are shown as mean ± SEM percent contents; minor components are not shown. The degree of fatty acyl unsaturation is indicated by the unsaturation index (= Σ [% fatty acyl X number of unsaturated bonds]). The ratio of 20:4/18:2 acyls is shown as an index of hepatic desaturation activity, because the diet contained no 20:4 fatty acid. Values of p were calculated from group comparisons by Student's t test.

contents in both groups of animals (Table I), the fatty acyl contents of mitochondria prepared from the livers of hypothyroid rats were abnormally distributed.

Liver microsomal fatty acyl composition was measured in extracted total lipids (Table III). The distribution of the polyunsaturated fatty acyl groups in the microsomes from hypothyroid rats was much like that in the mitochondrial phospholipids, with increased 18:2 and 20:3, decreased 20:4 acyl residues, and decreased overall unsaturation index (-11%). Because total microsomal protein, in g recovered/100 g wet wt of liver, was the same in hypothyroid rats (0.85 ± 0.10 SEM; n = 9) and control rats (0.88 ± 0.12 SEM; n = 8), the fatty acyl contents of liver microsomes of hypothyroid rats were abnormally distributed. Microsomal lipids have not yet been further resolved for fatty acid analyses; however, we reported elsewhere (18) that hypothyroidism doubles the cholesterol content.

The excess 18:2 and the depleted 20:4 in liver mitochondria (Table II; refs. 2,3), microsomes (Table III) and nuclei (11) of hypothyroid rats point to a defective conversion of 18:2 to 20:4. To examine this hypothesis in vivo, we injected [1-^{14}C]-linoleate into normal or hypothyroid rats and measured the hepatic incorporation and distribution of the label in lipids 6 hr later (Table IV). Hypothyroid rats had lower body weights and a lower liver:body-wt ratio than normals, but hypothyroid livers contained almost the normal proportion of lipid. The hypothyroid animals incorporated much less (7-50%) of the label into liver lipids but, as in normals, the label appeared in phospholipids and triglycerides. Hypothyroids converted much less 18:2 to the incorporated 20:4. While normal rats converted the 18:2 almost exclusively to 20:4, hypothyroid animals instead transformed large portions of the 1-^{14}C of 18:2 into saturated fatty acids; much more label was found in 16:0 and 18:0 than in 20:4. Taken together with the production of 20:4, the liver of the hypothyroid animal appeared to metabolize more of the 18:2 than did the liver of the normal rat.

DISCUSSION

The data obtained for hepatic organelle fatty acyl composition serve to eliminate some alternative mechanisms for the membrane abnormalities observed in hypothyroid rats. For example, the well known anorexia of hypothyroidism (19) might suggest a nutritional deficiency of essential fatty acids. Comparisons of food intake between controls and hypothyroids were not possible because the animals were fed ad lib. in open-bottomed cages. However, when hypothyroid rats were fasted 48 hr and then given access to a 20% sucrose solution, they ingested as much sucrose/g body wt as did control animals; the hypothyroids failed to induce the microsomal Δ9-desaturase (12) but maintained the relative excess of linoleate in their liver mitochondria and microsomes either when they were refed sucrose or when they were just starved (F.L. Hoch and T.R. Chavis, unpublished data). These observations make dietary intake an unlikely cause of the results obtained here in the hypothyroid rats.

Because dietary 18:2 was the only source of the ω6 fatty acids in these rats, the

TABLE III

Fatty Acyl Contents of the Total Lipids in Liver Microsomes Prepared from Control and Hypothyroid Rats

Fatty acyl	Controls (12)	Hypothyroids (12)
16:0	14.5 ± .5	15.6 ± .6
18:0	18.3 ± .5	18.7 ± .7
18:1	17.2 ± .7	17.6 ± .6
18:2	15.5 ± .4	19.6[b] ± .7
20:3	0.8 ± .07	2.3[b] ± .2
20:4	26.4 ± .9	17.3[a] ± .8
22:6	3.7 ± .4	3.9 ± .4
Unsaturation index	191 ± 2.3	170[b] ± 4.7
20:4/18:2	1.7 ± .09	0.9[b] ± .06

$p<{}^{a}0.005$; $^{b}0.001$.

Liver microsomes were prepared, extracted and analyzed as described in Materials and Methods and in Table II. N is shown in parentheses. Mean % of total fatty acids ± SEM are shown.

TABLE IV

Distribution of the [1-^{14}C] of Labeled Linoleate into Liver Lipids 6 hr after Injection

	Normal rats		Hypothyroid rats		
	A	B*	C	D	E
Body weight (g)	344	345	175	191	170
Liver: body weight (g/100 g)	3.8	3.7	2.8	2.7	3.1
Lipid: liver (mg/g)	48.3	44.5	32.6	36.8	44.2
^{14}C in total lipid (dpm/g liver)	256,782	143,261*	25,645	88,512	91,202
(% of dose)	4.53	2.49	0.33	1.10	1.32
^{14}C in phospholipids (% of total lipid dpm)	63.9	62.3	73.2	79.4	74.0
^{14}C in triglycerides (% of total lipid dpm)	32.9	34.5	24.8	19.1	24.0
^{14}C in fatty acid methyl esters of total lipids (% of total lipid dpm)					
16:0	0	9.4	43.9	14.3	24.1
18:0	0	0	13.3	8.0	2.4
18:2	74.8	66.0	42.8	65.8	59.1
20:4	25.2	24.6	0	11.8	14.3

Rats were injected intraperitoneally with [1-^{14}C]linoleate, 0.10 μCi/g body wt (except for normal rat B*, which received 0.022 μCi/g; corrected values for dpm/g liver are shown) and killed 6 hr later. Extracted total lipids of the livers were counted; aliquots were resolved by TLC, or subjected to methanolysis and resolved by GLC combined with radio-assay of the fatty acid methyl esters.

concomitant depletion of membrane 20:4 acyl groups implicates a defective biosynthesis from 18:2, or, given the adequacy of this conversion, an increase in the amount of phospholipids that are normally selectively acylated to favor 18:2 rather than 20:4 acyl groups. The latter process normally occurs in the synthesis of cardiolipins, which contain 55% 18:2 and only 6% 20:4 acyl groups, as compared to the 5-10% 18:2 and the 25% of 20:4 acyl residues found in the other mitochondrial phospholipid classes (Table II). An increase in the relative amount of CL would in this way contribute to the hypothyroid fatty acyl pattern, and this is what was observed (Table I); further, the CL fatty acyl content of hypothyroid mitochondria resembled that in controls. However, even in the hypothyroid mitochondrial membranes, cardiolipin fatty acids make only a minor contribution to the overall fatty acid distribution. In addition, CL is mostly a mitochondrial component (20) and so would not be significantly involved in the similarly abnormal fatty acyl compositions of microsomes (Table III) and nuclei (11). In liver mitochondria of hypothyroid rats, as in controls, PC and PE represented 75% of the phospholipid content (Table I), and their abnormally distributed polyunsaturated fatty acids predominated in producing the overall pattern (Table II). The severity of the abnormal distribution appeared in the order PC>PE>PS(PI)>CL. The exchange of the PL classes follows this order, with PC> PE and CL not exchanging at all (21). In contrast, the rapid replacement of PL, as measured by the appearance of injected labeled phosphate in hepatic organelle membranes, is faster in PE than in PC (22).

The observation that, in hypothyroid rats, the liver microsomes contained polyunsaturated fatty acyl residues that were distributed (Table III) in the same pattern that was seen in the liver mitochondria (Table II; refs. 2,3) and nuclei (11) suggests that this general pattern reflects a polyunsaturated fatty acid pool accessible to organelle membranes through acylation-deacylation reactions (23). It makes unlikely the alternative that the mitochondrion, which appears to possess some degree of autonomy in forming its own membrane phospholipids, as it does with polypeptide synthesis (22), is the source of the lipid defects. This conclusion is supported by observations that liver mitochondrial contributions to the synthesis of unsaturated fatty acids, i.e., the chain elongation, are insensitive to the hypothyroid state (10).

The diminished ability of hypothyroid rats to incorporate the label of [1-^{14}C] 18:2 into liver lipids (Table IV) may be connected with their decreased liver coenzyme A

concentrations (24). Little, if any, label was incorporated into saturated fatty acyl groups by normal rats under our conditions, whereas a major portion was incorporated by the hypothyroid animals. This excessive conversion appears surprising, as did Gordon and Goldberg's (25) observation that hypothyroid humans convert labeled 16:0 to CO_2 faster than controls, in view of the decreased fatty acid oxidation and high RQ in this condition. Gordon and Goldberg suggested that diminution and slowed turnover of the endogenous "active" FFA pool minimally dilutes administered fatty acid and that absolute rates of conversion are not necessarily increased. This explanation seems applicable to our data on 18:2 and to the apparent anomalies of absolute accumulation of 18:2 in liver lipids (Tables II and III), and depressed fatty acid synthesis in hypothyroidism (26). Theoretically, two alternatives might explain the pattern of label distribution in hypothyroidism: either defective conversion of 18:2 to 20:4 diverts the label toward acetyl CoA and 16:0 (and perhaps CO_2, which we did not measure), or accelerated β-oxidation of 18:2 to acetyl CoA competes with the conversion of 18:2 to 20:4. However, many observations since 1928 (27) have confirmed that β-oxidation is slowed in hypothyroidism, not accelerated.

Therefore, the most likely mechanism for the observed alterations of hepatic organelle membrane fatty acyl composition in hypothyroidism is a defect in the microsomal conversion of 18:2ω6 to 20:4ω6 fatty acyls, which involves a chain elongation and Δ6- and Δ5-desaturations. Hypothyroidism has been reported to slow microsomal chain elongation by -35% (10), although others find no change (28). However, the elongation 18:3ω6$\rightarrow$20:3ω6 normally proceeds 5.6 times faster than the Δ5-desaturation and 4.5 times faster than the Δ6-desaturation (29), and so would not seem to be rate-controlling in any case. Preliminary data on hypothyroid rats show decreased liver microsomal Δ6- and Δ5-desaturase V (maximal velocity, determined by extrapolation to infinite [S]) and unchanged Km (18); the Δ6-desaturase V but not the Km rises, and liver microsomal 20:4 acyl content falls within 4 hr after injection of L-triiodothyronine; Δ5-desaturase activity has not yet been measured this early after hormone injection.

deGomez Dumm et al. (28) report that hypothyroidism (using female rats fed propylthiouracil) significantly decreases the 18:2 content of liver fatty acids and does not alter either the 20:4 content or the microsomal Δ9- or Δ6-desaturase activities. Their data on fatty acid composition disagree with Ellefson and Mason's (30) on liver total lipids and ours (Tables II, III; refs. 2,3) on liver organelle fatty acids. We agree that hypothyroidism leaves the Δ9-desaturase activity unchanged but find that it blocks the normal dietary induction of this activity (12). As already noted, we find decreased Δ6-desaturase activity in hypothyroid rats. Presumably, the different in vivo and in vitro experimental conditions account for these apparent divergencies.

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Effects of Acute Administration of Chlorinated Water on Liver Lipids

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ABSTRACT

An acute administration of chlorinated water to rats caused "fatty liver" and indicated a more than 2-fold increase in liver triacylglycerols at 2 days after administration. The acyl group composition of triacylglycerols and phospholipids in both liver mitochondria and liver whole homogenate were also altered by the chlorine treatment. Among the phospholipid acyl groups, there was an increase in the proportion of 20:4 but a decrease in most other polyunsaturated acyl groups. The acyl group changes were more obvious with phosphatidylcholines than with phosphatidylethanolamines. Other phospholipids, including cardiolipin in the mitochondrial membranes, were not greatly altered. Both morphological and biochemical changes were maximum at 2 days after the treatment and were fully recovered after 10 days. The disturbance of a number of enzymatic processes in the liver membranes may account for a large part of the changes observed.

INTRODUCTION

It is known that haloalkanes, such as CCl_4 and $BrCCl_3$, may cause fatty liver and peroxidative decomposition of liver cell structural lipids (1,2). Recently, liver nuclear preparations were found to activate chloroform and carbon tetrachloride to reactive metabolites that bind to lipids and proteins (3). In a previous work from our laboratory (4), acute administration of sodium hypochlorite solution to rats resulted in accumulation of chlorinated hydrocarbons, especially chloroform, in liver and other organs. Either the hypochlorite ion itself or the resulting chlorinated hydrocarbons may produce the hepatotoxicity. In this study, we report the change of lipid content in liver and the alterations of fatty acid profiles in various lipid fractions of liver after an acute dose of chlorinated water in the form of a dilute solution of sodium hypochlorite.

METHOD AND MATERIALS

Adult male Sprague-Dawley rats (Charles River, Wilmington, MA) were divided into 4 groups with 4 in each group. Groups II, III and IV were each given, intragastrically, 5 ml of sodium hypochlorite solution containing 1% of equivalent free chlorine. The animals were sacrificed by decapitation at 2, 5 and 10 days, respectively, after treatment. Controls in group I were fed the same volume of saline 12 hr prior to sacrificing.

Analysis of Tissue Lipids

Whole liver homogenate. After decapitation, rat livers were weighed and samples were rinsed slightly with ice-cold saline solution. The tissue was homogenized in 5 ml of 0.8% NaCl, after which 5 vol of chloroform/methanol (2:1, v/v) was added and mixed thoroughly. After phase separation, the organic layer was taken to dryness by rotary evaporation, and the lipid residue was redissolved in 10 ml of chloroform.

Triacylglycerols. For isolation and separation of the neutral glycerides, aliquots of the lipid extract were spotted on Silica Gel G plates. A solvent system containing hexane/ether/15 N NH_4OH (80:20:0.1, by vol) was used to separate different neutral glycerides, cholesterols and cholesteryl esters. The phospholipids which are more polar would remain in the origin. After solvent development, thin layer plates were sprayed with 2′,7′-dichlorofluorescein, and lipid spots were visualized under UV. The triacylglycerol band was scraped from the plate and their acyl groups were converted to methyl esters by alkaline-methanolysis together with 50 μg of 20:0 methyl ester as an internal standard (5).

Phospholipids. For separation of the phospholipids, portions of the lipid extract were applied to another thin layer plate which was developed with a solvent system containing chloroform/methanol/15 N NH_4OH (135:60:10, by vol). After development, the plate was visualized by spraying with 2′,7′-dichlorofluorescein. Two types of glycerophospholipids, i.e., phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were taken for alkaline-methanolysis together with 20 μg of 17:0 as an internal standard. The fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) using a Hewlett-Packard Model

5860A which is equipped with dual flame ionization detectors and an automatic integration device. Stainless steel columns, 1.8 m x 3 mm id, packed with 10% EGSS-X on Gas-Chrom P, were used. The column oven was temperature-programmed from 175 to 205 C at 2 C/min. Carrier gas was nitrogen at ca. 20 ml/min.

Liver mitochondria. Mitochondria were isolated by the method of Schnaitman and Greenawalt (6), and the lipids were extracted in the same manner as the whole homogenate. Because a one-dimensional separation cannot resolve the cardiolipins from the PE, a two-dimensional thin layer chromatography (TLC) separation was applied, instead. The solvent systems were: I. chloroform/methanol/15 N NH_4OH (135:60:10, by vol); II. chloroform/methanol/acetone/glacial acetic acid/0.1 M ammonium acetate (140:60:55:3.5:10, by vol). Five types of phospholipids, i.e., cardiolipins, PE, PC, phosphatidylinositols (PI) and phosphatidylserines (PS), were taken for alkaline-methanolysis and subsequent GLC analysis.

RESULTS

The liver samples obtained from rats 2 days after the administration of an acute dose of sodium hypochlorite solution containing 50 mg equivalent free chlorine indicated obvious morphological changes resembling the "fatty liver" syndrome. However, the pale-colored appearance of the liver gradually disappeared. By 10 days after treatment, there was no obvious difference between treated livers and controls. The total triacylglycerols in each group were calculated relative to the internal standard, and a 250% increase was found in the samples at 2 days after treatment (Fig. 1). Corresponding to the morphological changes, the triacylglycerol level started to decline after reaching a peak at 2 days and at 10 days had reached control levels.

Analysis of the acyl group composition of liver triacylglycerols revealed the presence of small but significant amounts of long chain polyunsaturated fatty acids (PUFA) such as 20:4 and 22:6. As shown in Table I, there is an increase in the proportions of 18:2, 20:4 and 22:6 and a decrease in 16:0, 16:1 and 18:1 in the liver triacylglycerols with respect to hypochlorite treatment. Although all samples indicated changes in the acyl group composition at 2 days after the acute treatment, the rate of recovery seemed to vary somewhat. Thus, 5 days after the treatment, half of the animals were already recovered from the abnormality judging from the changes in acyl

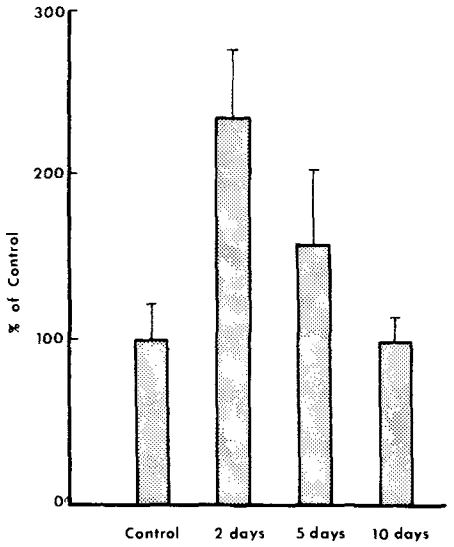

FIG. 1. Accumulation of liver triacylglycerols after administration of 50 mg equivalent-free chlorine. Rats were sacrificed at each indicated time after chlorine administration and liver triacylglycerols were determined using TLC separation of the lipids and subsequent GLC analysis of the fatty acid methyl esters from the triacylglycerols. For quantitation purpose, a known amount of the standard (20:0 methyl ester) was added to each sample. Results are expressed as percentage change relative to the controls, which indicated a mean ± SD of 0.61 ± 0.05 μmol of triacylglycerols/g tissue as determined from 7 samples.

group composition, whereas obvious differences were observed in the other half of the samples. All acyl group changes returned to nomal 10 days after the hypochlorite treatment.

The PC in liver are highly enriched in 20:4, which comprised about 20% of the total acyl group content. Some prevailing changes were again shown in the acyl group composition of this phospholipid. These changes include an increase in 18:0, 20:4 and 22:6 and a decrease in 16:0, 18:1, 20:3 and 20:5 (Table II). Among them, the proportion of 20:5 decreased 4-fold and 20:4 increased ca. 40%. The acyl group composition of the PE, another major membrane phospholipid, also showed an increase in 18:2, 20:3 and 20:4, and a decrease in 16:0, 18:1, 20:5 and 22:5 (Table III). All acyl group changes returned gradually to normal after 10 days.

The mitochondrial phospholipids were also investigated for comparison. In general, the PC in mitochondria (Table IV) showed changes similar to those in whole liver homogenate with the exception of 18:2, which showed no change, and 22:6, which is not definitive

because of the variance. The cardiolipin fatty acids, which are composed of a high proportion of 18:2, were not affected by the hypochlorite treatment. A typical acyl group composition of the PE, PI, PS and cardiolipins from the liver mitochondria is given in Table V. These data were obtained from a group of controls which were not treated with chlorine.

DISCUSSION

Many chemicals, when administered to the body, are known to cause transient changes in liver morphology and chemistry. Examples of these include ethanol (7), morphine (8) and the chlorinated hydrocarbon compounds such as carbon tetrachloride (CCl_4) and chloroform ($CHCl_3$) (1,2). A common feature of the morphological changes is the development of a "fatty" appearance in the liver. In the case of CCl_4 poisoning, fatty accumulation in the liver can be detected as early as the first hour after an acute dose of the compound (9) and is attributed mainly to an increase in tissue triacylglycerols (TG).

We have reported that in vivo administration of sodium hypochlorite can result in the formation of $CHCl_3$ in tissues and organs (4). Hepatotoxicity similar to $CHCl_3$ or CCl_4 poisoning was observed in this study. In addition, TG accumulated more than 2-fold in the liver 2 days after an acute dose of sodium

TABLE I

Acyl Group Composition (wt % of total) of Triacylglycerols in Rat Liver after Acute Administration of Chlorine[a]

Acyl groups	Control		Days after administration			
			2	5		10
16:0	35.5 ± 1.8		34.5 ± 2.4	31.6	36.3	37.1 ± 2.2
16:1	7.7 ± 0.8		a	a	10.6	8.4 ± 0.2
18:0	1.4 ± 0.2		1.1 ± 0.1	0.9	1.2	1.2 ± 0.4
18:1	32.9 ± 0.3	$p<0.01$	30.0 ± 0.2	30.6	31.5	33.5 ± 1.3
18:2	16.6 ± 1.3	$p<0.005$	27.4 ± 1.9	25.4	15.8	15.3 ± 1.0
20:4	1.5 ± 0.1		2.4 ± 0.2	2.3	1.3	1.5 ± 0.4
20:5	1.0 ± 0.1		0.9 ± 0.1	0.8	0.6	0.8 ± 0.2
22:5	1.5 ± 0.6		1.6 ± 0.6	3.3	1.0	1.0 ± 0.2
22:6	2.2 ± 0.5		3.6 ± 0.9	5.1	1.9	1.6 ± 0.3
	(n=3)		(n=4)	(n=2)	(n=2)	(n=4)

[a]When the amount of 16:1 was small, it was eluted as a shoulder peak together with 16:0. Rats were either administered 50 mg equivalent free chlorine as sodium hypochlorite or an equal volume of saline as in controls. Animals were decapitated at 2, 5 and 10 days after chlorine administration. Lipids were extracted from liver and the triacylglycerols separated by TLC as described in text. Acyl groups of triacylglycerols were analyzed as methyl esters by GLC. Results are mean ± SE from 4 samples in each group. Analysis of variance was performed comparing results between group II and controls.

TABLE II

Acyl Group Composition (wt % of total) of Phosphatidylcholines in Rat Liver after Acute Administration of Chlorine[a]

Acyl groups	Control		Days after administration		
			2	5	10
16:0	24.6 ± 1.7	$p<0.025$	19.9 ± 0.8	22.4 ± 1.5	23.1 ± 2.8
18:0	20.5 ± 0.9	$p<0.01$	23.6 ± 0.5	20.5 ± 1.7	20.2 ± 0.9
18:1	10.7 ± 0.8	$p<0.005$	7.3 ± 0.2	9.8 ± 0.8	10.7 ± 0.5
18:2	15.2 ± 0.8		13.8 ± 0.6	15.4 ± 0.7	16.4 ± 1.3
20:3	1.1 ± 0.1		0.8 ± 0.3	0.7 ± 0.3	1.5 ± 0.5
20:4	19.4 ± 0.9	$p<0.005$	26.8 ± 0.4	22.3 ± 1.5	19.4 ± 2.4
20:5	1.7 ± 0.2	$p<0.01$	0.4 ± 0.4	1.1 ± 0.7	1.8 ± 0.5
22:5	0.9 ± 0.6		0.6 ± 0.3	0.6 ± 0.2	0.9 ± 0.2
22:6	6.4 ± 1.0		7.3 ± 0.4	7.4 ± 0.8	6.3 ± 0.8
	(n=4)		(n=4)	(n=4)	(n=4)

[a]Experimental conditions were same as in Table I.

TABLE III

Acyl Group Composition (wt %) of Phosphatidylethanolamines in Rat Liver after Acute Administration of Chlorine[a]

Acyl groups	Control		Days after administration 2	5	10
16:0	17.0 ± 0.4	$p<0.025$	15.0 ± 0.7	16.8 ± 1.2	17.9 ± 1.1
18:0	21.1 ± 0.2		21.0 ± 0.4	18.9 ± 1.4	20.0 ± 0.6
18:1	8.1 ± 0.8		6.7 ± 0.6	8.3 ± 0.6	7.8 ± 0.3
18:2	12.4 ± 1.3		14.4 ± 0.7	15.0 ± 1.0	12.6 ± 0.5
20:3	0.1 ± 0.1	$p<0.05$	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
20:4	21.7 ± 1.4	$p<0.05$	24.5 ± 0.4	21.9 ± 1.1	21.9 ± 1.0
20:5	1.7 ± 0.5	$p<0.05$	0.9 ± 0.1	1.3 ± 0.4	1.8 ± 0.4
22:5	2.2 ± 0.3	$p<0.05$	1.7 ± 1.3	1.9 ± 0.5	2.3 ± 0.5
22:6	15.8 ± 0.8 (n=4)		15.9 ± 0.6 (n=4)	15.8 ± 0.7 (n=4)	15.3 ± 0.7 (n=4)

[a]Experimental conditions were same as in Table I.

TABLE IV

Acyl Group Composition (wt %) of Phosphatidylcholines in Liver Mitochondria after Acute Administration of Chlorine[a]

Acyl groups	Control		Days after administration 2	5	10
16:0	21.7 ± 1.3	$p<0.05$	18.2 ± 1.4	19.8 ± 3.1	21.3 ± 1.5
18:0	21.0 ± 1.7		23.5 ± 2.4	21.2 ± 1.4	20.6 ± 1.1
18:1	8.8 ± 0.4		7.5 ± 0.8	8.7 ± 0.6	9.6 ± 0.5
18:2	12.4 ± 0.8		12.3 ± 2.0	12.5 ± 1.5	12.5 ± 0.8
20:3	1.5 ± 0.2		1.0 ± 0.1	1.4 ± 0.6	1.7 ± 0.6
20:4	22.1 ± 0.4	$p<0.05$	29.0 ± 0.6	25.4 ± 2.7	22.3 ± 2.0
20:5	3.0 ± 0.6	$p<0.05$	0.9 ± 0.2	1.8 ± 0.3	2.4 ± 0.6
22:5	1.4[b]		1.0[b]	1.3 ± 0.3	1.6 ± 0.2
22:6	8.1 ± 0.7 (n=4)		7.7 ± 1.1 (n=4)	8.4 ± 0.8 (n=4)	8.5 ± 0.7 (n=4)

[a]Experimental conditions were same as in Table I.
[b]n=2.

TABLE V

Acyl Group Composition (wt % of total) of Phospholipids in Liver Mitochondria[a]

Acyl groups	Cardiolipin	Phosphatidylethanolamine	Phosphatidylinositol	Phosphatidylserine
16:0	2.7	11.2	6.3	0.8
16:1	–	–	–	–
18:0	1.3	25.4	39.3	40.6
18:1	17.6	6.2	8.0	10.0
18:2	72.1	3.9	2.9	1.4
20:3	2.1	–	2.1	–
20:4	2.3	29.3	36.7	25.9
20:5	–	1.3	–	0.8
22:5	–	3.1	1.8	2.0
22:6	1.8	19.6	3.0	18.5

[a]Total lipid extract from liver mitochondria was separated by two-dimensional TLC as described in text to resolve the individual phospholipids. Mitochondrial samples were obtained from rats without chlorine treatment.

hypochlorite. Liver TG accumulation was transient, and this effect disappeared after 10 days. The TG accumulation was also accompanied by a change in the acyl group composition which showed a large increase in the proportion of 18:2 and a smaller increase in the long chain PUFA such as 20:4 and 22:6. The increase in long chain PUFA level is surprising because TG in liver normally contain only trace amounts of these fatty acids. Although the exact biochemical basis of the TG accumulation is not yet understood, there is good evidence showing that the chlorinated compounds cause a derangement of the lipoprotein secretion system, perhaps due to induction of lipid peroxidation (10) which in turn alters membrane transport function (11). Benedetti et al. (12) showed that CCl_4 could induce free radical attack on the PUFA of membrane phospholipids. The peroxidation process was further correlated to a decrease in the arachidonic level in some membrane phospholipids (13). Whether the excess TG are derived within the liver in situ or transported to the liver from an extrahepatic source is not known.

The administration of sodium hypochlorite to rats also gives rise to alterations of mitochondrial respiration activity and membrane cation transport functions (14). The changes in membrane transport activites may result from an alteration of the membrane acyl group composition. Previous studies regarding $CHCl_3$ and CCl_4 poisoning suggested that the PUFA of membrane phospholipids were altered due to free radical attack induced by the chlorinated compounds (12). This phenomenon may well explain the decrease in PUFA such as 20:5 and 22:5 in the phospholipids but not the increase in 20:4 and 18:0 in PC from both liver homogenates and mitochondria. This increase probably results from synthesis of new PC species which are enriched in these fatty acids. Perhaps it is a measure to compensate for the degradative changes. An increase in PC synthesis may also be due to an increase in acylation of lysolecithin, because a blockage of the lipoprotein transport system is likely to occur in liver due to the hypochlorite treatment. This may be the underlying cause for the accumulation of TG in the system.

In both tissue homogenate and mitochondria, fatty acid changes were more obvious in PC than in PE. Apparently, the acyl groups of PC are engaged in more rapid turnover. The morphological and compositional changes observed in this study have demonstrated that ingestion of sodium hypochlorite may have deleterious effects on enzymatic processes in the body. Although most of the changes observed are transient in nature, some of the events may render the liver susceptible to more permanent damage.

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Temperature Acclimation in the Crayfish: Effects on Phospholipid Fatty Acids[1,2]

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ABSTRACT

Acclimation to different temperatures by a poikilothermous animal must include modification of its membrane lipids to maintain the proper physical properties. The simplest way to achieve this acclimation would seem to be by modification of the phospholipid fatty acids. In a freshwater crayfish, *Procambarus clarkii*, rapid changes in the degree of unsaturation of newly synthesized phospholipid fatty acids were correlated with changes in environmental temperature, both in whole animals and in slices of hepatopancreas tissue. At 5 C, the rate of fatty acid synthesis was about half that occurring at 23 C. Hepatopancreas tissue from animals acclimated to either 5 C or 23 C, when incubated for 2 hr at 5 C, incorporated a higher percentage of exogenous [1-^{14}C] acetate into polyunsaturated acids (27-38% of the radioactivity in total fatty acids) than when incubated at 23 C (12-14%); conversely, more saturated fatty acids were synthesized at 23 C (73-80% vs 51-73%). The higher average unsaturation of the fatty acids biosynthesized at 5 C constitutes an effective response to the animal's need for modification of lipids to maintain adequate membrane function at the lower environmental temperature.

INTRODUCTION

The most detailed information about lipid-associated effects in temperature acclimation of multicellular organisms exists in two areas: (a) studies of certain membrane-associated lipoprotein enzymes (e.g., succinate dehydrogenase [1] and sarcoplasmic reticulum Ca^{++}-transport ATPase [2,3]), and (b) measurements of the general physical properties of membranes (4). The important lipid type involved in each case is phospholipid; and the parameter most often measured experimentally is the change in fatty acid composition with temperature.

To function satisfactorily, the membrane must maintain the proper fluidity, the liquid-crystalline state. Chapman (5) has pointed out that varying the degree of unsaturation of the component phospholipid fatty acids is only one of many ways by which membrane fluidity can be changed. The strong influence on membrane function of the unsaturation of its lipids has been shown in both living cells and model systems (6-10). The long-chain polyunsaturated fatty acids characteristic of most aquatic animals have melting points well below the freezing point of water. Thus they are well suited to play an important role in regulating the physical chemical properties of membranes in aquatic poikilotherms. In fact, increased amounts of C_{20} and C_{22} polyunsaturated acids in the phospholipids of cold-exposed fishes and invertebrates have been reported many times (11-17).

The experiments presented in this paper were designed to determine the pattern of fatty acid biosynthesis in an organ known to actively metabolize fatty acids, the hepatopancreas, of a poikilotherm subjected to abrupt changes in environmental temperatures. Our results with the freshwater crayfish *Procambarus clarkii* show that the adjustment of the fatty acid composition of the phospholipids to temperature begins rapidly.

MATERIALS AND METHODS

We purchased crayfish from a commercial live bait supply house; they were collected from fresh water in southern California or possibly northwestern Mexico. The only species reported to occur in this area is *P. clarkii* (18). The 50-70 g animals were maintained in aerated tap water at room temperature, 22-23 C, or at +4-5 C in the cold room, for at least 3 days. They were fed chopped chicken liver, but all food was withdrawn one day before the experiments began. The 23 C animals were subject to the natural diel cycle of illumination, whereas the 5 C animals were subject to a 12-hr light, 12-hr dark cycle in a cold room.

For in vivo experiments, groups of 4 warm-acclimated animals were held at either 5 or 23 C for 2 days before 5 μCi sodium [1-^{14}C]-acetate (4.52 mCi/mmol) in sterile saline was injected into the ventral lymph sac and the

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puncture wound cauterized. The animals were maintained at the experimental temperature for an additional 24 hr, when they were killed by decapitation, their hepatopancreases removed and the total lipid extracted with chloroform/methanol (2:1, v/v). Analyses in triplicate were made on pooled samples from 4 animals. For in vitro experiments, animals acclimated at either 5 or 23 C were sacrificed and their hepatopancreata immediately sliced thinly with a razor blade. Pooled tissue (500 mg) from 3 animals in 3 ml of pH 7.4 Ringer's solution modified for freshwater crustaceans (19) was incubated with 1 μCi sodium [1-^{14}C]acetate at either 5 or 23 C, as detailed in Tables I and II. Incubation was terminated by homogenizing the tissue in chloroform/methanol. The chloroform extract was washed free of radioactivity with Folch et al. upper phase (20) containing 0.1 M unlabeled sodium acetate.

Triglycerides and phospholipids were separated by preparative thin layer chromatography on Silica Gel G plates, using petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v) as solvent and locating the zones by staining with iodine vapor. The phospholipids remained at the origin in this system but were resolved from cholesterol and free fatty acids. Methyl esters were prepared from total fat, triglycerides or phospholipids by heating with 5% HCl in absolute methanol at 80 C for 30 min in a sealed glass ampoule. After partitioning between petroleum ether and water, the crude methyl esters, containing any nonsaponifiable material (largely cholesterol, see below), were separated by argentation thin layer chromatography (TLC), developing with 25% diethyl ether in petroleum ether. The zones were visualized by iodine vapors or rhodamine 6G spray (21). Usually, 4 bands were detected: saturated, monounsaturated, di- plus triunsaturated and polyunsaturated methyl esters. The separation was checked by gas liquid chromatography (GLC), using as markers inactive esters prepared from the same organ.

The individual zones were scraped from the thin layer plates and counted in a toluene-based scintillation cocktail, either directly (as a suspension) or after extracting the methyl esters from the adsorbent with four 2.5-ml portions of diethyl ether, transferring to counting vials and evaporating the solvent. Counting was done in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3003.

Gas chromatography was performed with a Barber-Coleman Model 20 instrument using a 1.83 m x 3.2 mm od column of 12% DEGS on 80/100 mesh Gas-Chrom P (Applied Science Laboratories, Inc., State College, PA).

RESULTS

Effect of Cold Exposure in vivo on the Fatty Acid Composition of Phospholipids

Warm-acclimated crayfish exposed to cold for 10 days had increased amounts of eicosapentaenoic and docosahexaenoic acids in their phospholipids, with a concomitant de-

TABLE I

Distribution of Radioactivity in Crayfish Hepatopancreas Total Lipid Fatty Acids after Incubation with Sodium [1-^{14}C]Acetate

	Incubation temperature (C)					
	5[a]	23[b]				23[c]
	Incubation time (min)					
	120	15	30	60	120	120
No. of double bonds						
0	56.0 ± 5.6	49.6 ± 1.2	54.6 ± 1.0	62.1 ± 1.5	68.3 ± 3.2	73.1 ± 1.2
1	17.1 ± 5.5	32.1 ± 0.1	28.0 ± 1.9	23.1 ± 0.9	17.8 ± 0.9	14.4 ± 0.4
2 + 3	19.5 ± 8.2	8.5 ± 2.6	12.2 ± 3.5	10.5 ± 1.5	10.1 ± 2.3	8.7 ± 1.0
>4	7.4 ± 1.3	9.8 ± 3.9	5.2 ± 0.6	4.3 ± 0.2	3.8 ± 1.0	3.8 ± 0.5
Total ^{14}C (cpm X 10^{-3}/mg)	3.6 ± 0.4					6.8 ± 0.8

Values are the mean ± SD of duplicate experiments, each using pooled tissue from 3 animals, expressed as percent of total radioactivity recovered.

[a]Hepatopancreas slices of 23 C animals incubated at 5 C.

[b]Hepatopancreas slices of 23 C animals were preincubated at 5 C for 120 min without radioactivity, warmed to 23 C, [^{14}C]acetate added and incubation continued at 23 C for 15-120 min.

[c]Hepatopancreas slices of 5 C acclimated animals incubated at 23 C.

TABLE II

Distribution of Radioactivity in Crayfish Hepatopancreas Phospholipid Fatty Acids Following Incubation with Sodium [1-^{14}C] Acetate

Treatment	Animals exposed to cold for (days): 0	3	10	20	0	3	10	20
	Incubation temperature (C) 5				23			
Number of double bonds								
0	50.7 ± 1.3	66.7 ± 0.6	70.0 ± 1.0	73.2 ± 0.8	78.0 ± 1.2	85.7 ± 1.5	85.5 ± 0.7	79.9 ± 1.2
1	10.8 ± 0.4	12.3 ± 0.9	11.4 ± 0.6	12.8 ± 1.0	7.6 ± 0.8	10.2 ± 0.3	4.9 ± 0.4	11.1 ± 0.7
2 + 3	29.4 ± 3.1	16.9 ± 1.7	15.8 ± 1.2	11.2 ± 0.7	12.2 ± 0.7	2.0 ± 0.4	7.7 ± 1.0	7.9 ± 1.2
>4	9.1 ± 1.7	4.1 ± 0.5	2.7 ± 0.6	2.8 ± 0.9	2.1 ± 0.8	2.1 ± 1.1	1.6 ± 0.4	1.1 ± 0.2

Warm-adapted animals were held at 5 C for 3, 10 and 20 days. Pooled hepatopancreas tissue from each group (3 animals) was incubated with labeled acetate for 120 min at both 5 C and 23 C. The results are the means ± S.D. of triplicate determinations, expressed as percent of total radioactivity recovered.

crease in palmitic and stearic acids. The ratio of saturated to unsaturated fatty acids decreased from 0.47 in warm-acclimated animals to 0.33 in crayfish exposed to cold. Values for the major fatty acids are given in Table III.

In vivo Incorporation of [^{14}C] Acetate into Hepatopancreas Fatty Acids

In warm-exposed animals, more than 70% of the total radioactivity was present in the saturated fatty acid fraction and only a low percentage in polyenoic acids (Table IV). In the cold-exposed specimens, however, the polyunsaturated fatty acids accumulated considerably more label with a concomitant decrease in labeling of the saturated fatty acids. The specific activity of hepatopancreas lipids of 23 C animals was 2.4 times that of 5 C animals. Differences in the distribution of activity among the fatty acids of triglycerides compared to phospholipids were also seen, particularly in 5 C animals. More of the newly labeled polyunsaturated fatty acids were incorporated into phospholipids than into triglycerides.

Effect of Incubation Temperature on the in vitro Incorporation of [^{14}C] Acetate into Phospholipid Fatty Acids

When slices of the hepatopancreas from warm-acclimated animals were incubated for 2 hr at 5 C and 23 C with sodium [1-^{14}C]-acetate, incorporation of radioactivity into hepatopancreas total fatty acids was linear with time, and the tissues incorporated 3.6 ± 0.4 × 10^3 and 6.8 ± 0.8 × 10^3 cpm/mg fat, respectively (Table I). In some experiments,

TABLE III

Major Phospholipid Fatty Acids of Crayfish Acclimated to Different Temperatures

Temperature (C)	4[a]	23
	(wt %)	
Fatty acids		
16:0	13.5 ± 0.5	17.0 ± 1.5
16:1	5.5 ± 0.4	6.5 ± 0.1
18:0	7.2 ± 0.6	8.5 ± 0.3
18:1	11.6 ± 0.4	9.5 ± 0.2
18:2	8.5 ± 0.3	7.5 ± 0.8
18:3	1.5 ± 0.1	2.3 ± 0.2
20:4	10.0 ± 0.4	9.5 ± 0.4
20:5	21.7 ± 0.6	16.4 ± 0.5
22:6	3.5 ± 0.3	2.0 ± 0.7
Satd/unsatd	0.33	0.47

Phospholipid fatty acids from 5 animals were analyzed separately; values given are means ± SD.

[a]Warm-adapted animals were exposed to 4 C for 10 days.

the slices were preincubated in the cold for 2 hr in the absence of radioactivity, then warmed quickly, the labeled acetate added, and the tissues incubated for varying intervals at 23 C. For hepatopancreas slices from warm-acclimated animals, 2 hr incubation at 5 C was sufficient to change the distribution of activity to a pattern similar to that obtained in vivo with 5 C animals. At this lower temperature, hepatopancreas slices accumulated more label in polyenoic acids and less in saturated acids. For slices incubated with [^{14}C] acetate following preincubation at 5 C without label, the distribution of radioactivity gradually changed with the time at 23 C from a pattern resembling cold-incubated tissue (15 min) to a pattern similar to that of warm-incubated tissues (after 120 min at 23 C).

A change in distribution of radioactivity with time was also obtained when hepatopancreas slices of cold-exposed animals were incubated at both 5 C and 23 C; see Table II. The longer the cold exposure of the living animals before sacrifice, the less was the accumulation of label in polyenoic acids. This was true for tissues incubated both at 5 C and 23 C, although the 5 C slices in every case incorporated a higher percentage of the radioactivity into polyenoic acids.

DISCUSSION

The amounts of components other than methyl esters present in the crude "methyl esters" fractionated by argentation TLC are unknown, since the composition of the total lipids of *P. clarkii* was not determined. Components which might be more than trace constituents are hydrocarbons, including squalene (22), cholesterol (22,23), dimethyl acetals and glyceryl ethers. The first two are presumably not biosynthesized from acetate by crayfish (22), and so would be unlabeled in *P. clarkii*. Any dimethyl acetals present, derived from tissue alkenyl glyceryl ether lipids, would have R_f values very similar to those of methyl esters of the same unsaturation, and so would not affect the conclusions drawn from Tables I, II and IV. Glyceryl ethers, derived from any alkyl glyceryl ether lipids of *P. clarkii* (no ether lipids have been reported for crayfish), could add extraneous counts to the polyunsaturated methyl esters (glyceryl ethers are barely moved off the origin by 25% diethyl ether). However, we believe that *P. clarkii* lipids contain less than 5% alkyl glyceryl ether lipids and expect that incorporation of radioactivity from acetate into these components would be slower than into phospholipid fatty acids. In the extreme case,

TABLE IV

Distribution of Radioactivity in Fatty Acids of Different Crayfish Hepatopancreas Lipids Following in vivo Administration of Sodium [1-^{14}C] Acetate

	Total lipids		Phospholipids		Triglycerides	
Temperature (C)	5	23	5	23	5	23
No. of double bonds						
0	50.3 ± 5.6	79.4 ± 10.1	20.5 ± 3.5	72.7 ± 9.8	49.4 ± 7.0	80 ± 4.3
1	14.4 ± 3.2	13.6 ± 4.5	11.3 ± 4.8	11.6 ± 3.8	28.4 ± 4.0	10 ± 2.5
2 + 3	13.8 ± 2.5	4.7 ± 2.0	32.5 ± 6.4	11.7 ± 3.0	22.1	10
>4	21.4 ± 2.8	2.3 ± 1.5	35.6 ± 5.8	4.0 ± 1.8		
Total ^{14}C (cpm × 10^{-3}/mg)	1.8 ± 0.5	4.3 ± 0.8	—	—	—	—

Values are the means ± SD of triplicate determinations using pooled hepatopancreas from 4 animals, expressed as percent of total radioactivity recovered.

then, counts observed in polyunsaturated esters might be exaggerated by a maximum of 5% (of the total lipid radioactivity), most significantly with warm-acclimated tissue. Since generally less than 5% of the lipid radioactivity of 23 C in vitro experiments was found in methyl esters with four or more double bonds, we believe that our conclusions are not invalidated by the uncertainty of any glyceryl ether contribution.

Cossins (14) did not detect significant changes in the level or composition of phospholipid classes in the muscle of the crayfish, *Austropotamobius pallipes*, acclimated to 4 C compared to 25 C. The phospholipid fatty acid patterns of the two species were generally similar, with 20:5, 16:0, 18:1 and 20:4 the major components (9-29%) and smaller amounts of 18:0, 16:1, 18:2 and 22:6 (2-8%). The phospholipids of a third species, *Orconectes rusticus*, differed only in having 5-7% 20:4 (23). The response of the muscle phospholipid fatty acid composition of *A. pallipes* to temperature acclimation (14) was more limited than we observed for *P. clarkii* hepatopancreas, confirming our expectation that the most immediate temperature effects would be seen in a tissue normally metabolizing exogenous dietary lipid.

Our investigations confirm earlier results with fish (12) and show that the increased degree of unsaturation of the lipids observed in cold-acclimated crayfish is achieved, in major part, by preferential biosynthesis of unsaturated fatty acid chains. We assume that much of the ^{14}C-radioactivity from acetate incorporated into polyunsaturated acids of the phospholipids of 5 C animals (Table IV) is accounted for by chain elongation of dietary 18:2 and 18:3 components and further desaturation to C_{20} and C_{22} tetra-, penta- and hexaenoic acids, as is known for marine crustaceans (24,25). Temperature acclimation in *P. clarkii* includes changes in the lipid metabolism of the hepatopancreas which are manifest on two time scales: an immediate response, which in vitro produces different fatty acid labeling patterns from the same hepatopancreas tissue depending on the temperature of incubation (Tables I and II); and complete acclimation, which requires at least 3-10 days (Table II). Clearly, the immediate response to incubation temperature is determined by the relative quantities and properties of the enzymes of fatty acid metabolism already present in the tissue and does not require the synthesis of new enzyme molecules.

In crayfish, the monounsaturated fatty acids play only a subordinate role in the temperature acclimation processes. There was only a slight increase in the labeling of the monoenes upon cold exposure in vitro (Tables I and IV) and the level of these fatty acids did not change in vivo in either *P. clarkii* (Table III) or *A. pallipes* (14). Brenner has suggested that the Δ^6-desaturase acting on 18:2(n-6) and 18:3(n-3) is a key regulatory step in higher poikilotherms in the biosynthesis of the C_{20} and C_{22} tetra-, penta- and hexaenoic acids (25,26). The activity of this enzyme apparently is increased at lower temperatures (16,27). Kasai et al. (28) propose that, in *Tetrahymena pyriformis*, the activities of fatty acid desaturases are modulated by the physical state of the membranes so that membrane fluidity is self-regulating.

Temperature acclimation of a mature crayfish would seem to require some mechanism for rapidly incorporating newly synthesized fatty acids into existing membrane phospholipids under conditions in which growth and new membrane formation would be slow. The specificities of acyl transferases (29,30) acting on lysophospholipids—formed in turn by phospholipase A hydrolysis of existing phospholipid molecules—coupled with a means of exchanging newly modified phospholipid molecules with existing membrane phospholipids could provide such a route (31,32).

These concepts are sufficient to account for our observations, but their application to *P. clarkii* is speculative, since we did not measure enzyme activities. Nevertheless, the greatest incorporation of [^{14}C]acetate into polyunsaturated acids (18 or 33%) was observed for tissue from 23-C-acclimated animals incubated at 5 C and the lowest (7.5 or 12.5%) for 5-C-acclimated animals incubated at 23 C. Similarly, the least amount of saturated acids were synthesized by hepatopancreas from 23-C-acclimated animals incubated at 5 C (50 or 53%). These findings are all in accord with the accumulated observations of many investigators (6-17).

Probably the maximal differences would be observed by shortening the time of incubation of the hepatopancreas tissue (cf. the 15- to 120-min incubations of Table I), since we did not add 18:2(n-6) or 18:3(n-3) fatty acids, and 2-hr incubations might have exhausted these endogenous precursors of the polyunsaturated acids, particularly at the higher incubation temperature. That is, the highest rates of biosynthesis would be obtained by extrapolation to time zero, when substrates are in highest concentrations, the enzymes are most active, and the cells are most vigorous. Indeed, some evidence in support of this may be deduced

from Table I, by calculating the amounts of acetate incorporated into tetra- to hexaenoic acids. At both 5 C and 23 C 260-270 cpm, or about 2.1 nmol, of acetate was incorporated into these polyenes, suggesting that the endogenous precursors were exhausted in both experiments in 2 hr. Morris and Sargent (24) emphasize the paradox of low levels of C_{16} and C_{18} polyunsaturated precursors vs the comparatively heavy labeling of C_{20} and C_{22} polyenoic acids in crustaceans fed [^{14}C]palmitate, when accepted wisdom rules out de novo synthesis of polyenoic acids by these animals. In *P. clarkii*, the 2% of endogenous 18:3(n-3) seems commensurate with the radioactivity found in the polyenes.

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Free Radical Polymerization and Lipid Binding of Lysozyme Reacted with Peroxidizing Linoleic Acid

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ABSTRACT

Insolubilization and polymerization of proteins exposed to peroxidizing lipids may be due either to cross-linking with incorporation of fragments of the lipid oxidation products, or to free radical transfer from lipid to protein and subsequent free radical polymerization of protein. The second mechanism which has been proposed was inferred from measurements of electron spin resonance signals in proteins. In this study, uniformly labeled linoleic acid, [^{14}C(U)] LA, was reacted with lysozyme. Volatile oxidation products of LA were also used in some experiments. Incubation was done in the absence of water. Oligomers of lysozyme, as well as the monomer, were isolated after incubation, and the [^{14}C] label incorporated into each fraction was determined. The results show that the dominant mechanism of protein polymerization after exposure to peroxidizing linoleic acid is the transfer of free radical from lipid to protein, and subsequent free radical polymerization.

INTRODUCTION

Exposure of proteins to peroxidizing lipids can produce changes in proteins, including loss of enzyme activity, polymerization, insolubilization, scission and formation of lipid protein complexes. Polymerization and insolubilization of protein may be explained by either or both of the following mechanisms: (a) formation of protein-centered free radicals, followed by free radical polymerization of the proteins, without incorporation of lipid fragments; (b) crosslinking of proteins by bifunctional products of lipid peroxidation, e.g., malondialdehyde (1-5).

In previous studies, we showed that protein-free radicals formed when lysozyme was exposed to γ-radiation (6), to autoxidized methyl linoleate (4,7), and to volatile decomposition products of autoxidized methyl linoleate (8).

This study was designed to prove that polymerization of proteins, without incorporation of cross-linking segments of the peroxidized lipid, does occur. We reacted lysozyme (LYS) with uniformly labeled [^{14}C(U)] linoleic acid (LA), separating the resulting lysozyme oligomers and demonstrating absence of label in quantities sufficient to be involved in cross-linking. Roubal and Tappel (1) used a similar approach with cytochrome C and [$^{14}C_1$]-labeled linolenate. However, the most likely cross-linking agents resulting from lipid peroxidation would not include the [$^{14}C_1$] label. Hence, the low level of incorporation of [$^{14}C_1$] label in the cytochrome oligomers did not prove absence of lipid-derived cross-linking fragments.

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EXPERIMENTAL PROCEDURES

In our study, two types of experiments were done: chicken egg white lysozyme (LYS) (crystallized three times; ICN Nutritional Biochemicals, Cleveland, OH) incubated in direct contact with labeled linoleic acid [^{14}C-(U)] LA (New England Nuclear, Boston, MA); and LYS exposed to the volatile decomposition products of [^{14}C(U)] LA.

Experiments in which protein and lipid were in intimate contact were performed as follows: 2.4 mg of LYS in 1 ml of water in a small glass vial were freeze-dried to obtain a large surface of the solid protein. The samples were kept in dry conditions (over $CaSO_4$), then 1.5 ml of a hexane solution of [^{14}C(U)] LA (1.4 mg LA, 75 μCi) was added to the protein, drop by drop. The solution was allowed to dry under N_2 before adding more lipid. The samples were stored for 10 and 20 days in the dark at 37 C. LA was then extracted with 3 portions of 1 ml of Cl_3CH/MeOH (1:1).

Experiments in which LYS was reacted with volatile products of oxidation of LA were conducted as follows: 2.4 mg of LYS was dispersed on small pieces of filter paper by wetting the papers with 0.5 ml of an aqueous solution of LYS and then freeze drying the papers. On other pieces of filter paper, 1.2 mg (150 μCi) of [^{14}C(U)] LA was dispersed by wetting the papers with hexane solution of LA and evaporating the hexane. The two systems were sealed within the same 20-ml vial in such a way that the only contact between them was through vapor phase. The vials were stored at 37 C. The protein was extracted from filter paper at 10, 20 and 40 days by 3 extractions with 1.5 ml of water. The total extract was then freeze-dried.

Separation of LYS and its soluble polymerization products was done using a SDS-polyacrylamide gel electrophoresis (8) after denaturation of the sample with 8 M urea and reduction of disulfide bonds with dithiothreitol. In each case, at least three gels were run. The gels were fractionated in slices of 1 mm, then each slice was transferred to a 20-ml glass scintillation vial and treated with 10 ml of 3% Protosol in Econofluor overnight at 37 C for radioactivity analysis. The vials were counted in a Beckman 25-250 Scintillation counter; [^{14}C] was counted with 87-90% efficiency. The remaining gel was stained with Coomassie Brilliant Blue and scanned at 550 nm using a Hitachi Perkin UV-VIS gel scanner attachment on a Hitachi-Perkin Elmer Model 200 spectrophotometer.

The insoluble fraction of the protein was estimated by the difference between initial protein content and spectrophotometrically determined soluble protein after quantitative aqueous extraction. This determination was made using unlabeled LA samples.

We also performed the following three control experiments: (a) In a sample containing 2.4 mg LYS and 1.2 mg [^{14}C(U)] LA (150 μCi), we extracted the lipid immediately after adding it to the protein to test LA extraction before incubation. The protein was then processed electrophoretically, as already described. (b) A sample containing 2.4 mg LYS and 3×10^{-2} μCi [^{14}C(U)] LA was run electrophoretically to verify the position of LA in the gel in case some lipid remained in the protein. (c) A sample containing 3×10^{-2} μCi [^{14}C(U)] LA was run electrophoretically to verify the position of LA in the gel without protein interference.

From these control experiments, we learned that before incubation, LA was recovered completely under our extracting conditions. If any non-bound LA remained in the sample, the lipid would have appeared in the gel with a mobility of approximately 1.1 with respect to LYS. We also observed that the position of LA in the gel is unaffected by the presence of LYS.

RESULTS AND DISCUSSION

Table I shows the weight fractions of the different oligomers of LYS after incubation with LA or its volatile oxidation products. It also shows the mole fraction of LA bound to these fractions, a ratio based on the molecular weight of LA. We wanted to estimate the relative extent of the following reactions (P = LYS and L = LA or its breakdown products):

$$P\cdot + P\cdot \rightarrow P\text{-}P \quad [I]$$

$$P + L \rightarrow P\text{-}L \quad [II]$$

or

$$P + L\cdot \rightarrow P\text{-}L \quad [IIa]$$

$$(P\text{-}P)\cdot + P\cdot \rightarrow P\text{-}P\text{-}P \quad [III]$$

$$(P\text{-}P) + L \rightarrow P\text{-}P\text{-}L \quad [IV]$$

or

$$(P\text{-}P)\cdot + L\cdot \rightarrow P\text{-}P\text{-}L \quad [IVa]$$

We used the data in Table I to estimate the relative concentrations of P-P, P-L, P-P-P and

TABLE I

Linoleic Acid (LA) Binding to Polymerization Products of Lysozyme (LYS)[a]

Sample composition and days	Lysozyme (monomer) (%)	Lysozyme (monomer) mol LA / mol LYS ($\times 10^{+3}$)	Dimer (%)	Dimer mol LA / mol LYS ($\times 10^{+3}$)	Trimer (%)	Tetramer (%)	Insoluble (%)
LYS t = 0 days	94.0	–	5.0	–	1.0	0.0	0.0
LYS + LA t = 0 days	94.0	0.0	5.0	0.0	1.0	0.0	0.0
LYS + LA t = 10 days	54.2	45.1	24.1	0.0	6.0	1.7	14.0
LYS + LA t = 20 days	37.5	68.0	20.1	10.3	5.8	1.3	35.3
LYS + VOL[b] t = 10 days	60.0	1.8	28.0	0.0	4.0	2.0	7.0
LYS + VOL t = 20 days	45.0	6.5	17.1	2.9	9.2	5.4	23.3
LYS + VOL t = 40 days	23.4	4.4	9.0	0.0	0.6	–	67.0

[a]Storages were done at 37 C, a_w = 0.0 and darkness.

[b]VOL: volatile oxidation products of LA.

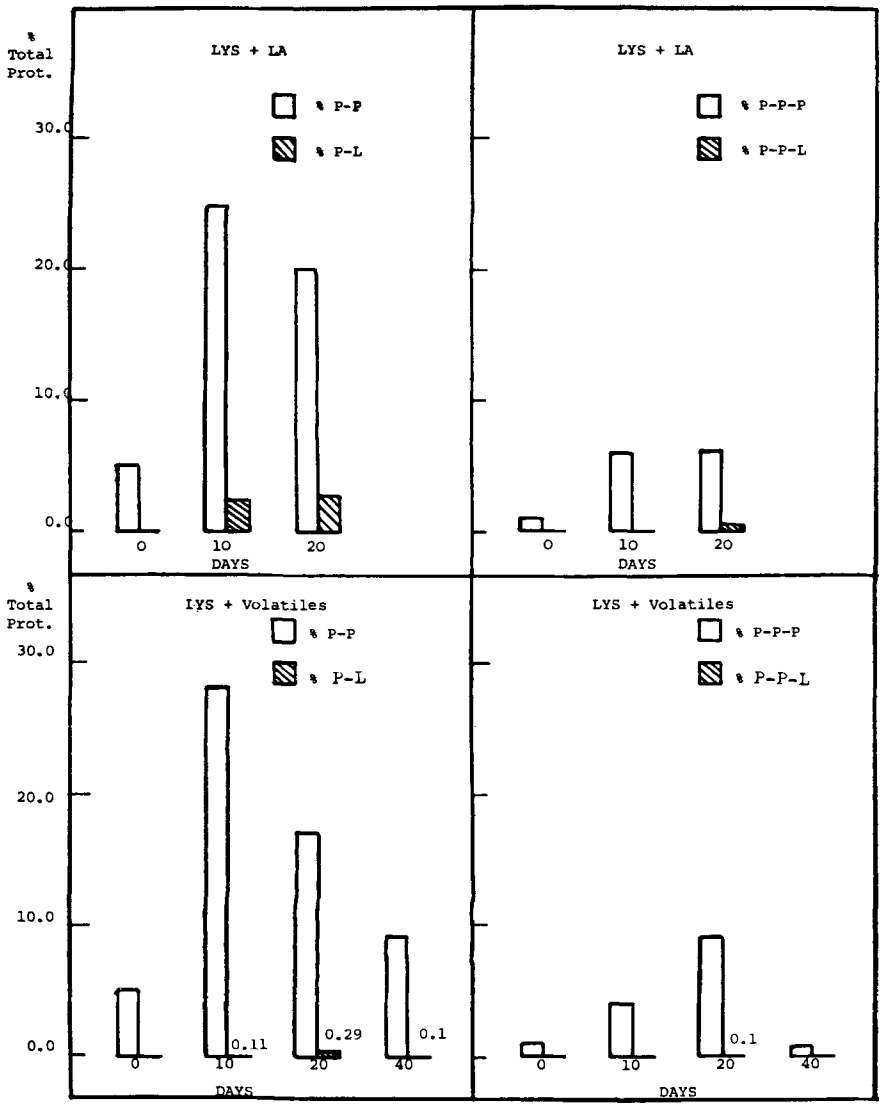

FIG. 1. Relative concentrations of lysozyme reaction products P-P, P-L, P-P-P and P-P-L, resulting from reaction with peroxidizing linoleic acid, or its volatile breakdown products.

P-P-L. Because of the cost considerations, variability of results could not be determined on [^{14}C]-labeled samples. However, work on similar systems showed that dimer and trimer formation gave results with standard deviations of 5.0 and 1.5%, respectively. The estimation of label incorporation has a standard deviation of 3×10^{-3} mol/mol in the case of direct contact, and 10^{-3} mol/mol in the case of volatile compounds. Results are shown in Figure 1. They clearly show that the LYS dimer P-P and trimer P-P-P are formed predominantly even at a lipid:protein molar ratio of 25, which exists in our system (wt ratio 0.5). The calculated molar yield of P-L formation is based on molecular yield of linoleate; if the cross-linking fragments were much smaller, the molar yield could be up to 10 times higher. That increase would still not be sufficient to invalidate the conclusion that the predominant mechanism of polymerization is that shown in Equations I and III.

Other investigators have also studied lipid incorporation into protein exposed to peroxidizing lipids (1). However, these studies could not provide definitive information on the

occurrence of reaction I either because [$^{14}C_1$] (1,9,10) or [$^{14}C(U)$] label was used, but the protein fractions were not separated (11).

It is interesting that the authors just mentioned, who worked in aqueous systems, found higher levels of incorporation than we found in our dried system, as would be expected on the basis of previous experiments (4,8). It is also expected that any contribution of malonaldehyde (MA) to cross-linking would be higher in systems containing linolenate, and at high water contents, because aldehydeamine condensations are known to be inhibited at low water contents (12-15). The effect of local environment on reactions between proteins and lipids is therefore important and the results of this study are appropriate to lipid-protein systems at low local water contents. Which system is most appropriate to biologically significant structures, such as membranes in which some regions are aqueous and others nonpolar, is still unknown.

LYS exposed to volatile oxidation products of LA shows similar polymerization and binding pattern to LYS reacted in contact with LA. This agrees with our previous work showing formation of P• from LYS exposed to volatile products of methyl linoleate oxidation, to hexanal or to 2,4-decadienal, or to 2-heptenal.

Results in Table I show that volatile products of LA are less likely to bind to the protein than LA in direct contact. Yet, volatile products remain quite effective in generating P•, as evidenced by formation of P-P and higher oligomers.

We also obtained evidence on the formation of noncovalently bound label. Samples of LYS + LA (direct contact) showed a small peak corresponding to unbound LA, which remained with the protein through solvent extraction, but was apparently released by treatment with 8 M urea.

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Eicosa-5,11,14-trienoic and Octadec-5-enoic Acids of the Reproductive Tract of the Male House Cricket (*Acheta domesticus*) and Field Cricket (*Gryllus* spp.)[1]

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ABSTRACT

The reproductive tracts (testes, seminal vesicles, accessory glands, ejaculatory duct) of male house crickets (*Acheta domesticus*) and field crickets (*Gryllus* spp.) contain ca. 6% of eicosa-5,11,14-trienoate in total tissue fatty acids. This fatty acid is concentrated primarily in the phosphatidylcholine fraction of phospholipids (12.6%) and occurs in no more than trace amounts (<0.1%) in neutral lipids. The 18:1 fatty acid fraction of total tissue lipid fatty acids consisted of 2 isomers with unsaturation in the Δ5 and Δ9 positions in proportions of ca. 33 and 67 mol %, respectively.

Reports of the occurrence of 20-carbon polyenoic acids in insects are rare. Kinsella (1) reported arachidonic acid (20:4) in the sphingomyelin fraction of egg cases and nymphs of cockroaches (*Periplaneta americana*) in which it comprised up to 15% of total fatty acids. Wood et al. (2) reported 20:4 in both larvae and adults of tobacco budworm (*Heliothis virescens*) reared on a semipurified diet which also contained 20:4. Arachidonic acid was conserved in adults during fasting, an observation which was interpreted by the authors to imply a non-energy function for 20:4 in the tobacco budworm. Lambremont and Dial (3) reported a 20-carbon unsaturated acid in testicular phospholipids of the house cricket. These authors also observed an acid which was tentatively identified as *trans*-18:1. Dadd and Kleinjan (4) recently demonstrated a minimal requirement of 0.005% arachidonic acid in the diet of mosquito (*Culex pipiens*) larvae for development of normal adults. Other evidence presupposing the presence of 20-carbon polyenoic acids in insects and other arthropods includes the identification of prostaglandins in the reproductive tract of the male house cricket (*Acheta domesticus*) (5-7) and in the salivary glands and reproductive organs of the tick (*Hyalomma anatolicum excavatum* Koch) (8).

In a recent investigation of the fatty acid profile of lipid obtained from the reproductive tissues of male house and field crickets (*Gryllus* spp), we reported the presence of four 20-carbon fatty acids, including 2 polyenoic acids, 20:3 and 20:4 (9). The 20:4 acid was identified as arachidonic acid; however, the 20:3 acid did not correspond to either of the eicosatrienoate isomers commonly found in animal systems. We report here the identification of this fatty acid as the 5,11,14-isomer previously identified in plants (10) and as an end product of linoleate metabolism in the cat (11). Identifications were based on results of catalytic hydrogenation and ozonolysis of the isolated methyl esters and on gas liquid chromatographic (GLC) retention behavior on 3 column systems.

MATERIALS AND METHODS

House crickets were obtained from a local supplier and maintained in the laboratory on Purina mouse chow (7). Field crickets were caught from the native habitat near Athens, GA. Reproductive tissues of males (testes, seminal vesicles, accessory glands and ejaculatory duct) were removed as described previously (5) and immediately frozen.

Lipid standards consisting of methyl esters of 16:0, 18:0, 20:0, 22:0, 24:0, 5,8,11,14-20:4, 11,14,17-20:3 and 8,11,14-20:3 were purchased from Nu-Chek-Prep, Elysian, MN. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine were purchased from Avanti Biochemicals, Birmingham, AL. *Caltha* seeds containing 5,11,14-20:3 were furnished courtesy of Dr. R. Kleiman (USDA Northern Regional Research Center, Peoria, IL).

GLC packing materials were purchased from Applied Science, State College, PA. Acid-washed Florisil was prepared in the laboratory as described by Carroll (12). Tissue lipids were extracted by the procedure of Bligh and Dyer (13) and separated into lipid classes on acid-washed Florisil columns as described by Miller

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and Landes (14). Identification of phospholipid fractions was based on thin layer chromatograms obtained with appropriate standards on Silica Gel G plates. Plates were developed in a solvent system containing chloroform/methanol/water (65:25:4). Spots were detected by spraying with a dye mixture (15) and viewing under UV light.

Fatty acid methyl esters were prepared from freeze-dried tissues and from lipid fractions obtained from Florisil chromatography by methods reported previously (16).

GLC procedures were done with a MicroTek 220 chromatograph equipped with an effluent splitter, dual flame ionization detector and an electronic integrator.

Analyses of methyl esters were performed on 180 x .4 cm id glass columns packed with 10% Silar 10C on 100/120 Gas chrom Q, 15% OV 275 on 80/100 Chromosorb W (AW) (DMCS), and 3% OV 225 on 70/80 Chromosorb W (AW) (DMCS). The Silar and OV 225 columns were maintained at 180 C and the OV 275 column at 170 C. Equivalent chain length (ECL) values of sample fatty acids were determined by comparison with semilog retention time plots of the C_{16} to C_{24} homologous series of saturated fatty acid methyl esters (17).

The tempreature of the GLC effluent splitter was adjusted to 275 C with a thermostated resistance heater. Eluting fatty acid methyl esters were captured by inserting a 6" glass capillary (1.1 mm od) into the splitter exit port and allowing the eluting compounds to condense inside the capillary. Condensate was eluted from the capillary with dichloromethane and purity was verified by GLC. A portion of condensate was dissolved in high pressure liquid chromatography grade methanol and reduced in the presence of platinum oxide (Adams catalyst) in a micro-hydrogenator (Supelco, Inc., Bellefonte, PA) according to manufacturer's instructions.

Ozonolysis of methyl esters samples was done at -70 C (Dry Ice/acetone bath) in purified hexane (18) using a micro-ozonizer (Supelco, Inc.) following the manufacturer's instructions. The ozonides were reduced to the corresponding aldehydes with triphenylphosphine and analyzed by GLC on 10% Silar 10C, aldehydes at 58 C and a helium carrier gas flow of 50 cc/min, and aldehydo-esters at 150 C and carrier gas flow of 100 cc/min. Peaks were identified by comparison with retention time semilog plots of homologous series of aldehydes and aldehydo-esters. Propanal, pentanal and heptanal were obtained from Eastman Kodak; nonanal was prepared from methyl oleate by ozonolysis. The 5-,8-, and 11-carbon aldehydo-esters were prepared by ozonolysis of methyl esters of 5,8,11,14-20:4, 8,11,14-20:3, and 11,14,17-20:3, respectively.

RESULTS AND DISCUSSION

GLC analysis of the total methyl esters prepared from reproductive tissues (testes, seminal vesicles, accessory glands, ejaculatory duct) of male *A. domesticus* and *Gryllus* spp. revealed a substantial peak (6%) (9) with ECL of 20:40, 21.35 and 22.22 on 3% OV 225, 15% OV 275, and 10% Silar 10C columns, respectively. This compound, which was shown previously (9) to be a 20-carbon triene fatty acid, proved to be chromatographically distinct from both 8,11,14-20:3 and 11,14,17-20:3 on 10% Silar 10C at 180 C.

Hydrogenation of the 20:3 methyl ester produced an ester with an ECL identical to that of methyl arachidate (n-20:0) on 10% Silar 10C, thus eliminating the possibility of a branched hydrocarbon chain. Ozonolysis of the 20:3 methyl ester produced a 6-carbon aldehyde, a 5-carbon aldehydo-ester, and one additional major peak with an aldehydo-ester ECL of 6.40. The latter peak was not positively identified but was assumed to be a C_6 dialdehyde.

These data indicated a straight-chain, 20-carbon acid with unsaturation in the 5 and 14 positions and one additional double bond in either the 8 or 11 position.

Cochromatography of cricket 20:3 methyl ester with methyl esters prepared from *Caltha* seed oil (10) revealed a retention time on OV 225; OV 275, and Silar 10C columns identical

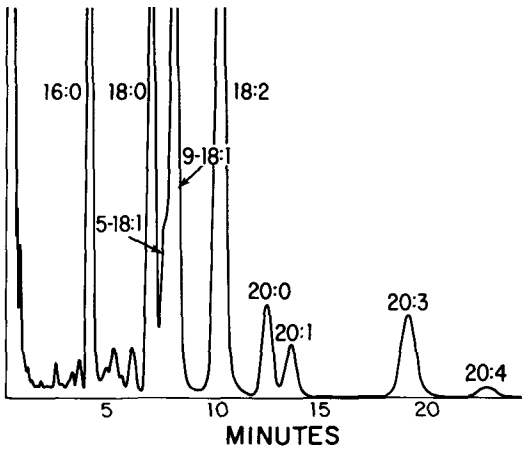

FIG. 1. Gas liquid chromatogram of total methyl esters prepared from *A. domesticus* reproductive tissues. Column packing–15% OV275. Operating conditions given in Materials and Methods.

TABLE I

Fatty Acid Composition of Neutral and Phospholipids of the Male House Cricket (*Acheta domesticus*) Reproductive Tract

	Fatty acids (wt %)							
	16:0	18:0	18:1	18:2 + 20:0	20:1	20:3	20:4	Other
Neutral lipid	23.9	6.8	26.1	36.4	1[a]	T	T	6.9
Phosphatidylethanolamine[b]	10.7	18.8	21.2	40.2	4.1	4.2	.4	.1
Phosphatidylcholine[c]	16.2	11.9	20.9	36.6	1.0	12.6	.5	.3

[a]T = less than 0.1%.

[b]Contained some phosphatidylcholine.

[c]Contained some phosphatidylethanolamine.

to that of *Caltha* seed 5,11,14-20:3. We conclude from these data that the cricket 20:3 is the 5,11,14-isomer.

GLC of total methyl esters on 15% OV 275 (Fig. 1) revealed 2 components in the 18:1 peak. Ozonolysis of this component produced a C_5 and C_9 aldehydo-ester in relative proportions of ca. 33 and 67 mol %, respectively. Thus, the 18:1 component consisted of both the Δ5 and Δ9 positional isomers. The Δ5 isomer probably corresponds to the atypical 18:1 isomer previously observed in house cricket testicular phospholipids (3).

The neutral and phospholipid fractions separated by Florisil chromatography were analyzed by GLC on Silar 10C columns and the weight percentages of fatty acids are given in Table I. The 20:3 acid was concentrated primarily in phosphatidylcholine. The GLC profile showed only traces of 20:1, 20:3 and 20:4 in the neutral lipid fraction. The unidentified 20-carbon unsaturated acid observed in house cricket testicular phospholipids by Lambremont and Dial (3) was probably the 5,11,14-20:3-isomer that we have identified in this study. The values reported by Lambremont and Dial for the unidentified acid agree closely with the 20:3 values found in this study (Table I).

The 5,11,14-20:3 fatty acid has not, to our knowledge, been previously reported in insects and only rarely in other animal systems. This compound is an end-product of linoleic acid metabolism in the cat (11), an animal that is apparently unique in its inability to convert linoleate to arachidonate. The 20:3 acid is also produced in vitro from 11,14-20:2 by human (19) and rat (20) testicular tissue and by rat liver microsomes (19). In vivo studies have shown rats to be unable to convert 5,11,14-20:3 to arachidonic acid (21). Mammalian species generally desaturate linoleic acid (9,12-18:2) to 6,9,12-18:3 which is then elongated to 8,11,14-20:3. Arachidonic acid is then produced by further desaturation at the 5 position.

The probable metabolic pathway for the production of 5,11,14-20:3 in the cricket is not apparent from the fatty acid profiles. Although this acid is known to occur in some plants, it is unlikely that it was obtained from the food supply. Dietary fatty acids influence the fatty acid profiles of tissue triacylglycerols (22) and the 20:3 acid was not detected in measurable amounts in the tissue neutral lipids. Analysis of the mouse chow failed to show the presence of 20:3.

Biosynthesis of 5,11,14-20:3 by desaturation of 18:2 to 18:3 followed by chain elongation would require a Δ3 desaturase. GLC analysis of tissue fatty acids on OV 225 revealed a trace of 18:3, but this peak appeared to be that of the 9,12,15-isomer (linolenic acid). On OV 275 and Silar 10C columns, 18:3 is coeluted with 20:1. An alternative pathway would require elongation of 9,12-18:2 to 11,14-20:2 followed by desaturation at the 5 position. However, 20:2 was not detected by either GLC (Fig. 1) or by gas chromatography/mass spectrometry (9).

The role of 5,11,14-20:3 in the cricket reproductive system is unknown to us. Experiments are in progress to determine if this fatty acid is the potent inhibitor of prostaglandin synthetase demonstrated earlier in the house cricket (6,7).

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METHODS

Separation of Wax Esters from Steryl Esters by Chromatography on Magnesium Hydroxide

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ABSTRACT

Chromatography of stearyl oleate and cholesteryl oleate on thin layer plates coated with magnesium hydroxide-celite, 1:1, or magnesia-celite, 1:1, showed that magnesium hydroxide had better resolving power for the separation of these wax ester and steryl ester model compounds than did magnesia, an adsorbent which has been used previously for this separation. By means of high pressure liquid chromatography on magnesium hydroxide, wax esters and steryl esters from the skin surface lipids of human, rat and monkey were separated completely and without hydrolysis.

INTRODUCTION

The need to separate wax esters and steryl esters arises in studies of mammalian skin surface lipids (1,2) and plant lipids (3,4). These separations are also of importance in metabolic and nutritional studies of fish (5,6) and terrestrial herbivores (7). Since chromatography on silicic acid fails to resolve wax esters and steryl esters, alternative means have been sought (8,9). The most satisfactory method reported to date involves column chromatography on magnesia (MgO) as described by Nicolaides (9). This method has been used to resolve wax ester-steryl ester mixtures from several natural sources (2-7). A disadvantage of the magnesia method for wax ester-steryl ester separation is the difficulty of achieving a complete separation. Furthermore, magnesia tends to cause partial hydrolysis of esters (9). This problem could be alleviated but not cured by prior removal of fines from the adsorbent (9).

While experimenting with separation of wax esters and steryl esters by thin layer chromatography (TLC) on magnesia, we observed that this adsorbent had a rather low capacity for steryl esters and it seemed possible that the separation was occurring on an inherent contaminant of magnesia, perhaps magnesium hydroxide. Magnesium hydroxide has, in fact, been found by Keefer and Johnson to be a satisfactory alternative to magnesia for the separation of α- and β-carotene (10) and to be useful also in separations of polynuclear hydrocarbons (11) and azaaromatic compounds (12). These authors did not, however, make any direct comparison of the resolving power or capacity of magnesia and magnesium hydroxide. When we proceeded to compare the behavior of wax ester and steryl ester standards on TLC plates coated with mixtures of magnesia or magnesium hydroxide with celite, we observed that magnesium hydroxide, like magnesia, retarded steryl esters, but did so much more strongly. However, low capacity appeared to be a characteristic of both adsorbents. We then attempted preparative scale separations of the wax esters and steryl esters from the skin surface lipids of the human, the rat and the Cynomolgus monkey using column chromatography on magnesium hydroxide performed with high pressure liquid chromatography (HPLC) equipment. The HPLC separation proved to be quick and complete and no hydrolysis of the esters was observed.

MATERIALS AND METHODS

TLC on Magnesium Hydroxide and Magnesia

Laboratory grade magnesium hydroxide was purchased from Fisher Scientific Co. and magnesia (MX-66 catalytic grade 200 mesh adsorptive powder) from Matheson Co. (Norwood, OH). For TLC, each adsorbent was mixed with an equal weight of celite (Fisher) and then coated as a slurry on 20 × 20 cm glass plates in 0.5 mm layers. The magnesia-celite slurry was mixed in a blender. The coated plates were allowed to air-dry for about 5 min and then placed in a 100 C oven for 2 hr. Before use, the layers were scored into 6-mm-

wide lanes. Chromatograms were developed with 1% ethyl acetate in hexane in paper-lined tanks. Developed chromatograms were charred by spraying with 50% aqueous sulfuric acid and then heating to 220 C on a hot plate.

HPLC on Magnesium Hydroxide

HPLC equipment consisted of an injector, pump, solvent flow programmer, refractive index detector, and an empty 7.8 mm id × 61 cm column, all purchased from Waters Associates (Milford, MA). Magnesium hydroxide was packed into the column dry and without prior treatment to increase or decrease the average particle size, since no fines had been seen when a sample of the substance was suspended in chloroform and grinding the magnesium hydroxide had been found to reduce its resolving power. The column was washed overnight with 2% ethyl acetate in hexane, flowing at 0.5 ml/min. For the separation of wax esters from steryl esters, a 5- to 10-mg sample was injected into the system and eluted with 2% ethyl acetate in hexane. The solvent flow was programmed from 5 to 10 ml/min along a concave curve (curve 10 on the Waters flow programmer) in order to improve the shape of the steryl ester peak. The maximal pressure required was 300 lb/sq in.

Analysis of HPLC Fractions

Wax esters and steryl ester fractions were dissolved in 0.5 ml of benzene and saponified by adding 1.0 ml of 1.0 M methanolic KOH and heating at 70 C for 2 hr. The fatty acids were then methylated by adding an excess of boron trichloride/methanol directly to the saponification mixture. The saponification products were analyzed on plates coated with 0.25 mm layers of Silica Gel G. The plates were developed twice to the top with hexane/ether/acetic acid, 70:30:1, and the spots were detected by charring. To isolate sterols and alcohols for gas chromatographic analysis, saponification products were applied to 1-mm-thick Silica Gel G plates and, after development as for the analytical chromatograms, the bands were located by spraying with a solution of 2′7′-dichlorofluorescein (Eastman Kodak Co.) in ethanol and viewing under UV light. The bands were then scraped from the plates and the lipids eluted from the adsorbent with ether. Gas chromatographic analysis of the sterols and alcohols was done on a 3 ft column of 3% OV 17 in an F&M 402 instrument (Hewlett-Packard Co.). The temperature was 270 C for separating alcohols and 300 C for separating sterols or checking for their presence.

Isolation of Skin Surface Monoesters (Wax Esters + Steryl Esters)

Human skin surface lipid was extracted with chloroform from the hair and scalp of volunteers. The hair had been washed 3 days previously with a 2% solution of sodium dodecyl sulfate. Rat surface lipid was recovered by partially immersing anesthetized, mature male rats in acetone. Monkey (*Macacca fascicularis*) surface lipid was extracted with chloroform from the body fur of a tranquilized animal. Monoesters were isolated from total surface lipid by TLC on 1-mm-thick layers of Silica Gel G. For human and monkey lipids, the plates were developed with hexane and then with toluene (13); for rat lipids, development was with hexane/benzene, 1:1 (14). The monoester bands were detected using dichlorofluorescein and then eluted with ether.

RESULTS

Keefer has noted that it is easier to produce satisfactory thin layers of magnesium hydroxide than of magnesia (11) and we have confirmed his observation. However, by mixing magnesia with an equal weight of celite and preparing a slurry of this mixture in a blender, we were able to spread uniform layers which did not crack when dried. Addition of celite was not necessary to obtain satisfactory thin layers of magnesium hydroxide, but in order to compare this adsorbent with magnesia under similar conditions, magnesium hydroxide was also mixed 1:1 with celite before being used to prepare TLC plates. An advantage of adding celite was that it permitted detection of spots by charring, since it reduced the tendency of both magnesia and magnesium hydroxide to peel away from the glass when the plate was heated.

In Figure 1, the TLC separation of stearyl oleate from cholesteryl oleate on magnesium hydroxide/celite, 1:1, developed with 1% ethyl acetate in hexane, is compared to the separation of these substances on magnesia/celite, 1:1, using the same solvent system for development. Cholesteryl oleate was considerably more strongly retarded on magnesium hydroxide/celite than on magnesia/celite, even though the difference in migration of stearyl oleate on the two adsorbents was small. Thus, the resolution of the two substances was better on magnesium hydroxide/celite. Our speculation that a small amount of magnesium hydroxide occurring in magnesia might be the active adsorbent was not supported by the chromatograms in Figure 1. The increase in band width as the amount of cholesteryl oleate was increased from 1 μg to

20 μg was about the same on both magnesia and magnesium hydroxide, suggesting that the number of binding sites is similar in both adsorbents. A greater affinity of the binding sites on magnesium hydroxide for steryl esters may account for the better resolution on this adsorbent.

Because of the relatively low capacity of magnesium hydroxide, column chromatography, rather than TLC, was used for developing a preparative scale separation of wax esters and steryl esters. Since solvent flow rates of up to 10 ml/min did not cause packing down of the magnesium hydroxide, we were able to take advantage of the speed and convenience of HPLC. Figure 2 shows the results when human, rat, or monkey monoesters were subjected to HPLC on magnesium hydroxide. Complete separation of the wax esters from the steryl esters appeared to have been obtained in all 3 cases, even with the monkey monoesters, in which the proportion of steryl esters is high.

The completeness of the separations was confirmed by examination of the products obtained after saponification and methylation of the material recovered in each peak. TLC of the products from the wax ester peaks from all three species showed spots corresponding in mobility to fatty acid methyl esters and wax alcohols only. The steryl ester peak from the human yielded spots corresponding to fatty acid methyl esters and cholesterol, as well as a small quantity of another, unidentified sterol. Methyl esters and 4 sterols were detected in the steryl ester peak from the rat. The monkey steryl ester peak yielded methyl esters and a sterol spot which appeared to be mostly or entirely cholesterol as judged by the pink color which appeared during charring. The fractions collected between the HPLC peaks in the human and rat chromatograms contained no lipid. An intermediate fraction was not obtained in the monkey chromatogram. Because of overlapping of the rat sterol spots with the location where alcohols would appear if present, a further check on the purity of the HPLC fractions of the rat skin lipids was made by separating the fatty alcohols and sterols from the fatty acid methyl esters by preparative TLC and then subjecting them to gas chromatography. No peaks corresponding to sterols were found in the alcohols and no peaks corresponding to alcohols were found in the sterols.

The possibility that the monoesters could have been partially hydrolyzed during chromatography on magnesium hydroxide, as has been reported for the magnesia method (9), was checked after the column had been used for

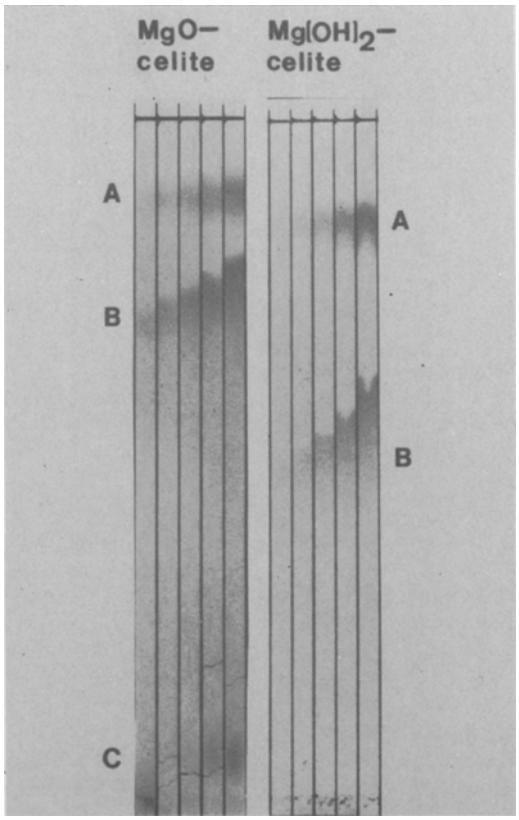

FIG. 1. Separation of stearyl oleate (A) from cholesteryl oleate (B) and from hydrolysis products (C) on magnesia/celite, 1:1, or magnesium hydroxide/celite, 1:1. The lanes on each plate were spotted with 1, 2, 5, 10, or 20 μg each of stearyl oleate and cholesteryl oleate and the plates were developed in 1% ethyl acetate in hexane.

chromatography of several batches of monoesters. The column was eluted with several times the volume of 100% ethyl acetate found to be necessary to elute lauryl alcohol. The eluate was evaporated to a small volume and a portion subjected to TLC. No alcohols were detected. Possibly the rapid flow rates made possible by HPLC equipment and the inclusion of a hydrolyzable solvent, i.e., ethyl acetate, in the eluting solvent, assists in avoiding hydrolysis on this adsorbent. However, magnesium hydroxide may be inherently less active in hydrolyzing esters than is magnesia. Indeed, it can be seen in Figure 1 that some material, possibly hydrolysis products, is left at the origin on the magnesia plate whereas there is nothing detectable at the origin of the magnesium hydroxide plate.

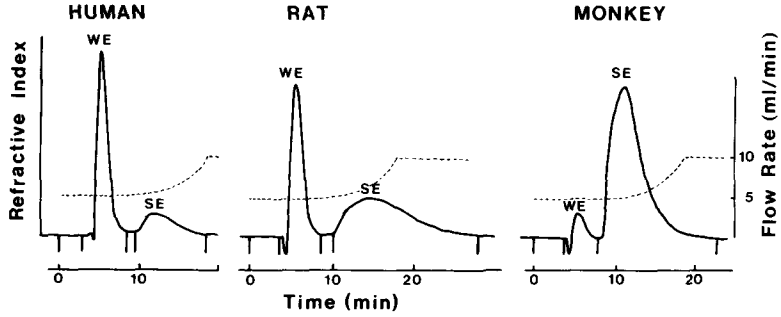

FIG. 2. Separation of the skin surface monoesters of the human, the rat, and the monkey into wax esters (WE) and steryl esters (SE) by HPLC on magnesium hydroxide. Five mg of human or monkey monoesters or 10 mg of rat monoesters was injected. The rate of flow of the eluting solvent (2% ethyl acetate in hexane) was programmed along the curve shown (dotted line). Fractions were collected as indicated by the event marks.

DISCUSSION

The mechanism of the separation of wax esters and steryl esters on magnesium hydroxide is a matter for speculation. The separation is qualitatively similar to the separation on magnesia, i.e., steryl esters are retarded relative to wax esters, although more strongly so on magnesium hydroxide than on magnesia. This behavior is unlike that which occurs on silica gel TLC plates, where, in a suitable solvent (e.g., benzene), cholesteryl oleate has a slightly larger R_f value than stearyl oleate and the broader bands formed by natural mixtures of wax esters and steryl esters overlap. The wax ester-steryl ester separation on magnesium hydroxide or magnesia appears, therefore, not to be based on polarity, as are separations on silica gel. Nicolaides, considering the relative strength of binding various carotenoids and porphyrins on magnesia, as well as the retardation of steryl esters relative to wax esters, proposed that relative molecular flatness is the property involved in separations on magnesia (9). This flatness effect may apply also to separations on magnesium hydroxide, since wax esters and steryl esters do not have the differences in oxygen functions or unsaturation that Keefer and Johnson consider to be the bases for separations on this adsorbent (10).

In the wax ester-steryl ester separation, magnesium hydroxide had several advantages over magnesia. A major one was the stronger retardation of steryl esters on magnesium hydroxide, which made a complete separation of the 2 lipid classes much easier to achieve. Although both adsorbents have low capacity for binding steryl esters, more overloading of steryl esters should be tolerable on magnesium hydroxide because of the greater separation of the bands. This supposition is supported by the successful separation of monkey monoesters which consist mostly of steryl esters (Fig. 2). Another important advantage of magnesium hydroxide as used in the HPLC method described here is the lack of hydrolysis of esters. Whether the inclusion of ethyl acetate in the eluting solvent is important in avoiding hydrolysis is not known, but in any case, ethyl acetate may be preferable to acetone for use with a refractive index detector since acetone has been found to form condensation products on magnesia (9,15). Finally, magnesium hydroxide is more convenient to use than magnesia for both thin layer and column chromatography. As reported by Keefer (11), magnesium hydroxide forms excellent thin layers without the addition of a binder. Columns packed with the material as it is supplied by the manufacturer presented only moderate resistance to flow and, since magnesium hydroxide did not become compressed under pressure, HPLC equipment could be used without the special techniques for column packing required when magnesia is used (16).

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Syntheses of Radioactive Furan Fatty Acids

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ABSTRACT

A novel route for the synthesis of naturally occurring furan fatty acids with particular emphasis on labeling with ^{14}C is described. Methyl [2-^{14}C] 9-(5-pentyl-3,4-dimethyl-2-furyl)nonanoate was synthesized from 3,4-dimethyl-2-pentylfuran by a new route. [3-^{14}C] 11-(5-Pentyl-3-methyl-2-furyl)undecanoate and [2-^{14}C] 9-(5-pentyl-2-furyl)nonanoate were prepared from their lower homologs. The label was introduced in all cases by means of the Arndt-Eistert method for chain elongation, using $^{14}CH_2N_2$. Comparisons of yields show that, with increasing number of substituents on the ring, the furan compounds are increasingly subject to uncontrollable side reactions.

INTRODUCTION

Furan fatty acids (F acids) with methyl groups on the ring (1) were first discovered in liver and testes lipids of northern pike *(Esox lucius)* (2). Subsequently, they were found in lipids of many fish species (3–6) and in some cases they may represent the major portion of the total fatty acids in these tissues. However, the occurrence of F acids is not limited to *Pisces*. Acids of the same or very similar structure have also been encountered in soft corals (7), rubber latex (8) and *Exocarpus* seed oil (9), and one may expect their detection in additional biological materials. Investigations of the biochemistry and biological role of the furan fatty acids require methods for their radioactive synthesis. The particular goal in the work reported here was to obtain labeled fatty acids which would be used to study biological conversions within this class of fatty acids.

Recently, we described syntheses of two of the naturally occurring furan fatty acids (10). The preparative sequences involved 4-methyl-2-pentylfuran or 3,4-dimethyl-2-pentylfuran as intermediates. They were converted via their α-Li compounds and additional steps to esters of 11-(5-pentyl-3-methyl-2-furyl)undecanoic, MeF(11,5) (see Appendix), or 11-(5-pentyl-3,4-dimethyl-2-furyl) undecanoic, diMeF(11,5) acids. We are now reporting synthesis of radioactive F acids by alternate routes.

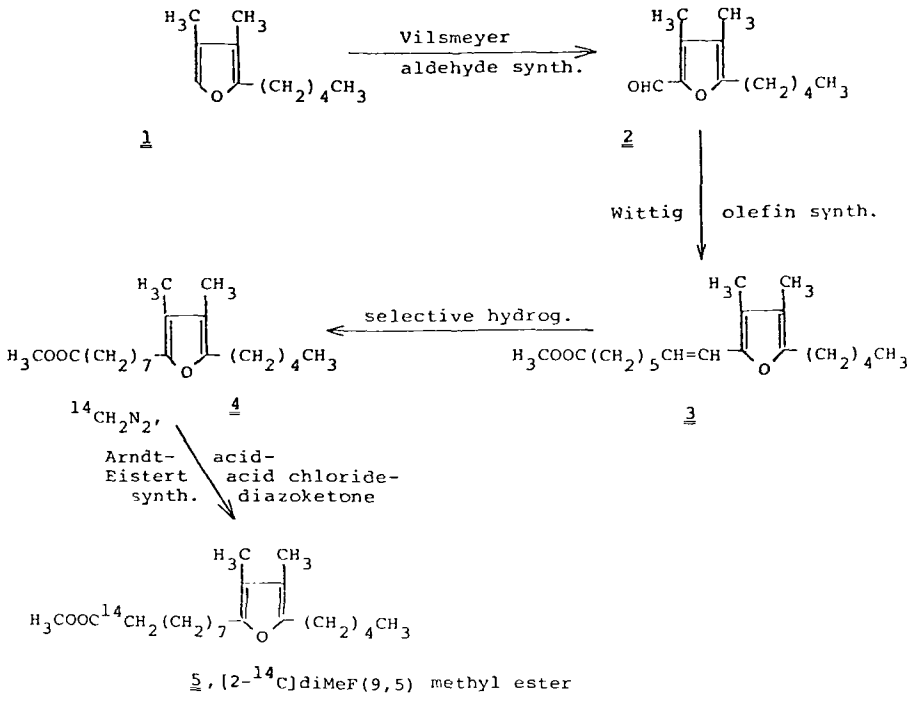
The di- and trialkylfurans to which we referred are accessible in satisfactory yield from commercial materials (10) and we used 3,4-dimethyl-2-pentylfuran 1 as the starting material (Scheme I). It was converted to the furfural 2 by reaction with dimethylformamide/$POCl_3$ (11, 12). The aldehyde is amenable to carbonyl olefination (12–14). The aldehyde 2 (Scheme I) was allowed to react with the ylid prepared from (6-carbomethoxyhexyl)triphenylphosphonium iodide to obtain the methyl ester of the olefinic furan fatty acid 3. Selective hydrogenation of the olefinic bond (12, 15) gave diMeF(8,5) 4 which is a lower homolog of the naturally occurring diMeF(9,5) in regard to the alkylcarboxyl chain. The reactants for these synthetic steps can be altered and yields from such variations are given.

The acid 4 with a chain length of 17 C atoms has not been encountered in nature. It was converted, as the acid chloride, by reaction with $^{14}CH_2N_2$ and rearrangement of the resulting diazoketone (16,17) to [2-^{14}C]-diMeF(9,5), 5. The ester was shown to be identical to that of diMeF(9, 5) from fish (F_3 in ref. 1). This method of labeling was applied also to furan fatty acids with one and without methyl substituent.

EXPERIMENTAL

3,4-Dimethyl-2-pentylfuran 1 was prepared in 45% yield as previously described from commercial 3,4-bis (acetoxymethyl)furan (10). 7-Bromoheptanoic acid was from ICN-K&K Laboratories Inc. (Plainview, NY), and N-^{14}C-methyl-N-nitroso-*p*-toluenesulfonamide from New England Nuclear Corp. (Boston, MA). MeF(9,5) acid was isolated from rubber latex (8) and contained ca. 6% of straight chain fatty acids. The furan fatty acid F(8,5) without methyl groups on the ring was synthesized from furan by a variation of known reactions (18, 19). Because furan compounds may be autoxidized, all procedures were done with the usual protection by nitrogen.

Silica Gel H (Merck) and hexane/diethyl ether/acetic acid (85:15:1, by volume) were used for thin layer chromatography (TLC). Gas liquid chromatography (GLC) was done in an aluminum column, 3.2 mm × 2.45 m on a phase of CS-5, 10% on Chromosorb WAW,



SCHEME I. Synthesis of methyl [2-^{14}C] 9-(5-pentyl-3,4-dimethyl-2-furyl)nonanoate.

100–120 mesh, from AllTech (Arlington Heights, IL). The temperature was 150–240 C with a gradient of 5 C/min. Low resolution MS was done under conditions previously described (10).

5-Pentyl-3,4-dimethylfurfural 2

One-half ml $POCl_3$ was added by drops at 0 C within ca. 1 min to 0.5 ml dimethylformamide with magnetic stirring. During further stirring for 1 hr at 0 C, the mixture became somewhat viscous. 3,4-Dimethyl-2-pentylfuran 1 (0.86 g, 5.18 mmol) was added, using a small amount of dimethylformamide for rinsing. The reaction began immediately, with generation of heat and HCl, turning the solution dark red. After stirring for one more hour at 0 C, the mixture was poured onto ice. Aqueous K_2CO_3 was added until pH 9 was reached. The oil which formed on the surface during 5 hr stirring at room temperature was extracted with diethyl ether (2 x 50 ml). The extract was washed with water, dried over Na_2SO_4 and the ether removed under vacuum.

Analysis by TLC revealed the presence of a considerable amount of strongly polar material in the crude preparation. This was removed by column chromatography on 15 g activated Unisil (100–200 mesh), 3.2 cm high, and eluting the desired compound with a mixture of hexane and diethyl ether, changing the ratio in 4 steps from 98:2 to 75:25 (total eluting volume, 150 ml). The aldehyde 2 (0.43 g, 43%) was recovered pure according to TLC and >99% pure according to GLC.

2-Pentylfuran (4.0 g, 29 mmol) under similar reaction conditions (2.7 ml $POCl_3$ and 2.2 ml dimethylformamide) gave 99% pure 5-pentylfurfural in considerably better yield (3.5 g, 73%).

Methyl 7-Iodoheptanoate

7-Bromoheptanoic acid (15 g, 71.7 mmol) was esterifed in 100 ml methanol + 1 ml conc. H_2SO_4. After 4 hr of reflux, most of the methanol was removed under vacuum. Water was added and the ester extracted with ether, washed with 1% aqueous $NaHCO_3$ and dried over Na_2SO_4. The yield of ester (16 g) was virtually quantitative.

Sodium iodide (16.1 g, 107.6 mmol) was dissolved in 100 ml methylethylketone by stirring for 1 hr under reflux. The bromo-ester was added and the mixture refluxed for 10 hr with stirring and breaking up lumps of NaBr which formed during that time. After standing overnight at room temperature, NaBr was filtered off and most of the methylethylketone

removed under vacuum. Water was added and the iodo-ester extracted with benzene (2 × 125 ml). After washing with water and 5% aqueous $Na_2S_2O_3$, the benzene solution was dried over Na_2SO_4. The product (18.5 g, 95% from bromo-acid) was, according to GLC, free of bromo-ester.

(6-Carbomethoxyhexyl)triphenylphosphonium Iodide

Methyl 7-iodoheptanoate (19.3 g, 71.5 mmol) and triphenylphosphine (22.5 g, 86 mmol) were dissolved in 80 ml benzene. After reflux for 10 hr, most of the solvent was removed under vacuum, leaving behind a highly viscous yellow oil. The (6-carbomethoxyhexyl) triphenylphosphonium iodide could not be brought to crystallization. To remove unreacted components, the material was extracted with 5 portions of diethylether by kneading it with a heavy glass rod.

Methyl 11-iodoundecanoate and triphenylphosphine yielded (10-carbomethoxydecyl) triphenylphosphonium iodide which, after washing with ether, crystallized as a white powder (yield 85%).

Methyl 8-(5-Pentyl-3,4-dimethyl-2-furyl) octanoate 4, diMeF(8,5)

The (carbomethoxyhexyl) triphenylphosphonium iodide (3.5 g, ∿5.6 mmol) was added in 10 ml dimethylformamide to compound 2 (0.43 g, 2.2 mmol) with stirring. Freshly prepared $NaOCH_3$ (20) (0.36 g, 6.67 mmol) was added in one portion at 0 C. The solution rapidly turned orange. After stirring at room temperature for 10 hr, it was poured on ice, extracted with ether (2 × 50 ml), washed with water and dried over Na_2SO_4. Analysis of the oily residue from the ether extract by GLC showed one peak corresponding to unreacted aldehyde 2 (∿10%) and two major peaks with longer retention times. Under the described conditions, the relative amounts of the two major peaks were in the order of 3:1, but their ratio changed with reaction time, temperature and other conditions. Results from the hydrogenation and other evidence (see following) suggest that these peaks represent the two stereoisomers of the olefinic furan acid methyl ester 3.

The crude preparation was hydrogenated in hexane for 2 hr at 2.7 atm H_2 over $Pd/CaCO_3$. Under these conditions, only the olefinic double bond is hydrogenated. GLC of the product showed only one major peak and TLC gave 4, diMeF(8,5) (140 mg, yield 20% from the aldehyde 2) in 98% purity.

The structure of 4 was confirmed by the diagnostic MS peaks, m/z (rel. int.):322 (M^+, 42); 265 ($M-C_4H_9$, 52); 179 ($M-C_6H_{12}COOCH_3$, 100); and 123 ($M-C_4H_9-C_6H_{12}COOCH_3$, 12). The ions correspond to those from diMeF(9,5) and diMeF(11,5) of fish (F_3 and F_6 in ref. 1) when the homology of structures is taken into account (10).

The methyl ester of a higher homolog, diMeF(12,5), 12-(5-pentyl-3,4-dimethyl-2-furyl) dodecanoate was prepared using furfural 2 and (10-carbomethoxydecyl) triphenylphosphonium iodide (already described). The product again gave two major GLC peaks. Their mass spectra were identical, confirming the assumption of stereoisomers. After hydrogenation, GLC with methyl stearate as an internal standard indicated overall yields of 65–80%.

Methyl [2-^{14}C] 9-(5-Pentyl-3,4-dimethyl-2-furyl) nonanoate, [2-^{14}C] diMeF(9,5), 5

The Arndt-Eistert chain elongation as described for polyunsaturated fatty acids (17) was applied with minor modifications. Ester 4 was saponified and the acid recovered. Reaction of the acid (140 mg, 0.45 mmol) with oxalyl chloride (1.8 mmol) was done in 3 ml benzene with stirring for 1 hr at a temperature close to the freezing point of the mixture and for an additional hour at room temperature. The acid chloride was recovered, dissolved in ether and immediately used for the following reaction.

The solution was added slowly at −18 C to $^{14}CH_2N_2$ in ether, prepared from labeled Diazald (0.59 g, 2.7 mmol, 1.0 mCi). Within 2 hr, the temperature was raised to 10 C with occasional magnetic stirring. ^{14}C-Methylchloride and excess $^{14}CH_2N_2$ were removed by a stream of nitrogen. The diazomethane was used for esterification of nonlabeled F acids to obtain labeled F esters which can be used as internal standards or for other analytical purposes.

The ether was evaporated and the crude diazoketone dissolved in 7.5 ml methanol. Freshly prepared silver benzoate (30 mg) in trimethylamine (0.5 ml) was added at room temperature by drops to the stirred solution. After 1 hr, the mixture was refluxed for 5 min and then worked up by filtration, addition of water, mild acidification with 2 N H_2SO_4 and thorough extraction with ether. After washing with aqueous $NaHCO_3$ and water, the solution was dried over Na_2SO_4.

The oily residue from the ether solution did not dissolve completely in hexane. After filtration, the preparation (154 mg) still contained polar contaminants which did not

migrate with methyl esters in TLC using a nonpolar solvent. It was purified by column chromatography on 15 g activated Unisil, 13.5 cm high. Elution with hexane/ether, 100:2 (600 ml) gave 38.1 mg [2-^{14}C] diMeF(9,5) ester. According to analytical GLC, it contained 12% starting material, diMeF(8,5) methyl ester. This contaminant does not interfere with biological use of the radioactive preparation since radio-GLC showed that it contained less than 0.5% of the total ^{14}C. The desired ester 5 represented 97% of the radioactivity (33.5 mg, 23.1% yield, 0.37 mCi/mmol). MS, m/z (rel. int.): 336 (M^+, 41), 279 (M-C_4H_9, 51), 179 (M-$C_6H_{12}COOCH_3$, 100), 123 (M-C_4H_9-C_6H_{12}-$COOCH_3$, 11). The data are in full agreement with those reported for this compound (1,2) and compatible with those of its homolog (10).

The method of labeling with $^{14}CH_2N_2$ was applied also to F(8,5) (0.5 g, 1.8 mmol) to obtain [2-^{14}C] F(9,5) ester (yield 80%) in chemical and radioactive purity of 95%. Chain elongation by 2 C atoms was done with MeF(9,5) (0.31 g, 0.96 mmol) using first $^{14}CH_2N_2$. The product [2-^{14}C] Me(10,5) was subjected to another elongation using nonlabeled CH_2N_2. [3-^{14}C] MeF(11,5) ester was obtained and was identical in analytical properties with one of the major F acids previously identified from fish (F_5 in ref. 1). The overall chemical yield was 47%, contaminated by 3.8% of the intermediate ester and 6% of other radioactive esters derived from acids that had been in the preparation from latex. In all cases, the molar radioactivities of the products were very close to those of the labeled Diazald used.

DISCUSSION

Several expedient procedures are available for synthesis of furan fatty acids with methyl substituents (see ref. 5a-m in ref. 10). However, syntheses of the biological F acids met with considerable difficulties due to the methyl substitution on the ring (10). This was also encountered in the reactions described here, in which we synthesized from the trialkylfuran 1 the tetrasubstituted diMeF(9,5) acid 5 in labeled form. Each of these synthetic steps was explored first with simpler reactants. Comparison of yields shows the tendency of multisubstituted furan compounds for loss by side reactions (see Experimental). Moreover, in the synthesis described in Scheme I, the heptanoate-phosphonium iodide necessary for the Wittig olefin synthesis, 2 → 3 could not be purified by crystallization and this seems to be of further disadvantage, since in several reactions with the crystalline undecanote phosphonium iodide, the yields were higher.

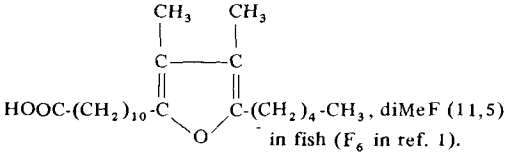
In view of these experiences, it is particularly desirable to introduce a tracer atom late in the sequence of reactions. The most common procedure for preparation of labeled fatty acids makes use of [^{14}C] cyanide and alkylhalides or mesylates (21). The natural F acids are readily reduced by $LiAlH_4$ to alcohols, but mesylates could not be prepared from them in satisfactory quality due to the instability of the furan ring. F acid chlorides, however, could be prepared so that chain elongation with $^{14}CH_2N_2$ was feasible. The sequence of reactions in the Arndt-Eistert method gives overall yields of better than 90% with polyunsaturated fatty acids, but with F acids, the yields decreased with increasing substitution of the furan ring.

Reaction of acids with carbon tetrachloride and triphenylphosphine has been recommended as a particularly mild method for preparation of organic acyl chlorides (22). We found this applicable to fatty acids and furan fatty acids, with carbon tetrachloride as well as hexachloroethane. The formation of acyl chlorides was proven by derivatization to anilids (23) and their identification by TLC. However, the acyl chlorides could not be purified from the phosphine compounds and these interfered when the elongation reactions were applied to the crude mixtures.

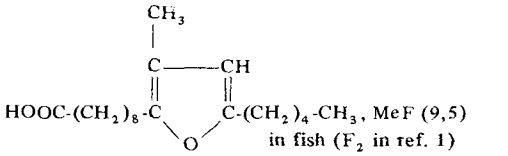
The radioactive acids, [2-^{14}C] diMeF(9,5), [3-^{14}C] MeF(11,5) and [2-^{14}C] F(9,5), were important for demonstrating some previously suggested biological interrelationships of furan fatty acids (3) by addition or loss of 2 C atoms at the proximal moiety (to be published). In addition, the synthetic sequence involving carbonyl olefination will contribute to structure assignments of olefinic F acids which have been detected by GLC-MS-SIM as trace components in some fish lipids (Krick et al., unpublished).

APPENDIX

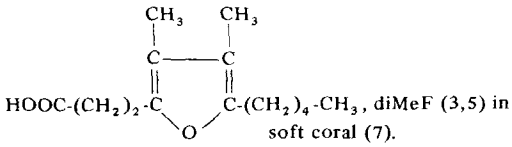
The following abbreviated notation is used: F, to indicate the furan ring; prefix Me or diMe, to indicate one or two methyl groups at the ring, one methyl being in position 3 or two methyls being in positions 3 and 4 of the ring; affix (m,n) to indicate, in this sequence, the chain lengths of the alkylcarboxyl (proximal) and of the alkyl (terminal) substituents, at ring positions 2 and 5, respectively. The notation is applicable to most furan fatty acids so far reported from natural sources. For example:



diMeF (11,5) in fish (F_6 in ref. 1).



MeF (9,5) in fish (F_2 in ref. 1) and in rubber latex (8).



diMeF (3,5) in soft coral (7).

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Dry Column Method for the Quantitative Extraction and Simultaneous Class Separation of Lipids from Muscle Tissue[1]

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ABSTRACT

A method for lipid isolation is presented that is alternative to the traditional chloroform/methanol extraction methods. This new method allows lipid isolation by solvent elution of a dry column composed of a tissue sample, anhydrous sodium sulfate, and Celite 545 diatomaceous earth ground together. To isolate total lipids, the dry column is eluted with a mixture of dichloromethane/methanol (90:10, v/v). Alternatively, the lipids may be isolated and simultaneously separated into neutral and polar fractions by a sequential elution procedure; neutral lipids free of polar lipids are eluted first with dichloromethane, followed by elution of polar lipids with the dichloromethane/methanol (90:10) mixture. The two dry column methods–isocratic or sequential elution–were compared with the traditional chloroform/methanol methods by gravimetric, thin layer chromatographic and phosphorus analyses.

INTRODUCTION

Although many methods have been proposed for the extraction of lipids from biological tissue, only two, originally proposed over two decades ago, have met with great acceptance because their use presumably allows lipid to be isolated quantitatively, unaltered and free of nonlipid contamination. These two methods–the Folch et al. (1) and the Bligh and Dyer (2)–require the use of mixtures of chloroform and methanol as the extraction solvent. Despite widespread use of these methods, work in this and other laboratories during the past several years (3-5) has shown limitations in both and in their more recent modifications (6): (a) chloroform is a suspected carcinogen; (b) excessive amounts of solvent are required; (c) the procedures are tedious and time-consuming, especially for multiple extractions; (d) use of expensive tissue homogenizing equipment is required; (e) emulsion problems often are encountered; (f) separation of the lipid into its subclasses requires subsequent chromatographic procedures.

We recently developed a dry column method for the determination of the total fat content of meat and meat products (3). The method was proposed as a replacement for the laborious Soxhlet ether extraction techniques currently used by food analysts to determine fat content. We now have found that, with some modifications, this same method can be used for the isolation of intact lipids from muscle and adipose tissue. In this report, we show that the same values for lipid content of muscle and adipose tissue may be obtained by the dry column method as those obtained by the traditional chloroform-methanol methods, but more rapidly and without many of the limitations encountered with the latter techniques. Moreover, we demonstrate that by a sequential application of solvents to the dry column, the lipid can be extracted from the column bed and separated simultaneously into major neutral and polar fractions.

EXPERIMENTAL

Reagents, solvents and apparatus were the same as discussed previously (3). Smaller columns (16 mm id × 25 cm with 8 mm id × 5 cm drip tip) were used for extraction of 1 g tissue samples. For these smaller samples, the ratio of tissue to Celite 545 to sodium sulfate was maintained as given previously for 5-g tissue samples.

Although the procedure will accommodate minced tissue, random sampling, for the purposes of this comparative study, of 5 g of tissue from much larger tissue sources demanded initial comminution of the entire tissue. This was accomplished with ease by use of a Cuisinart Model CFP5A home food processor (Robot-Coupe S.A., Stamford, CT 06902). A 5-g sample of comminuted tissue (2 g for adipose tissue) was weighed to the nearest 0.1 mg. When multiple extractions were to be done, complete subsets of 5-g portions were set aside at the same time. Samples of 1 g size were handled similarly. Because of difficulties in

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uniform sampling, it is preferable to handle the larger sample sizes when sufficient tissue is available.

Tissue Extraction: Isolation of Total Lipid by Isocratic Elution

Tissue (5 g) was ground in a 750-ml porcelain mortar with granular anhydrous sodium sulfate (20 g) and then with Celite 545 (15 g), and the resulting mixture was packed above a 1:9 $CaHPO_4$/Celite 545 trap (10 g) in a glass chromatography column (id 35 mm), as previously described (3). In this study, the columns were charged with CH_2Cl_2/MeOH (90:10) and 150 ml of eluate was collected in a 200-ml volumetric flask, whose contents then were brought to volume so that measured aliquots could be withdrawn for subsequent gravimetric, phosphorus and chromatographic analyses. Extracts of 1-g tissue samples were collected in 25-ml volumetric flasks.

Tissue Extraction: Isolation of Neutral and Polar Lipids by Sequential Elution

This procedure is similar to that described previously (3) except that two separate solvent systems are required–dichloromethane and the 90:10 mixture already described. The column was packed in the same manner, but it was charged first with 150 ml (for 5 g of tissue) of dichloromethane instead of the 90:10 solvent mixture. At the point when the last of the dichloromethane reached the top of the column packing, the flask containing the collected eluate (neutral lipid) was replaced by a second 200-ml volumetric flask, and the column was charged with 150 ml of the 90:10 solvent mixture. The eluate was collected until the column was stripped of solvent (60-90 min). This second fraction contained the polar lipids from the tissue sample. The contents of both volumetric flasks were brought to volume, and aliquots were removed as already described.

TABLE I

Variations in Elution Solvents to Optimize Efficiency of Method[a]

A. To minimize nonlipid coelution (isocratic procedure):

Solvent		Results
CH_2Cl_2/MeOH	80:20 (v/v)	Excessive nonlipid coelutes with lipid.
	90:10[b]	Complete elution of lipid. Nonlipid <0.1% of tissue weight with 1:9 trap (cf. text).
	99:5	Incomplete elution of very polar phospholipids.
	99:1	Incomplete elution of all classes of phospholipids.
CH_2Cl_2/i-PrOH	80:20	Some coelution of nonlipid. Some retention of phospholipid.
	90:10	Some retention of phospholipid. Excessive amount of solvent required.
CH_2Cl_2/acetone	80:20	All neutral lipids eluted by 10 ml; polar lipids elute after 100 ml, but incompletely.
CH_3CN		Phospholipids and nonlipid elute first, but incompletely; neutral lipids appear after 60 ml.

B. To optimize separation of neutral lipid from polar lipid (sequential elution procedure):

Solvents, numbered in their sequential order		Results
1. CH_2Cl_2 neat, 150 ml	b	Essentially complete elution of neutral lipid. No phospholipid coelution.
2. CH_2Cl_2/MeOH 90:10, 150 ml	b	Complete elution of phospholipid. Trace amount (ca. 3 mg) of neutral lipid (even after excess solvent is used in prior elution step).
1. CH_2Cl_2/MeOH (or i-PrOH) 99.75:0.25, 100 ml		Neutral lipid elutes with trace of phospholipid.
2. CH_2Cl_2/MeOH (or i-PrOH) 99.75:0.25, 50 ml		Trace of neutral and polar lipid.
3. CH_2Cl_2/MeOH (or i-PrOH) 99.75:0.25, 50 ml		Trace of neutral and polar lipid.
4. CH_2Cl_2/MeOH 90:10, 75 ml		Elution of polar lipid; coelution of some neutral lipid.

[a]All experiments done on 5-g tissue samples.

[b]Indicates preferred procedure.

Thin Layer Chromatography (TLC)

Adsorbent and development systems are given in Figures 1 and 2. All TLC visualizations were accomplished first by spraying the developed plate with ninhydrin (0.3% in absolute ethanol) with subsequent warming to reveal amino-group-containing lipids and nonlipids as pink spots. The same plate then was cooled and sprayed with Phospray (Supelco, Inc., Bellefonte, PA) to visualize phospholipids as heteropolyphosphomolybdate blue spots. (Some substances used in TLC such as Phospray and chloroform are toxic and must be used with care and proper ventilation.) Finally, the plate was heated in a fume hood to reveal all the material as charred spots.

For quantitative recovery of neutral lipid contaminants from polar fractions, lipid was applied quantitatively to 20 x 20 cm 250-μ-thick plates of Silica Gel H and 5% $(NH_4)_2SO_4$ (L/S Redicoats; Supelco, Bellefonte, PA) using a TLC sample streaker (Applied Science, State College, PA). Development was in chloroform/methanol/water (65:35:5), and visualization was with iodine vapor. Bands of neutral lipids were scraped, packed into chromatography columns, and eluted with dichloromethane to recover lipid from the adsorbent. Subsequent elution with dichloromethane/methanol (1:2) failed to remove any additional neutral lipid, as confirmed by examination of aliquots by TLC.

Phosphorus Analysis

Aliquots of lipid solutions were analyzed for phosphorus according to the method of Vaskovsky et al. (7). Quantitation was accomplished by concurrent determination of seven pairs of solutions of inorganic standard KH_2PO_4 ranging in content from 0 to 3.39 μg P. Absorb-

TABLE II

Variations in Column Packing to Optimize Efficiency of Method[a]

Modification	Results
A. To optimize column flow rate	
Column packed dry to height of 60-70 mm, then charged with solvents and allowed to drip without assistance[b].	Column loading is rapid and facile; elution is unattended and complete within 2 hr.
Loosely compressed packing.	Channeling; incomplete elution of lipid.
Column packed wet (dry packing added to column filled with CH_2Cl_2).	Polar lipids eluted with neutral lipids during sequential extraction.
Eluates drawn off under vacuum.	Occasional vapor lock; increased amounts of nonlipid coeluted; poor class resolution during sequential extraction.
B. To optimize separation of neutral from polar lipid (sequential procedure):	
Non-washed Celite 545[b].	Optimal flow as well as best separation of lipid classes.
Acid-washed Celite 545.	Same results as above, but more expensive.
Celite 545 washed by solvent (CH_2Cl_2/MeOH 92:8 azeotrope; Soxhlet extraction).	Same results as above, but requiring additional time for extraction and subsequent drying. Negligible removal of contaminants.
Fine-mesh ("Analytical grade") Celite.	Column flow rate severely limited.
Celite 545 pre-equilibrated with water in water-saturated atmosphere.	Poor separation of lipid classes during sequential elution.
Celite 545 pretreated with 1% water.	Poor separation of lipid classes during sequential elution.
Celite 545 heat-treated at 600 C.	Poor separation of lipid classes during sequential elution.
$NaHCO_3$ instead of Celite 545.	No separation of lipid classes.
Acid/base washed and silylated Celite 545.	No improvement in class separation and slight retention of phospholipid.
C. To eliminate nonlipid coelution:	
Trap at base of column = 10 g $CaHPO_4$/Celite 545 1:9[b].	Retains all but trace amounts of nonlipid.
Same trap as above, in 2:8 ratio.	Column flow retarded; incomplete removal of lipid.
No trap.	Coelution of nonlipid >0.1% of tissue weight.

[a]All experiments done on 5-g tissue samples.

[b]Indicates preferred procedure.

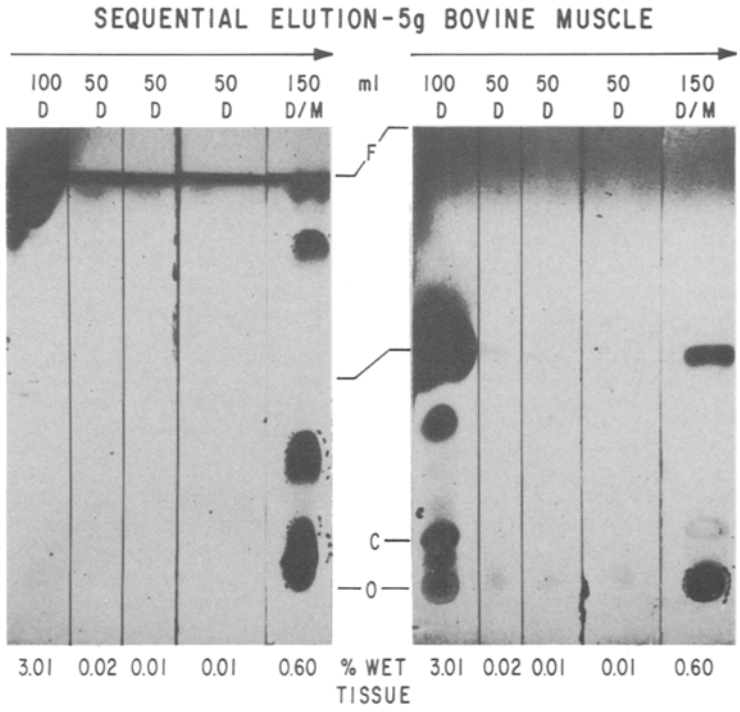

FIG. 1. TLC of 1% aliquots from sequential extracts of 5 g bovine muscle. TLC on Supelco L/S Redicoat plates, thickness 250 μ. Order of elution indicated with arrows. Eluants: dichloromethane (D) and then dichloromethane/methanol (D/M), 90:10. Left-hand plate developed in chloroform/methanol/acetic acid/water (85:15:10:3) to demonstrate absence of polar lipid (R_f = 0-0.8) until change of eluant. Visualization: same as Fig. 2. Neutral lipids appear near solvent front (F). Right-hand plate developed in hexane/ether/acetic acid (80:20:1) to demonstrate apparent exhaustion of neutral lipid (triglyceride R_f = 0.5; cholesterol indicated as "C") with initial eluant (D) and then emergence of additional neutral lipid upon change of eluant (D/M). Polar lipids remain at origin (O).

ances were read at 830 nm in a Spectronic 21-DV spectrophotometer (Bausch and Lomb; Fisher Scientific Company, King of Prussia, PA).

Determination of Calcium in Lipid Eluate

To determine whether calcium ion is leached from the column trap during solvent elution of lipid from the column, the eluates were analyzed for calcium by atomic absorption. Four parallel elutions were done: (a) standard procedure–5 g medium fat beef with column trap (trap = 9 g Celite 545 + 1 g $CaHPO_4 \cdot 2H_2O$); (b) same beef, no trap; (c) no beef, but trap present; (d) no beef, no trap. Elution was performed sequentially, and only the second eluates–150 ml dichloromethane/methanol (90:10, v/v)–were collected for analysis. Each of the four eluates was brought to 200 ml in a volumetric flask, and aliquots were removed for phosphorus analysis. Solvent was evaporated from the remaining portions of eluate, and the residues then were hydrolyzed with 8 N nitric acid at 100 C for several hours. The resulting solution was diluted to 1.6 N acid and analyzed for calcium content using a Perkin-Elmer Model 306 atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

The conditions given for the dry column method (see Experimental) represent the optimal conditions that we have found for lipid isolation and were based on results from experiments designed to minimize neutral lipid carryover into the polar lipid fraction and to prevent the coelution of nonlipid materials. The range of experiments applied to optimize those conditions is outlined in Tables I and II.

Although the neutral fraction from the sequential elution was free of polar lipid, a small but consistent amount of neutral lipid was isolated in the polar extracts using the best conditions found for the dry column method. Evidence for this neutral lipid carryover was shown by the following experiments. In the

TABLE III

Extractable Lipid[a] by the Dry Column and $CHCl_3$/MeOH Methods

Tissue (5-g samples)	Dry column procedure: Sequential elution — % Neutral lipid	Polar lipid[c]	Sum	Dry column procedure: Isocratic elution — Total lipid	$CHCl_3$/MeOH extraction[b] — Total lipid
Lean pork	L[d]: 4.97 (2)	0.82 (2)	5.79	5.94 ± 0.07 (4)	5.77 ± 0.13 (4)
	PL[d]: 0.00 (2)	0.64 (2)	0.64	0.70 ± 0.00 (4)	0.67 ± 0.02 (4)
Fatty beef	L: 29.35 (2)	0.67 (2)	30.02	29.96 ± 0.04 (4)	29.87 ± 0.22 (3)
	PL: 0.01 (2)	0.46 (2)	0.47	0.49 ± 0.01 (4)	0.50 ± 0.01 (3)
Medium beef	L: 10.08 (2)	0.72 (2)	10.80	10.84 ± 0.11 (4)	10.70 ± 0.11 (3)
	PL: — (0)	0.56 (2)	0.56	0.62 ± 0.02 (4)	0.61 ± 0.01 (3)
Lean beef	L: 3.43 ± 0.05 (5)	0.80 ± 0.03 (5)	4.23	4.36 ± 0.10 (7)	4.38 ± 0.18 (6)
	PL: 0.01 (1)	0.68 ± 0.01 (5)	0.69	0.69 ± 0.03 (5)	0.69 ± 0.01 (6)
4 Tissues, average	L: 11.96	0.75	12.71	12.78	12.68
	PL: 0.01	0.58	0.59	0.62	0.62
Bovine subcutaneous adipose 1	L:			82.12 ± 1.20 (5)	81.56 ± 1.05 (4)
	PL:			0.17 ± 0.00 (4)	0.19 ± 0.01 (4)
Bovine subcutaneous adipose 2	L:			85.26 ± 1.32 (4)	86.52 ± 1.84 (3)
	PL:			0.11 (1)	0.11 (1)

[a]As percentage of total tissue weight.

[b](6).

[c]Includes ca. 10% carryover of neutral lipid.

[d]L = lipid; PL = phospholipid, calculated as 25 × % phosphorus. Standard deviations are included for runs in triplicate or greater; number of replicates is shown in parentheses.

sequential elution, the dichloromethane eluates (three 50-ml fractions) following the first 100 ml contained only traces of neutral lipid (0.5 mg or less). However, the change of solvent to the 90:10 mixture unexpectedly caused a release of neutral lipid into the eluate, and thus in the polar fraction, as seen by TLC analysis (Fig. 1).

This neutral lipid carryover consistently comprised ca. 10-15% of the polar fraction as determined by gravimetric analysis of polar fractions subsequently separated by TLC. The two neutral lipid mixtures, one from the dichloromethane eluate and the other separated from the polar fraction, also were compared by an examination of gas liquid chromatography (GLC) profiles of their fatty acid methyl ester derivatives. The GLC traces were similar in all major components and only minor differences could be observed. It would appear, therefore, that the neutral lipid carryover into the polar lipid fraction differs little in composition from the major netural lipid fraction. The possibility that this holdback of neutral lipid (cf. Tables IB, IIB) may result from an affinity of Celite for neutral lipid was supported by the following experiment: tallow, containing no phospholipid, was mixed with dichloromethane and passed over a bed of 10 g of Celite 545 in a 35-mm id chromatography column. The column was flushed with dichloromethane until no further lipid was detected in the eluate. Subsequent elution with 100 ml of the dichloromethane/methanol (90:10) mixture gave, after solvent removal, 3 mg of additional lipid. This amount of neutral lipid corresponds to that ordinarily found in the polar fraction from sequential extractions of muscle tissue.

To determine whether the calcium ion of the column trap (cf. Table IIC) was immobile, polar fraction eluates from sequential elutions of four columns were examined for calcium content by atomic absorption spectrophotometry. Eluates from blank columns—with or without the trap, but containing no bovine muscle—showed no calcium. Extract from a column with a trap had 1.38 μmol calcium/5 g muscle (and 33.4 μmol phosphorus/5 g muscle). Muscle extract from a column without a trap had 0.82 μmol calcium/6 g muscle. Therefore, 0.56 μmol calcium/5 g muscle, or 1 mol calcium for every 60 mol phosphorus (or phospholipid) was eluted from the trap. This amount of calcium is inconsequential for most lipid isolations, but is removable by ion exchange during a wash of the lipid extract with 0.88% aqueous KCl ("Folch wash" of reference 6).

The dry column extraction method, using

the preferred procedures (Experimental and Tables I and II), has been used on a variety of muscle and adipose tissues. Comparisons of the total lipid content and phospholipid recoveries obtained by the new method with those of a $CHCl_3$/MeOH (modified Folch) procedure (6) are shown in Table III. The modified Folch procedure was used for comparison because it yielded a lipid extract that was free of nonlipid contaminants. Typical determinations were made in duplicate for sequential elutions and in quadruplicate for isocratic elutions by the dry column method. For purposes of comparison, replicate extractions by the $CHCl_3$/MeOH method were done simultaneously with the dry column experiments. The results obtained by the two methods–isocratic elution by the dry column method vs the $CHCl_3$/MeOH extraction–agree well for percentage lipid and percentage phosphorus in muscle tissue (Table III).

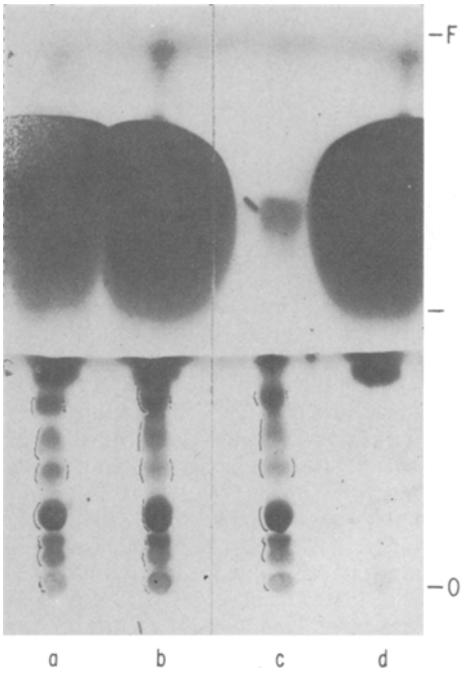

FIG. 2. TLC of 0.5% aliquots of modified Folch extract (a) and dry column extracts (b-d) of 5 g bovine muscle. Column b = total lipid eluate from isocratic elution of dry column; d and c = neutral fraction and polar fraction, respectively, from sequential elution of dry column. TLC on Analtech (Newark, DE) Silica Gel G Uniplates, thickness 250 μ. Double development, initially with chloroform to indicated solvent front F in order to draw the heavy triglyceride spot away from the remaining lipid, and then with chloroform/methanol/water (65:35:5) about half way up to separate the remaining lipids. Visualization: ninhydrin (positive spots indicated with right-hand parentheses), then Phospray (. . . left-hand parentheses), then charring. Cholesterol appears at R_f = 0.4; "O" = origin.

Results of the sequential elutions to obtain neutral and polar fractions also were compared with those of isocratic elutions by the dry column method (Table III). In all cases, the sum of the weights of the sequential fractions was nearly identical to the weight of the extract obtained by isocratic elution. All neutral lipid fractions were examined by TLC and phosphorus analysis for the presence of polar lipid, but only negligible amounts were detected. The phosphorus content of the polar fraction was in each case essentially identical to that found in the isocratic extract.

Analysis of excised adipose tissue, i.e., without skeletal muscle tissue, presented some difficulty by the sequential method. Class separations were in some cases erratic, and data for these fractions are not included in Table III. The separation of adipose tissue lipids into classes has long been recognized as a problem (8). Nevertheless, data shown in Table III confirm that the total lipid and the phospholipid contents of adipose tissue essentially were identical by the dry column (isocratic elution) and the $CHCl_3$/MeOH methods.

To demonstrate the applicability of the dry column method to smaller sample sizes than those discussed in Table III, total lipid was extracted (isocratic elution) from 1-g tissue samples by CH_2Cl_2/MeOH (90:10). Although all lipid was removed in the first 20 ml of eluate, for the convenience of collecting in a volumetric flask, 25 ml would be suitable. Comparative results of parallel extractions of total lipid from bovine muscle were as follows: 5-g tissue samples, 4 runs: 3.96 ± 0.02% lipid and 0.67 ± 0.02% phospholipid; 1-g tissue samples, 4 runs: 4.15 ± 0.23% lipid and 0.65 ± 0.03% phospholipid.

Finally, a $CHCl_3$/MeOH extract was separated by TLC (Fig. 2a) in parallel with three dry column extracts (Fig. 2b-d) of the same muscle. One aliquot (Fig. 2b) was from the isocratic elution with 150 ml of the 90:10 solvent mixture. Results of a sequential elution of a dry column are shown (Fig. 2c and d), in which d represents the neutral lipid fraction and c the polar fraction. Intensities of all separated components in Figure 2a were similar to those of Figure 2b. The large spots in the upper half of the plate are triglyceride. Cholesterol appears at R_f = 0.4. Seven subclasses of phospholipids are separated; phosphatidylcholine appears at R_f = 0.1 and phosphatidylethanolamine (ninhydrin positive) at R_f = 0.2. (Ninhydrin also detected only negligible nonlipid in both extracts.) Neutral lipid isolated

by sequential elution is free of polar lipid. The polar lipids, Figure 2c, are identical in composition to the polar lipids seen in columns a and b, along with a trace of triglyceride (R_f = 0.7) and cholesterol (R_f = 0.4).

In summary, though quantification of lipid components and determination of their structural integrity await further study, the TLC patterns, the weight recovery, and the phosphorus analysis data indicate that the dry column method may be an acceptable and advantageous alternative to the traditional chloroform/methanol extraction methods for lipid analysis of muscle and adipose tissue.

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LETTER TO THE EDITOR

Modification of Phenol-Sulfuric Acid Method for the Estimation of Sugars in Lipids

Sir:

Total sugars in glycolipids and phosphoglycolipids have generally been determined by the phenol-sulfuric acid method (1) either on the intact lipid or on water-soluble lipid hydrolysates (2). This method gives good results with water-soluble glycolipid hydrolysates and with intact acyl ester glycolipids (e.g., see ref. 3) but was found to give inconsistent and irreproducible results when applied to intact glycolipids of extremely halophilic bacteria (4) or methanogenic bacteria (5), which are derived from C_{20}-phytanylglycerol diether or C_{40}-glycerol tetraether, respectively. It was found that maintaining the temperature of the reaction with conc. sulfuric acid at 100 C was an important factor in obtaining reproducible results.

The procedure was then modified as follows: pipet an aliquot of lipid solution or aqueous hydrolysate containing 30-60 μg sugar (as hexose) into a 35-ml Lewis-Benedict sugar tube and evaporate the solvent to dryness under a stream of nitrogen; to the residue add 2 ml of water and 1.0 ml of 5% phenol solution, and mix gently by vortexing, making sure that the film of lipid at the bottom of tube is undisturbed. Add 5 ml of conc. sulfuric acid with vortexing and then heat for 5 min in a boiling water bath. Vortex the mixture briefly and allow it to cool for 30 min. Read the absorbance of the orange color at 490 nm against a reagent blank. For calibration, use standards containing 20, 40 and 80 μg of hexose or an appropriate mixture of different hexoses, depending on the kind of hexoses and their molar ratios in the original lipid. Beer's law holds in the range 0-80 μg hexose; 20 μg glucose gives an absorbance of 0.112. The modified method has been tested in this laboratory on several glycerol-ether-derived glyco-

TABLE I

Estimation of Total Sugar in the Lipids from *M. hungatei* GP1, *H. cutirubrum* and *M. marismortui*

Lipids[a]	Sugar (%)		
		Found	
	Calcd	Original procedure[b]	Modified procedure
M. hungatei GP1 (5)			
Phosphoglycolipid-I (PGL-I)	19.9	12.5, 15.7	20.3
Phosphoglycolipid-II (PGL-II)	19.9	14.9, 15.0	19.5
Diglycosyl tetraether-I (DGT-I)	22.2	–	21.5
Diglycosyl tetraether-II (DGT-II)	22.2	–	21.6
Diglycosyl diether-I (DGD-I)	36.9	24.7, 28.5	37.3
Diglycosyl diether-II (DGD-II)	36.9	–	36.5
H. cutirubrum			
Glycolipid sulfate (GLS)	43.7	42.8, 42.3	43.9
Triglycosyl diether (TGD)	47.4	–	47.7
H. marismortui (4)			
Triglycosyl diether (TGD)	47.4	–	47.1

[a]Calculated for: ammonium salt forms of PGL-I or PGL-II, $C_{101}H_{199}O_{21}$ PN·H_2O; GLS, $C_{60}H_{117}O_{21}$ S·NH_4; TGD, $C_{61}H_{118}O_{18}$; DGD-I or DGD-II, $C_{55}H_{108}O_{13}$; DGT-I or DGT-II, $C_{98}H_{189}O_{16}$.

[b]Values are given for duplicate analyses. Data for GLS are from B. Smallbone (unpublished results).

lipids and has been found to yield reproducible and satisfactory results (Table I). Deviation from the theoretical values is in the range ± 0.5 to 3%, compared to 3-37% for the original procedure. The method has also been found to give good results with fatty-acid-containing glycolipids, and we recommend it as a suitable procedure for sugar determination in intact glycolipids in general.

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COMMUNICATIONS

Relationships between Levels of Essential Fatty Acids and Zinc in Plasma of Cystic Fibrosis Patients

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ABSTRACT

Possible relationships between zinc and essential fatty acid metabolism have been assessed using plasma samples obtained from 39 cystic fibrosis patients. The fatty acid composition of plasma phospholipids was analyzed by capillary gas liquid chromatography and zinc levels were measured by atomic absorption spectrometry. Computer analyses revealed positive correlations between zinc levels and (a) arachidonic acid ($P<0.001$), (b) the arachidonate/linoleate ratio ($P<0.02$), and (c) eicosatrienoic acid (n–6) ($P<0.001$). These observations suggest that conversion of linoleate to arachidonate may be modulated by zinc.

INTRODUCTION

Both zinc and fatty acids of the linoleic acid family are essential in the diets of man and experimental animals. The deficiency signs for these two nutrients, which include growth retardation, delayed sexual maturation and infertility, dermal lesions, alopecia, and decreased rates of wound healing, show remarkable similarities. Only recently have possible metabolic connections between zinc and essential fatty acids (EFA) become apparent. Animal studies have shown relationships between zinc, EFA and prostaglandins (1–3), but the precise interaction remains unclear. Two clinical observations further support a possible relationship. In the hereditary disease acrodermatitis enteropathica, patients are deficient in plasma zinc, probably secondary to an inherited defect in zinc absorption (4); also, a decrease in serum levels of arachidonic acid (20:4 [n–6]) with normal levels of its precursor, linoleic acid (18:2 [n–6]), has been reported (5), although others (6) have reported normal levels of all plasma fatty acids. Dietary zinc supplements reverse the symptoms of the disease and affected patients then begin to thrive well on normal diets. Patients receiving prolonged total parental nutrition also may develop signs characteristic of zinc or EFA deficiencies when these nutrients are not regulated in the alimentary fluids; upon supplementation with zinc and EFA, such signs are rapidly alleviated (7).

We have sought additional evidence to elucidate a relationship between zinc and EFA by examining blood samples from cystic fibrosis (CF) patients. Lower than normal levels of EFA in plasma have been reported for some CF patients and other studies have indicated abnormally low levels of zinc (8). Accordingly, we reasoned that there might be a better opportunity of demonstrating correlations between zinc and EFA in a CF group than in a general population where deficiencies might be less frequent or less severe.

MATERIALS AND METHODS

Blood (5–10 ml) was collected from 39 CF children, 3–18 years old, in heparinized plastic syringes. Plasma was separated by centrifugation and held at −20 C until analyzed. A 1.0-ml aliquot of plasma was deproteinized with an equal volume of 10% trichloroacetic acid. The supernatant was analyzed for zinc concentration (9) by atomic absorption on a Techtron Model AA-4 spectrophotometer. The average range for duplicate analyses was ± 5% of the mean.

Plasma lipids were extracted (10) from an aliquot of plasma and separated into lipid classes on a silicic acid column (11). The phospholipid fraction was transmethylated with 10% BF_3 in the methanol (w/v) (12). Extracted fatty acid methyl esters were separated at 180

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C on a 50 m × 0.25 mm stainless steel capillary column coated with Silar 7CP liquid phase in a Hewlett-Packard Model 5711A gas liquid chromatograph equipped with flame ionization detectors. Methyl esters were identified by comparison to authentic standards (Supelco, Inc., Bellefonte, PA) and quantitated using a Hewlett-Packard Model 3380A integrator-recorder. Replicate analysis of methyl esters showed <2.5% variability for minor peaks (<5% of total response) and <1.5% variability for major peaks.

Data were analyzed with the assistance of a CDC 6400 computer using an SPSS program to evaluate and plot relationships among variables.

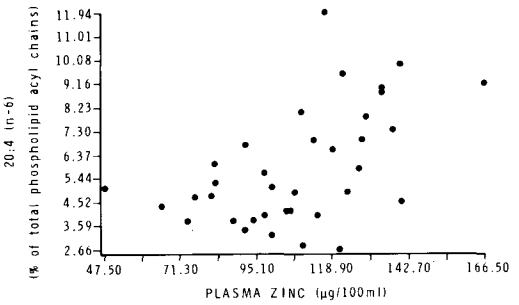

FIG. 1. Relationship between levels of plasma zinc and arachidonic acid, 20:4 (n–6), for 39 cystic fibrosis patients.

RESULTS

Relationships between levels of plasma zinc and various fatty acids or ratios that reflect EFA deficiency were assessed from the independently obtained zinc and fatty acid composition data. Mean values and standard deviations for 18:2 (n–6), 20:3 (n–6), 20:3 (n–9), 20:4 (n–6) and zinc were, respectively, 10.1 ± 2.4%, 1.2 ± 0.4%, 0.3 ± 0.2%, 5.9 ± 2.2% and 108 ± 24 µg/100 ml.

Positive correlations were observed between zinc and arachidonic acid (20:4 [n-6]) (Fig. 1) and eicosatrienoic acid (20:3 [n–6]) levels ($p<0.001$). On the other hand, no significant correlation could be demonstrated between plasma zinc levels and linoleic acid (18:2 [n–6]) or the triene:tetraene ratio (20:3 [n–9]/20:4 [n–6]), frequently used as indicators of EFA deficiency (Table I).

DISCUSSION

In this study, potential correlations between zinc and EFA, as suggested by several clinical and experimental observations, were examined directly in a patient population likely to express a range of these parameters. The mean value and ranges from our 39 patients were in general agreement with those from other studies (13); extreme deficiencies for either zinc or EFA were not observed. Phospholipid fatty acyl compositions were used in this analysis because they are less influenced by immediate fluctuations in dietary supply of lipids, contain a higher proportion of essential fatty acids and derivatives, and generally show greater composition changes in the longer term deficiency state than does the neutral or total plasma lipid.

Dietary 18:2 (n–6) of plant origin is converted to further metabolites by an alternating sequence of desaturation and chain elongation reactions followed in some instances by oxygenation, rearrangements and/or esterification. The positive correlation between plasma levels of zinc and arachidonic and eicosatrienoic acids (products formed following an initial Δ6 desaturation of linoleic acid), but no significant correlation with linoleic acid, suggests that zinc may have a role in Δ6 desaturation of dietary linoleate.

A specific relationship between zinc and Δ6 desaturation might explain why no correlation was found between zinc and the triene:tetraene ratio. This ratio is frequently used as an indicator of EFA deficiency when linoleic acid supply is restricted. As competition for the Δ6 desaturase is reduced by low levels of linoleate in the EFA deficiency state, the eisosatrienoic acid isomer 20:3 (n–9) is formed in levels much higher than normal, through the pathway involving desaturation and chain elongation of oleic acid, 18:1 (n–9). If, however, Δ6 desaturation were reduced by low levels of zinc, desaturation of oleic acid and thus synthesis of 20:3 (n–9) (as well as 20:3 [n–6] and 20:4 [n–6]) would be impaired and not display the increase observed with general EFA deficiency.

TABLE I

Summary of Computerized Linear Regression and Statistical Analyses of Relationships between Levels of Plasma Zinc and Phospholipid Fatty Acids

Fatty acid or ratio	Correlation coefficient	Significance (p)
18:2 (n–6)	+0.207	0.212
20:3 (n–6)	+0.498	0.001
20:3 (n–9)	+0.202	0.223
20:4 (n–6)	+0.517	0.001
20:4/18:2	+0.391	0.015
20:3 (n–9)/20:4 (n–6)	-0.120	0.475

Thus, our data support the possibility of a close relationship between the metabolism of EFA and zinc and suggest that zinc may interact at the level of Δ6 desaturation. An alternative that cannot be further assessed in this model is that zinc may influence the utilization of arachidonic and eicosatrienoic acids in other reactions such as prostaglandin, thromboxane and leukotriene production. Further studies, now in progress with experimental animals, will more directly address the effects that zinc may have on specific enzymatic reactions involved in the sequence of EFA metabolism and utilization.

ACKNOWLEDGMENTS

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Formation of γ-Ketols from 13- and 9-Hydroperoxides of Linolenic Acid by Flaxseed Hydroperoxide Isomerase

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ABSTRACT

A reexamination of the flaxseed hydroperoxide isomerase reaction showed that a minor enzymic product (ca. 5%), identified as a γ-ketol, was present. The substrates were the 13- or 9-hydroperoxides of linolenic acid, which were converted to 9-hydroxy-12-oxo-*cis*-15-*trans*-11-octadecadienoic acid, respectively. These compounds were formed in addition to the major products reported earlier: a 12,13-α-ketol and 12-oxo-*cis*-10,15-phytodienoic acid from the 13-isomer, and a 9,10-α-ketol from the 9-isomer.

Hydroperoxide isomerase, first demonstrated in flax by Zimmerman (1), catalyzes the isomerization of 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoic acid (13-OOH-18:2) to an α-ketol, 13-hydroxy-12-oxo-*cis*-9-octadecenoic acid (12,13-ketol-18:1). 9-Hydroperoxy-*cis*-12,*trans*-10-octadecadienoic acid (9-OOH-18:2) is converted to 9-hydroxy-10-oxo-*cis*-12-octadecenoic acid (9,10-ketol-18:1). Gardner et al. (2) showed that corn hydroperoxide isomerase forms both α- and γ-ketols from linoleic acid hydroperoxide: 12,13-ketol-18:1 and 9-hydroxy-12-oxo-*trans*-10-octadecenoic acid (9,12-ketol-18:1) were formed from 13-OOH-18:2, and 9,10-ketol-18:1 and 13-hydroxy-10-oxo-*trans*-11-octadecenoic acid (10, 13-ketol-18:1) were formed from 9-OOH-18:2.

Recently we reported on our investigations into the metabolism of linolenic acid in flaxseed. We showed that 13-hydroperoxy-*cis*-9,15-*trans*-11-octadecatrienoic acid (13-OOH-18:3) is converted to 13-hydroxy-12-oxo-*cis*-9,15-octadecadienoic acid (12,13-ketol-18:2) (3) and 12-oxo-*cis*-10,15-phytodienoic acid (12-oxo-PDA) (4); 9-hydroperoxy-*cis*-12,15-*trans*-10-octadecatrienoic acid (9-OOH-18:3) is converted to 9-hydroxy-10-oxo-*cis*-12,15-octadecadienoic acid (9,10-ketol-18:2) (3). In this report, we have reexamined the hydroperoxide isomerase reaction with these two substrates and now show that γ-ketols are also formed as minor enzymic products of the reaction. 9-Hydroxy-12-oxo-*cis*-15-*trans*-10-octadecadienoic acid (9,12-ketol-18:2) and 13-hydroxy-10-oxo-*cis*-15-*trans*-11-octadecadienoic acid (10,13-ketol-18:2) result from isomerization of 13-OOH-18:3 and 9-OOH-18:3, respectively.

MATERIALS AND METHODS

Chemicals

Materials used in this study were purchased as follows: linolenic acid 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); N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) from Pierce Chemical 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).

Derivatives

Esterification was accomplished with a minimum amount of diazomethane prepared from N-methyl-N-nitroso-*p*-toluenesulfonamide. Hydrogenation was performed by bubbling hydrogen through the sample dissolved in methanol with platinum oxide catalyst. Carbonyl groups were reduced with sodium borohydride in methanol. Trimethylsilyloxy derivatives were made by reacting 0.1 ml of BSTFA with ca. 0.5 mg of sample for 30 min at 22 C.

Preparation of Substrate Solutions

A stock substrate solution of linolenic acid (8 mM) was prepared according to Surrey (5). The 13-hydroperoxide of linolenic acid (13-OOH-18:3) was prepared by the reaction of soybean lipoxygenase (15 mg) with 330 ml of a solution of 0.35 mM linolenic acid in 10 mM borate buffer (pH 9) for 30 min at 22 C; the solution was then acidified to pH 4, and the hydroperoxide was extracted into dichloromethane. The 9-hydroperoxide of linolenic acid (9-OOH-18:3) was prepared with tomato lipoxygenase according to Matthew et al. (6). Both hydroperoxide isomer preparations were purified by thin layer chromatography (TLC) (hexane/ethyl ether/acetic acid, 65:35:1, v/v)

prior to use. Each isomer was dissolved in 1 ml of ethanol and then added to 50 mM K-phosphate buffer (pH 7) to give a hydroperoxide concentration of ca. 0.1 mM. The percentage of 13-OOH vs 9-OOH in each hydroperoxide preparation was estimated according to the method of Frankel et al. (7). The hydroperoxide from soybean lipoxygenase was 90% 13-OOH-18:3, and the hydroperoxide from tomato lipoxygenase was 99% 9-OOH-18:3.

Enzyme Reactions and Product Isolations

The enzyme extract was obtained from an acetone powder (1 g/10 ml K-phosphate buffer, pH 7) of flaxseed (*Linum usitatissimum* L., var. Summit). The extract (15 ml), which had hydroperoxide isomerase activity, was reacted with 300 ml of either 0.1 mM 13-OOH-18:3 or 0.1 mM 9-OOH-18:3 substrate solution for γ-ketol formation and isolation. After adjusting the solution to pH 4, the products were extracted with dichloromethane/methanol (2:1, v/v) and, after evaporation of the solvent, were dissolved in a minimal amount of diethyl ether.

Purification

Metabolites from the reaction of 13-OOH-18:3 and 9-OOH-18:3 with the flaxseed extract were purified by TLC. The diethyl ether solution of the products was applied to thin layer silica gel plates and developed 3 times in chloroform/acetic acid (100:2, v/v) solvent. Products were visualized by detecting absorbance of ultraviolet light (254 nm) or exposing a portion of the plate in a tank saturated with iodine vapor. Products of interest were eluted from the gel with diethyl ether.

Spectral Analyses

Infrared spectra were obtained on a Perkin Elmer 337 spectrophotometer with microliquid cells (0.50 mm path length) and carbon tetrachloride as the solvent. Ultraviolet absorbances were determined with a Beckman DK-2 recording spectrophotometer. Gas chromatography-mass spectrometry (GC-MS) analyses were performed with a Varian/MAT 112S GC/MS system operated at an electron potential of 70 eV. The glass column was 2 m long, 2 mm id, and was packed with 3% OV-210 on Gas Chrom Q, 100/120 mesh. The temperature was programmed from 180 to 230 C at 2 C/min.

RESULTS AND DISCUSSION

A minor, but enzymic, product was observed when 13-OOH-18:3 was reacted with a flaxseed extract containing hydroperoxide isomerase activity. We had previously shown that 12,13-ketol-18:2 and 12-oxo-PDA were the major products of this reaction (3,4). The minor product was not formed when a heat-treated enzyme extract (100 C for 5 min) was used. This compound comprised ca. 5% of the total products when examined by GC. The 12,13-ketol accounted for ca. 50% and 12-oxo-PDA ca. 20% of the total.

The R_f of the minor product on thin layer plates was 0.15, compared to R_f 0.59 for 12,13-ketol-18:2, and R_f 0.66 for 12-oxo-PDA. The UV absorption maximum at 220 nm was characteristic of the K-band of an α,β-unsaturated carbonyl. Another weak absorption maximum at 302 nm was characteristic of the R-band of a carbonyl (forbidden transition) (8). Infrared analysis of the methyl ester showed absorption bands at 3613 cm^{-1} (hydroxyl), 1736 cm^{-1} (ester carbonyl), 1692 cm^{-1}, 1674 cm^{-1}, and 1629 cm^{-1} (α,β-unsaturated carbonyl), and 975 cm^{-1} (*trans* double bond) (9).

The location of the functional groups was provided by mass spectral analysis of the methyl ester, trimethylsilyloxy (TMS) derivative (Fig. 1A). The identification of this product as methyl 9-trimethylsilyloxy-12-oxo-*cis*-15,*trans*-10-octadecadienoate was supported by mass fragments at m/e 396 $[M]^{\ddagger}$, and 381 $[M\text{-}CH_3]^+$. The location of the TMS group at carbon 9 was shown by the fragments at m/e 259 $[CHOTMS\ (CH_2)_7COOCH_3]^+$ and 239 $[M\text{-}(CH_2)_7COOCH_3]^+$. Graveland et al. (10) observed the same cleavage pattern in a γ-ketol resulting from the oxidation of linoleic acid by barley suspensions. Cleavage β to the carbonyl could account for the mass fragment at m/e 327.

Gardner et al. (2) established the position of functional groups in ketol compounds by the mass spectra of the hydrogenated TMS derivatives. Attempts by us to hydrogenate this pure compound in methanol with either platinum oxide or 10% palladium on charcoal catalyst resulted in the degradation of the ketol to 9 separate products after GC analysis. The TMS derivative of one of these products had a mass spectrum identical to that for the methyl ester of the hydrogenated TMS derivative of the 9,12-ketol. The characteristic fragments were m/e 243 (base peak), 259, 272, 279, 310, 315, 369, 385 and 400 (molecular ion).

The position of the oxo group in the ketol was better demonstrated by the mass spectrum of the sodium borohydride-reduced, TMS derivative of the compound (Fig. 1B). The molecular ion in this case was m/e 470, supported by ions at m/e 455 $[M\text{-}CH_3]^+$ and 439 $[M\text{-}OCH_3]^+$. The ion at m/e 387 $[M\text{-}C_6H_{11}]^+$

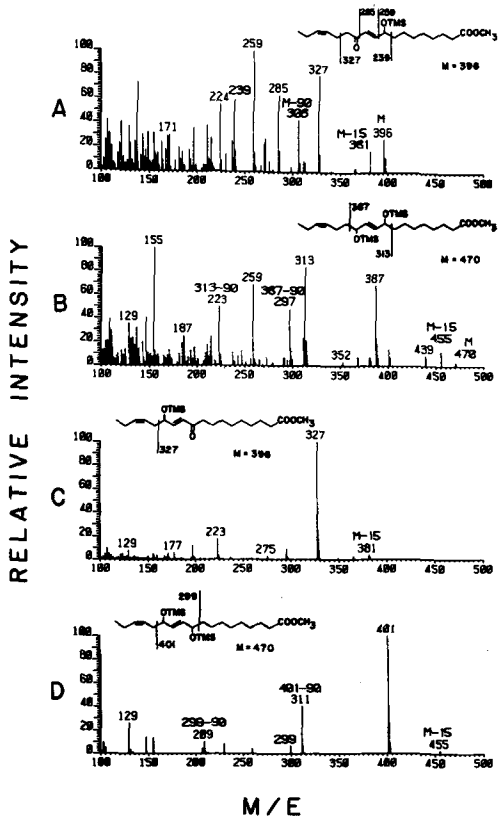

FIG. 1. Partial mass spectrum of: (A) the methyl ester, TMS derivative of the γ-ketol derived from 13-hydroperoxylinolenic acid; (B) the methyl ester, sodium borohydride-reduced, TMS derivative of the γ-ketol derived from 13-hydroperoxylinolenic acid; (C) the methyl ester, TMS derivative of the γ-ketol derived from 9-hydroperoxylinolenic acid; and (D) the methyl ester, sodium borohydride-reduced, TMS derivative of the γ-ketol derived from 9-hydroperoxylinolenic acid.

indicated that the second oxygen function, originally the oxo group, was at carbon 12. Since a double bond was known to be in conjugation with the oxo group, this fragment (m/e 387) also showed that the *trans* double bond must be at carbon 10, and not carbon 13. Thus, the underivatized compound was proposed to be a γ-ketol, 9-hydroxy-12-oxo-*cis*-15,*trans*-10-octadecadienoic acid (9,12-ketol-18:2).

The specific activity of flax hydroperoxide isomerase has been shown to be 36 times greater for 13-OOH-18:3 than for 9-OOH-18:3 (3). When 9-OOH-18:3 produced from tomato lipoxygenase was the substrate for flax hydroperoxide isomerase, the major product that separated on TLC was 9,10-ketol-18:2. No cyclic products were formed. The presence of 10,13-ketol-18:2 as a minor product of this reaction was demonstrated in the mass spectrum (Fig. 1C) of the methyl ester, TMS derivative of a polar product eluted from the TLC plate. The predominant mass fragment at m/e 327 $[M\text{-}C_5H_9]^+$ indicated that the TMS group was at carbon 13. Reduction of the compound with sodium borohydride prior to preparing the TMS derivative showed that the second oxygen, originally the oxo group, was at carbon 10. This was indicated by the mass fragments m/e 299 $[M\text{-}(CH_2)_8COOCH_3]^+$ and m/e 209 (299-TMSOH) (Fig. 1D). The mass fragment at m/e 401 again demonstrated the presence of an oxygen function at carbon 13.

Thus, the formation of a γ-ketol as a minor product appears to be a general property of hydroperoxide isomerases from various sources. The specific biological function of γ-ketols is unknown, but compounds with a related structure, 4-hydroxy-2-alkenals, exert marked inhibition of the sulfhydryl enzymes such as glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase (11). It may be that γ-ketols have a regulatory role during seed maturation or germination.

ACKNOWLEDGMENTS

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Lipoprotein Lipase in Cholesterol-Fed and Control Guinea Pigs

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ABSTRACT

A study of the in vitro activity of lipoprotein lipase of guinea pigs has shown that (a) the lipolytic activity of activated post-heparin serum is depressed in hypercholesteremic guinea pigs compared to the serum of normocholesteremic guinea pigs; and (b) this depressed lipolytic activity in hypercholesteremic guinea pigs is not due to the presence of an inhibitor.

INTRODUCTION

Triglyceride clearance from the plasma in vivo is mediated by a triacylglycerol lipase, functional at the surface of the capillary cells and solubilized by heparin (1-3). For maximal activity, it requires, as cofactor, apolipoprotein C-II (apo C-II), a constituent of very low density (VLDL) and high density (HDL) lipoproteins (4-6) in most species studied.

Studies on the mechanisms for the removal of triglyceride in vivo and for triglyceride hydrolysis by lipoprotein lipase (LPL) in vitro suggest that the cholesterol moiety of the substrate can have a substantial influence on the activity of LPL (7,8). Evidence for the physiological significance of cholesterol levels on lipolysis come from a study that demonstrated that clofibrate administration led to a decrease in serum cholesterol levels and an increase in LPL activity (9).

Adult guinea pigs have very low levels of HDL, and no analog to human apo C-II has been reported (10,11). Although there is no evidence that their utilization of triglycerides is defective, guinea pig post-heparin serum (post-HS) is almost devoid of LPL activity (12,13). Activity is, however, markedly stimulated by rat or human serum.

Guinea pigs respond to dietary cholesterol with hypercholesteremia and the appearance of large amounts of a lipoprotein with the characteristics of HDL rich in apo-E (10). VLDL are decreased and have an abnormally low electrophoretic mobility resembling β-VLDL of patients with broad β-disease (14). Their triglyceride content is decreased whereas their cholesterol content is greatly increased.

We are reporting the effects of hypercholesteremia on the activity of LPL in the guinea pig.

MATERIALS AND METHODS

Male guinea pigs (Simonsen Laboratories, Gilroy, CA) were fed ad lib Purina guinea pig chow supplemented with 5% cottonseed oil without (CONT) or with the addition of 1% cholesterol (CHOL). Post-heparin serum was obtained from overnight-fasted guinea pigs 10 min after jugular injection of 75 units of heparin/kg body weight. Rat serum for activation (RSA) of guinea pig serum was obtained from Purina rat chow-fed, nonfasted animals.

Serum total and free cholesterol was extracted by a modification of the methods of Mann (15) and Abell et al. (16), precipitated as the digitonide and assayed by the ferric chloride colorimetric methods of Zlatkis et al. (17).

Enzyme Assay

A 4% (w/v) bovine albumin (bovine albumin, Fraction V, Miles Lab., Chicago, IL) solution in 0.25 M Tris buffer was slowly tritiated at 4 C with 3 N HCl with constant mechanical stirring to a pH of 8.6 (BSA-Tris).

A triglyceride emulsion was prepared by a 1:1 (v/v) dilution of Intralipid[3] (10% Intralipid, Cutter Lab., Berkeley, CA) with BSA-Tris (TG-Prep). This emulsion was determined to be stable at 4 C for at least 7 days.

RSA was preincubated with BSA-Tris and TG-Prep at 37 C for 20 min prior to the addition of guinea pig serum. Volumes for a standard incubation were 0.2 ml post-heparin serum, 0.2 ml RSA, 0.3 ml TG-Prep, and 2.3 ml BSA-Tris. The final concentrations of Intralipid triglyceride, bovine serum albumin and Tris-HCl buffer were: 5.0 mg/ml, 30.7 mg/ml and 0.19 M, respectively, with a final pH of 8.6. This concentration of bovine albumin provides sufficient fatty acid binding sites to prevent the inhibition of lipolysis (18). Incubations were 30-45 min at 37 C.

Free fatty acids were extracted and tritiated as described by Dole and Meinertz (19).

Recrystallized palmitic acid in heptane served as standard. With each assay, a blank of 2.7 ml BSA-Tris and 0.3 ml of TG-Prep were incubated, extracted, and tritiated. One unit of lipolytic activity is defined as 1.0 meq of FFA

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TABLE I

Lipoprotein Lipase Activity (LPL) and Serum Cholesterol of Cholesterol-Fed and Control Guinea Pigs[a]

Type of guinea pig	LPL[b] (units of activity)	Unesterified cholesterol (mg/100 ml)	Total cholesterol (mg/100 ml)
Control (N)	47.1 ± 10.2[c] (6)	10.0 ± 2.8[d] (6)	35.7 ± 12.4[d] (6)
Cholesterol-fed (N)	16.9 ± 6.1[c] (7)	109.5 ± 38.3[d] (6)	213.7 ± 52.8[d] (6)

[a]Data are means ± SD.

[b]Units of activity: meq of FFA released/ml serum/hr incubation.

[c]Difference between groups is statistically significant at $p<0.05$ by Student's t-test.

[d]Difference between groups is statistically significant at $p<0.01$.

TABLE II

Lipolytic Activity of Mixtures of Guinea Pig Sera[a]

	Ser from:					
	CONT pre-H	CONT post-H	CHOL pre-H	CHOL post-H	N	Unit of activity
Expt. 1		X			2	34.7 ± 0.3
				X	3	22.2 ± 0.6
		X		X	2	28.5 ± 0.8[b]
Expt. 2		X			1	37.7
	X	X			2	37.4 ± 1.8
		X	X		2	37.8 ± 1.3
Expt. 3				X	1	20.5
			X	X	2	21.5 ± 0.8
	X			X	2	22.6 ± 1.8

[a]CONT: normocholesteremic; CHOL: hypercholesteremic; Pre-H: pre-heparin; post-H: post-heparin; N: number of assays. The mixtures contained 0.1 ml of each serum and the standard amounts of the other components (see Methods).

[b]The calculated activity for this mixture is 28.4 units.

released/ml of post-heparin serum/hr of incubation.

RESULTS

Post-heparin CONT serum, when activated with rat serum, hydrolyzed triglyceride substrate at ca. 3 times the rate than did similarly activated post-heparin CHOL serum (Table I). Both types of sera were inactive without prior activation.

To test for a possible inhibitory effect of the hypercholesteremia on the lipolytic activity of CHOL post-HS, mixtures of activated pre- or post-heparin CHOL and CONT sera were assayed. The lipolytic activity of a mixture of CHOL and CONT post-heparin sera did not differ significantly from the expected value that was computed from the mean of that of the individually assayed sera (Table II, expt. 1). This suggests that CHOL post-HS does not contain an inhibitor of the lipolytic activity of CONT post-HS.

CHOL pre-heparin serum also does not contain an inhibitor for the lipolytic activity of either CONT post-HS (Table II, expt. 2) or CHOL post-HS (Table II, expt. 3).

In order to test this conclusion further, activated CONT post-HS was incubated with incremental additions of CHOL pre-HS. Results indicate that additions of CHOL pre-HS did not affect lipolysis differently from that demonstrated by either pre-heparin CONT serum or saline-albumin (Fig. 1). The apparent increase of lipolytic activity in all 3 cases as some of the buffer is replaced is due to the decrease of buffering capacity of the incubation mixture.

DISCUSSION

Our study confirms previous reports that in the guinea pig (12,13), as in the pig (20), post-heparin serum does not exhibit lipolytic activity although an LPL is present that can be activated by a source of APO C-II and although there is no evidence that the utilization of

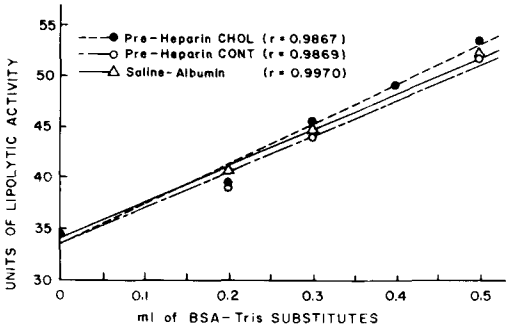

FIG. 1. Effect of the addition of pre-heparin CONT and CHOL sera on the lipolytic activity of post-heparin CONT serum. BSA-Tris was replaced by CHOL pre-HS, CONT pre-HS or saline-albumin as indicated. The final incubation concentration of albumin was held constant at 30.7 mg albumin/ml of incubation. The slopes of the 3 lines do not significantly differ from one another ($p>0.05$). r = Correlation coefficient.

triglycerides in vivo is defective. It has recently been reported that guinea pig VLDL can serve as a weak activator of bovine milk LPL whereas infranate from the centrifugation inhibits the activation (21). We have found that guinea pig VLDL does not stimulate activity of LPL in guinea pig post-heparin serum even at high concentrations (40 times physiologic) (unpublished observations). This can be due to differences in the LPL from the 2 different sources or to the inhibitory effects of the serum.

To our knowledge, there have been no reports on the lipolytic activity of serum from cholesterol-fed guinea pigs. We found the lipolytic activity of CHOL post-HS after activation with rat serum to be significantly lower than that of CONT post-HS.

Several studies have repoted an inhibition of LPL activity by elevated levels of cholesterol (7,8). Ganeson et al. (22) reported that apo-E ("arginine-rich" polypeptide) inhibits LPL in Type III (hypertriglyceridemic) patients. Normal guinea pig plasma has only trace amounts of apo-E whereas CHOL guinea pigs have very large amounts of this polypeptide, up to 60% of HDL (10).

We have not found any evidence for an LPL-inhibitory activity in the hypercholesteremic sera as tested against activated CONT sera. This indicates that the depression of LPL activity is not due to direct inhibition by elevated serum cholesterol or its accompanying apolipoproteins, including the elevated levels of apo-E.

Possible mechanisms for the decreased activity of LPL in CHOL post-HS include: synthesis of an LPL that partially resists activation; altered structure and accessibility of substrate; and impeded release by heparin. An in vitro study of the mechanism of milk LPL using a monomolecular film of 1,2-didecanoylglycerol as substrate has shown that changes in the surface density of the lipid affects LPL activity (23). An increase of cholesterol and/or apo-E at the surface of VLDL in the hypercholesteremic guinea pig may, therefore, be expected to similarly decrease LPL activity. Impeded release of LPL by heparin is another attractive hypothesis because it is compatible with the normal physiological clearance of triglyceride seen in cholesterol-fed guinea pigs. Furthermore, there is precedent for the specific blocking of the heparin-mediated release of lipoprotein lipase by, e.g., concanavalin A (24).

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Lack of Specificity in Accumulation of Sterols by *Phytophthora cactorum*

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ABSTRACT

The kinetics of sterol uptake in *Phytophthora cactorum* were examined. A V_{max} of 4.81×10^{-5} μmol/min/mg dry wt and an average apparent Km of 18.0 μM were determined for both cholesterol and sitosterol accumulation. Selectivity for specific sterols was not apparent in the accumulation, esterification, or distribution of sterols by *Phytophthora cactorum*.

INTRODUCTION

The Pythiaceous fungi, which include *Phytophthora cactorum*, lack sterol synthetic capability (1-4). Nevertheless, they exhibit vegetative growth in the total absence of sterol (5,6), a phenomenon that is unique among eucaryotes. When supplied in the growth medium, however, sterols are readily accumulated by these fungi (5). Accumulation of sterols results in two significant physiological modifications. The Pythiaceous fungi are incapable of both sexual and asexual sporulation in the absence of sterol. However, these modes of reproduction are fully functional in the presence of sterol (7-10). Addition of sterol to the culture medium also stimulates growth of the organisms (11-13). The mechanism by which sterols mediate these effects is not understood.

One important aspect of the study of the sterol effect in *Phytophthora cactorum* is the uptake of sterols by the organism. The original experiments of Leonian (6) in which mycelia were exposed to a pea infusion for varying lengths of time and then tested for sporulation capability were, in effect, the first kinetic studies of sterol uptake by this organism. Within one hr sufficient sterol was accumulated for the production of oogonia. The number of oogonia formed increased with extended exposure times of up to 8 hr.

Direct measurements of uptake of sterol by Phytiaceous fungi have been reported by two groups with significantly different results. Defago and Kern (14) observed very rapid uptake of [^{14}C]cholesterol from the medium of a nongrowing culture of *Pythium paroecandrum*, with 90% of the label being removed from the medium in 80 min. Elliott and Knights (15), on the other hand, observed much slower accumulation of [^{14}C]cholesterol in the acetone extracts of the mycelia of growing cultures of *P. cactorum*. They reported 90% of the labeled sterol was removed from the medium by 48 hr. Uptake of sterols was ascribed to simple absorption in both studies.

In order to elucidate the mechanism of sterol uptake, we have determined the kinetics of sterol accumulation in *P. cactorum*. Attempts to demonstrate selectivity in sterol accumulation and subsequent metabolism are also described.

MATERIALS AND METHODS

Organism and Culture Conditions

P. cactorum was obtained from E. Hansen, Department of Botany and Plant Pathology, Oregon State University. The organism was subcultured routinely on medium containing 10 g sucrose, 10 g tryptone, 5 g yeast extract and 15 g agar/ℓ.

Batch cultures were grown in a New Brunswick Scientific Co. Model MF-114 Microfermentor (14-ℓ capacity) containing 8-ℓ of basal medium. The basal medium was composed of 10 g sucrose, 2 g phthalic acid, 1 g KH_2PO_4, 1 g NH_4NO_3, 0.5 g $MgSO_4$, 0.1 g $CaCl_2$, 0.1 g thiamine·HCl, 10 μg H_3BO_3, 10 μg $CuSO_4 \cdot 5H_2O$, 1 mg KI, 50 μg $FeCl_3 \cdot 6H_2O$ and 70 μg $ZnSO_4 \cdot 7H_2O$/ℓ. The pH was adjusted to 6.0 with solid KOH. Where indicated, sterol (10 mg/ℓ) was added as a solution in ethanol containing 1% TWEEN-40. The maximal level of ethanol/TWEEN-40 added to the culture never exceeded 0.5%.

Growth was initiated by inoculating with ca. 10 mg of mycelial mat from a 20-ml still culture. Cultures were incubated at 25 C.

Uptake Experiments

Multiple aliquots yielding 50-100 mg wet wt mycelia per sample were removed from a log phase batch culture. Mycelia were harvested by filtration and resuspended in 20 ml 10 mM phthalate buffer (pH 6.0) containing 1% sucrose w/v. Due to the propensity for the mycelia to adhere to the sides of the incubation flasks, all uptake experiments were performed with slow shaking at 25 C in a New Brunswick

Model G24 Environmental Shaking Incubator. Samples were equilibrated ca. 30 min prior to the addition of sterol. Sterols were added to the indicated level in ethanol solutions containing 1% TWEEN-40, v/v. The total level of ethanol/TWEEN-40 added was constant throughout each experiment. After the desired time interval of incubation in the presence of sterol, the mycelia were harvested by filtration and washed 3 times with 10-ml volumes of a 0.5% Tergitol NP-40 solution to remove extracellular sterol. Samples were then washed 3 times with distilled water. The samples were lyophilized for dry wt determination and extracted for sterol.

In the sterol competition experiments, 4 different mixtures of sitosterol, campesterol and cholesterol were added to aliquots of mycelia. Sterols accumulated in the mycelia after 3 hr were determined as described in the next section.

Mitochondria Preparation

A batch culture was supplemented with a mixture of sitosterol, campesterol, stigmasterol and lanosterol. After 4 days' growth, an aliquot was removed for determination of whole-cell sterol composition. The remaining mycelia were harvested by filtration in a Buchner filtering apparatus, and resuspended at 0.8 g/ml in 0.6 M sorbitol, 10 mM Tris-HCl, and 0.5 mM EDTA (pH 6.0). Hyphae were distrupted in a French pressure cell at 1,500 psi. Intact hyphae and cellular debris were removed from the homogenate by centrifuging 3 times at 1,000 x g for 2 min. The supernatant was then centrifuged at 27,000 x g for 30 min to yield a crude mitochondrial pellet. Gradient purified mitochondria were prepared from this fraction according to the method of Bottema and Parks (16).

Sterol Quantitation

Sterols were extracted from lyophilized mycelia by either alkaline pyrogallol saponification (17) or a DMSO extraction regimen (18). Extracts were chromatographed on Silica Gel 60 F-254 (EM Laboratories) thin layer chromatography plates using the Skipski et al. system (19). The free sterol and the steryl ester bands were scraped and eluted with a mixture of chloroform/methanol (4:1). Steryl esters were saponified to free sterols prior to quantitation (18). Quantitation of free sterols was by integration of detector analog output from a Supelco SP-2250 column in a Varian 2700 Gas Chromatograph linked to a Varian CDS 111 integrator, using cholestane as an internal standard.

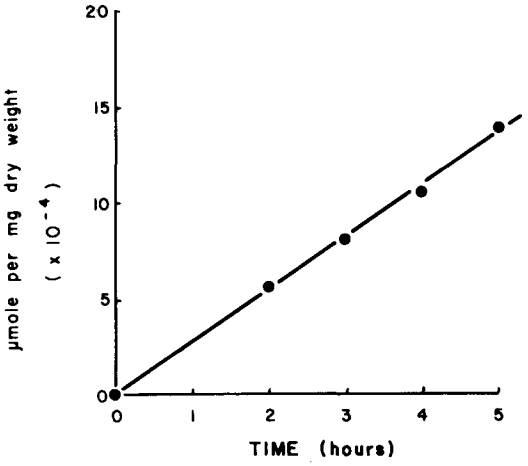

FIG. 1. Time course of sterol uptake by *P. cactorum*. A sterol mixture (sitosterol, campesterol and stigmasterol) was added to aliquots of mycelia to a final concentration of 10 mg/ℓ. The aliquots were harvested at intervals and sterol quantitated as described in Materials and Methods. The data points represent total sterol accumulated in the mycelia and have been corrected for the individual molecular weights of each sterol in the mixture.

Materials

Cholesterol, lanosterol, TWEEN-40 and Tergitol NP-40 were purchased from Sigma Chemical Co. Purified sitosterol (97%) was purchased from Applied Science Laboratories, Inc.

RESULTS

Time Course of Sterol Uptake

The time course of sterol uptake by *P. cactorum* is shown in Figure 1. Samples harvested at the zero time point were exposed to sterol for no more than 15 sec. The zero time point sample contained less than 0.2% of the sterol accumulated by a culture exposed to sterol for 3 hr. Therefore, it was concluded that the Tergitol NP-40 wash at the time of harvest removed essentially all of the nonabsorbed sterol. Accumulation of sterol is linear with respect to time for at least 5 hr. In the remaining experiments involving sterol uptake, mycelia were not exposed to the exogenous sterol in excess of 4.5 hr.

Effect of Sterol Concentration on the Rate of Accumulation

The effect of concentration on the accumulation of cholesterol and sitosterol by *P. cactorum* is shown in the double reciprocal plot of

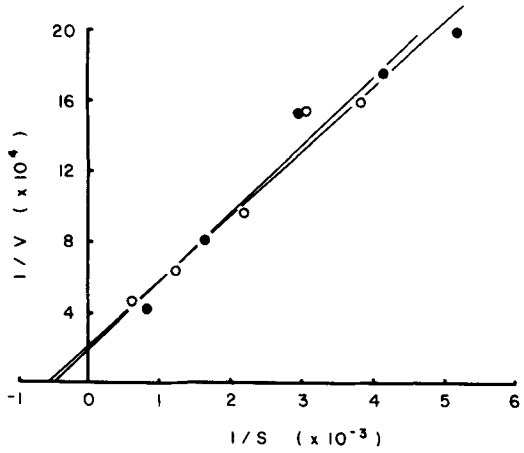

FIG. 2. Double reciprocal plots of rate of sterol uptake vs concentration of sterol. Purified cholesterol (○—○) or sitosterol (●—●) was added to aliquots of mycelia to the indicated concentration. The aliquots were harvested and sterol quantitated as described in Materials and Methods. The rate of uptake was reported as μmol/min/mg dry wt; sterol concentration as μmol/ℓ. The data points represent the mean of three replicate samples.

Figure 2. This experiment was performed with samples taken from the same batch culture to avoid any possible effect of culture conditions on sterol accumulation. The slopes of the curves for both sterols were essentially superimposable with an average apparent Km of 18.0 μM and a V_{max} of 4.81×10^{-5} μmol/min/mg dry wt.

Sterol Competition Experiments

The results of competition experiments are shown in Table I. Samples of *P. cactorum* were exposed for 3 hr to mixtures of sterols of known composition. Total sterols in the mycelia were determined from the nonsaponifiable fraction of lipid extracts. There was no consistent pattern of change in the composition of sterol accumulated from the original mixture present in the medium. The composition of the free sterol pool and the steryl ester pool also showed no consistent differences.

Selective Distribution

A batch culture, grown in the presence of a mixture of sterol, was harvested in log phase. Gradient purified mitochondria were isolated and the sterol composition determined. The results are shown in Table II. The composition of sterols extracted from the mitochondria was the same as that for a whole cell sample taken from the same culture. However, one of the minor sterols isolated from the mitochondria had been altered, as evidenced by a change in relative carbon number.

DISCUSSION

The interest in the Pythiaceous fungi for the study of sterol function is focused on 2 aspects. Because these fungi are capable of vegetative growth in the total absence of sterols, one aspect is the possibility of isolating eucaryotic membrane systems (e.g., mitochondrial) that previously have been unobtainable in forms that are absolutely devoid of sterols. The other aspect is the readiness with which these organisms incorporate exogenous sterol into their membranes. Thus, using Pythiaceous fungi, it is possible to examine a wider array of

TABLE I

Sterol Competition Experiments[a]

Experiment		Sitosterol	Campesterol	Cholesterol
1	Added	29.6	39.1	31.3
	total sterol	29.5	33.5	37.0
	free sterol	22.2	37.3	40.5
	steryl ester	30.8	35.6	33.6
2	Added	45.7	30.2	24.1
	total sterol	46.7	30.3	23.0
	free sterol	35.6	38.8	25.6
	steryl ester	47.6	31.3	21.1
3	Added	21.3	56.2	22.5
	total sterol	19.2	57.9	22.9
	free sterol	18.2	57.3	24.5
	steryl ester	22.9	54.9	22.2
4	Added	22.5	29.8	47.7
	total sterol	23.0	28.4	48.6
	free sterol	30.6	30.9	38.5
	steryl ester	25.3	27.6	47.1

[a]Data are reported as percentage composition.

TABLE II

Sterol Deposition in Mitochondria[a]

	Percentage of total sterol			
	Campesterol	Stigmasterol	Sitosterol	Lanosterol
Whole cell	16.0	31.0	47.2	5.8
Mitochondria	20.0	31.1	43.1	—*

[a]Sterol composition was determined from the nonsaponifiable fraction of lipid extracts as described in Materials and Methods.

*No lanosterol was detected in the mitochondria; instead, a new sterol representing 5.8% of the total mitochondrial sterol was isolated.

sterol effects on membrane properties than previously has been attainable in any one system.

A major consideration that must be made in studies concerning the effects of sterols in Pythiaceous fungi is the possible existence of an inherent mechanism of sterol specificity with regard to structure. Knights and Elliott (20) have shown that *P. cactorum* is capable of converting $\Delta^{5,7}$-sterols to Δ^{5}-sterols. The side chains of these sterols were not modified. It is not known if this is a reflection of a specificity for Δ^{5}-sterols or simply a conversion by remnants of a now lost sterol biosynthetic pathway.

There are 3 levels at which the organism may discriminate between sterols: (a) initial uptake of sterol from the medium, (b) differential esterification, and (c) differential distribution in specific membranes. Initial uptake of sterol by *P. cactorum* was shown to follow first-order kinetics. Double reciprocal plots of the rate of sterol uptake concentration in the medium are linear. The curves for cholesterol and sitosterol accumulation were essentially superimposable. These 2 sterols differ in structure only in the absence or presence of an ethyl group at position C-24 of the sterol side chain. Therefore, *P. cactorum* does not appear to exhibit specificity for sterol side-chain structure in initial uptake.

The absence of specificity in uptake was confirmed by competition experiments. The composition of the sterol extracted from the mycelia was the same as the composition of sterol mixtures added to the medium. In addition, these compositions were maintained in the free sterol pool and the steryl ester pool. Therefore, sterol specificity at the level of esterification is also lacking in *P. cactorum*.

The sterol composition of purified mitochondria was essentially the same as that for the whole cell, although at least one sterol or sterol derivative found in the mitochondria was structurally modified. It is not certain if the alteration of this one sterol was a reflection of specificity or a fortuitous result. Modification of the structure may have circumvented the criteria for exclusion from the membrane. If this is indeed the case, then it can be concluded that the presence of a preferred sterol is more important in the mitochondrial membranes than in the protoplasmic membrane. On the other hand, the enzymes in *P. cactorum* that modify the sterol ring structure may have simply altered the sterol prior to its availability for insertion into the mitochondria. Because no exclusion of individual sterols was observed in the mitochondria, a lack of specificity in sterol deposition in specific membranes is suggested.

Thus, the accumulation of sterol by *P. cactorum* appears to be unselective. Sterols with altered structures may be incorporated into target membranes with much less difficulty than is observed in many of the other systems used in similar studies. This is a distinct advantage for studies of the influence of sterol structure on biological membrane.

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Lack of Catabolism of Brain Cholesterol

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ABSTRACT

Since direct intracranial injections of precursors indicate that cholesterol is synthesized in the brain at all ages, there must be a mode of disposal also. The sterol nucleus itself is not degraded by mammalian systems but the side chain can be metabolized. [26-^{14}C]Cholesterol was therefore injected directly into the brain of 8- to 19-day-old rats which were sacrificed at the end of 24 hr, 1 week and 2 weeks after injection. The results indicate that, irrespective of the interval between injection and sacrifice, all of the radioactivity was found in the free cholesterol or the cholesterol component of the sterol esters. No radioactivity was found in the fatty acids of the phospholipids. We therefore conclude that the side chain of the cholesterol does not get metabolized to propionyl CoA, which in turn, could lead to fatty acid synthesis. Radioactivity in the serum, even after 2 weeks, indicates that there must be a slow but steady exchange between the brain and the blood that would explain the route of exit for brain cholesterol.

INTRODUCTION

Nervous tissue is known to contain a high concentration of cholesterol and within the central nervous system (CNS), the white matter and the myelin are rich in cholesterol. During myelination, cholesterol increases rapidly and, in the rat, the adult concentration is achieved ca. 30 days. Synthesis of cholesterol in this period seems to be far greater than uptake of intact cholesterol from blood into the brain (1,2). Once the cholesterol was deposited in the CNS, the turnover was assumed to be very slow, from early work by Davison et al. (3). Fumagalli et al. (4) using hypocholesteremic drugs found that brain sterol metabolism in adult animals is active. This led to the present concept of several pools of brain cholesterol all with different turnover rates (5).

Earlier work from our laboratory indicated that the adult brain is capable of total synthesis of cholesterol within 15 sec after an intracranial injection of [^{14}C]acetate (6). Since synthesis of cholesterol from precursors seems to be active even in adults, obviously there must be some system for degradation and removal of brain cholesterol. Normally, at least in the liver, cholesterol is converted to bile acids in the course of its catabolism (side chain degradation and hydroxyl group introduction) but no such transformation has ever been demonstrated in the CNS. Exchange of cholesterol from myelin to plasma has been suggested as a mechanism of removal (7). Another possibility is that the side chain is partially oxidized resulting in the formation of propionyl CoA in the liver as reported by Suld et al. (8). We undertook this investigation to determine if this pathway occurs in vivo in the brain and, if so, whether the propional CoA could be a precursor for fatty acid synthesis.

MATERIALS AND METHODS

Wistar rat pups, 8 to 21 days old, from mothers raised on rat chow, were used in the study. [26-^{14}C]Cholesterol, 50-60 mCi/mM, was obtained from New England Nuclear, Boston, MA. Five pups, 8 days old, weighing 14.7 ± 0.6 g, were given 5 μCi of labeled cholesterol/rat by direct intracranial injection (9) and sacrificed after 14 days. Another group of 5 pups, 19 days old, weighing 36 ± 3 g, was given a similar injection and sacrificed after 7 days. Finally, the last group of 5 pups weighing 24.5 ± 2.2 g was treated as just described and sacrificed after 24 hr. All pups were sacrificed by decapitation; blood was collected and immediately centrifuged to obtain plasma which was frozen in Dry Ice and stored at -80 C until analyzed. Brain and liver tissue was removed, washed, weighed, frozen and stored at -80 C until analyzed.

Total lipids (TL) were extracted by the Folch et al. method (10) and fractionation of total lipids was achieved by a SiO_2 column and Silica Gel 60 TLC plates (11). A Beckman scintillation counter, Model #LS 8100, was used to determine radioactivity. The efficiency was 96.7% for ^{14}C activity. A Packard Radio-GLC instrument was used to determine radioactivity in fatty acids (11).

RESULTS

Table I gives sp act (cpm/mg) of TL isolated from individual brain, liver and serum samples from rat pups sacrificed at different intervals following intracranial injection of [26-^{14}C]-cholesterol. The sp act of TL in the brain immediately after injection of labeled cholesterol (zero time) was 500 × 10^3 which dropped down to 46 × 10^3 in 24 hr, i.e., a

91% decrease. There was a further decrease in 1 week, leaving only 2% of the original injected activity in the brain. There seems to be only a minor change between 1 and 2 weeks.

The radioactivity introduced directly into the brain appeared in the liver undoubtedly via blood (24-hr interval). In a period of 7 days, there was a decrease from about 6,500 cpm/mg to only 900 cpm/mg (86% decrease). However, when the injection was given to 8-day-old pups sacrificed 2 weeks later, only 2% of the dose was recovered. In the circulating plasma, there was a decrease of ca. 50% in the sp act of TL in 1 week. In general, when 17- to 19-day-old animals (peak myelination period) were given labeled cholesterol, the 24-hr and 1-week intervals showed considerably more decline in sp act of brain, liver and serum total lipids. If the injection was given to 8-day-old pups which were sacrificed after 2 weeks, a higher sp act of liver and serum lipids was recorded compared to 19-day-old injected animals sacrificed after 1 week. Thus, the age of injected animals (in addition to the interval between injection and sacrifice) seems to influence retention of the injected dose.

The sp act of free cholesterol (cpm/mg) was much higher than TL because TL contained several components that contribute mass but very little radioactivity. The decrease in sp act of brain cholesterol in rats injected at 8 days and sacrificed 2 weeks later could also be due to dilution by newly synthesized cholesterol. Such a rapid buildup of cholesterol in the brain has been demonstrated earlier by several workers (5,12).

Table II gives the percentage distribution of ^{14}C activity in various lipid components. It was found that, irrespective of the time period or tissue source, over 90% of the total radioactivity was in the combined free and esterified cholesterol and the rest was distributed in other components such as free fatty acids (FFA), triglycerides (TG) and phospholipids (PL) (since the amount of radioactivity in lipid components other than free and esterified cholesterol was very minor, for brevity, it is given collectively). Surprisingly, when fatty acids (FA) were obtained by methanolysis (11) from these fractions, they contained negligible amounts of radioactivity.

The 90% or more of radioactivity found in the combined cholesterol fraction was not equally distributed between the free and esterified fraction; rather the distribution varied depending on the time interval and tissue source. In general, the brain and liver free cholesterol was far more radioactive but the serum esterified cholesterol was almost twice

TABLE I

Retention of Radioactivity in Brain, Liver and Serum, 24 Hr, 1 Week and 2 Weeks after Intracranial Injection of [26-^{14}C]Cholesterol

	17-Day-old pups sacrificed after 24 hr			19-Day-old pups sacrificed after 1 week			8-Day-old pups sacrificed after 2 weeks		
	Brain	Liver	Serum	Brain	Liver	Serum	Brain	Liver	Serum
Sp act of TL (cpm/mg)	46×10^3 ± 12×10^3	6.5×10^3 ± 2.1×10^3	11.5×10^3	9×10^3 ± 1.2×10^3	0.9×10^3 ± 0.2×10^3	4.5×10^3	10×10^3 ± 1×10^3	2.1×10^3 ± 0.2×10^3	5.9×10^3
Sp act of free cholesterol (cpm/mg)	221×10^3 ± 50×10^3	73.8×10^3 ± 23.6×10^3	43.5×10^3	33.8×10^3 ± 4.3×10^3	9.05×10^3 ± 2.7×10^3	12.6×10^3	23.8×10^3 ± 5.9×10^3	23.9×10^3 ± 0.16×10^3	52.5×10^3
Injected dose retained (%)	30.7 ± 23	1.3 ± 0.6	9.6	7.3 ± 1.3	0.9 ± 0.2	2.7	8.2 ± 0.9	2.1 ± 0.4	8.6

Average and standard deviations from 5 samples.

TABLE II

Percent Distribution of Radioactivity in Brain Liver and Serum Lipid Components at 3 Different Time Intervals Following Injection of [26-^{14}C]Cholesterol

	Sacrificed after 24 hr			Sacrificed after 1 week			Sacrificed after 2 weeks		
	Brain	Liver	Serum	Brain	Liver	Serum	Brain	Liver	Serum
				Total radioactivity in lipids (%)					
Free cholesterol	90.9 ± 2.5	78.6 ± 9.4	31.2	80.5 ± 1.7	78.2 ± 5.5	31.5	94.3 ± 1.4	58.5 ± 13.2	23.9
Esterified cholesterol	5.3 ± 1.9	9.8 ± 2.6	60.2	17.3 ± 1.7	12.7 ± 1.8	66.9	3.6 ± 0.5	39 ± 12.9	48.9
Other components (TG, FFA, PL)	3.3 ± 0.2	11.7 ± 1.5	8.6	2.2 ± 0.1	9.0 ± 1.5	1.5	2.1 ± 0.5	2.5 ± 0.2	25.3
Ratio: Free cholesterol/ esterified cholesterol	17.1	8.1	0.52	4.6	6.1	0.47	26.2	1.5	0.49

Average and standard deviations from 5 samples.

as active as free cholesterol. The same trend is seen after the longer period of 1 week although the magnitude differed appreciably in the brain. Two weeks after injection, the free cholesterol in the brain, again, had almost all the radioactivity and the esterified cholesterol had very small amounts of radioactivity. All through the experimental period, the serum esterified cholesterol contained far more radioactivity than the free cholesterol component.

DISCUSSION

Uptake and synthesis of cholesterol in the adult brain has been controversial. This stems from the fact that very small amounts of radioactive cholesterol were found in the brains of adult animals following parenteral administration (5 and references therein) of labeled cholesterol or labeled precursors. Similarly, turnover rates of brain cholesterol ranged from negligible to very rapid, depending on the identity of the cholesterol pool under investigation (12). Use of [4-^{14}C]cholesterol has already indicated that the sterol nucleus is not degraded in the CNS in situ (3). Using cholesterol labeled in the side chain, we wanted to learn about the catabolism of the non-nuclear portion. The catabolism was studied in rats covering the entire period of "brain growth spurt" (8-23 days) and a shorter interval involving the period of rapid myelination (17-23 days) (13). Developing brain has been shown to be capable of β-oxidation of intracranially administered fatty acids leading to formation of acetyl CoA followed by de novo synthesis of palmitate and cholesterol (9). Sun and Horrocks (14) have suggested that brain palmitate may be oxidized and the products reutilized for lipid synthesis in situ. Finally, Suld et al. (8) have demonstrated that the side chain of cholesterol is oxidized to provide propionyl CoA in the liver which, in turn, could be utilized for FA synthesis.

In our study, however, irrespective of the age of the rats or the interval between injection and sacrifice, only a negligible amount of radioactivity from the injected [26-^{14}C]cholesterol was found in the fatty acids of either the polar lipids or sterol esters. Thus, we conclude that the side chain of the injected cholesterol was not oxidized to provide precursors for fatty acid synthesis. The cholesterol radioactivity, however, decreased very rapidly, ca. 91% decrease, from time zero to 24 hr. This drastic decrease indicates that cholesterol is actually removed from the brain, possibly via blood. Such an exchange (up to 59%) from myelin to the plasma has been

reported by Graham and Green (7). Nicholas and Ramsey (15) have reported that at least 2 radioactive metabolites were excreted in the urine for more than 2 years after an intracranial injection of ^{14}C-labeled cholesterol, thus indicating a continuous, slow turnover of brain cholesterol. They report that the exact nature of the products is still not fully understood.

Normal adult brain contains very small amounts of esterified cholesterol, although just prior to myelination, rat pup brains contain detectable amounts (16). Thus, one would expect an esterification of the injected [^{14}C]-cholesterol which explains the radioactivity in cholesterol ester fraction of the brain from 5.3% (after 24 hr) to 17.3% after 1 week. Whether this conversion has any connection with the ultimate exit of cholesterol from the brain remains unknown at this time. The esterified cholesterol in the brain could not have its origin in the circulating plasma because of a restrictive uptake of intact cholesterol ester from blood into the brain (Blood-Brain Barrier Relationship) and the activity is too high to be accounted for by trapped blood. Higher activity in esterified cholesterol in the serum indicates the preferred form of transport in the blood. Even in the liver, the radioactivity of esterified cholesterol was confined to the cholesterol moiety; fatty acids again were almost free from radioactivity.

We conclude from this study that the side chain of the cholesterol does not produce precursors for lipid synthesis and that the brain cholesterol is removed as an intact cholesterol moiety and, as Nicholas and Ramsey have suggested, excreted by way of urinary products. However, if the brain cholesterol gets into the liver via blood, a major portion of it should be converted to bile acids and excreted in the feces; the urinary excretion product must be formed in the brain itself. Thus, the synthesis at various rates, depending on the age of the animal, and a corresponding rate of removal helps keep the cholesterol content of mature rat brain fairly constant.

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Fatty Liver of Growing Rats Fed Excess Lysine and Its Prevention by Adenine or Allopurinol

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ABSTRACT

Weanling male Sprague-Dawley rats were fed ad libitum 15% casein diets with and without 5.0% lysine-HCl, 0.25% adenine sulfate or 0.1% allopurinol for 2 weeks. Addition of lysine alone depressed 2-week growth from 94 to 65 g, increased average daily urinary orotic acid excretion from 0.39 to 1.77 mg and increased the percentage of total liver lipids from 3.6 to 11.2. Adenine or allopurinol did not change growth but markedly enhanced lysine-induced orotic aciduria and completely prevented lysine-induced fatty livers. Reports by others show that adenine and allopurinol also prevent fatty livers of rats fed arginine-free diets or excess orotic acid. The authors conclude that lysine-induced orotic aciduria results from arginine deficiency caused by antagonism of arginine function by lysine, and that lysine-induced fatty liver probably results from a lesion identical to that produced by feeding excess orotic acid.

INTRODUCTION

Growing rats fed excess lysine show signs of arginine deficiency. ("Argenine-free diet" in the present context means diets lacking the 3 urea cycle amino acids, arginine, ornithine and citrulline. Only arginine occurs in significant concentrations in common dietary proteins and therefore arginine intake becomes of practical concern. "Arginine deficiency" as used herein means diets lacking sufficient arginine for optimal growth. The term ammonia means $NH_3 + NH_4^+$). As is the case with diets lacking arginine (1,2), diets with 15% casein plus 5% lysine depress growth, cause a very significant rise in urinary orotic acid and an accumulation of lipids in the liver of growing rats (3; unpublished observations). These lysine-induced changes result from antagonism of arginine metabolism and are prevented by adding 1% arginine-HCl to the high-lysine diet (3; unpublished observations). Feeding 1% orotic acid also causes fatty liver (4) and simultaneous supplementation of high orotate diets with adenine (5) or allopurinol (6) prevents such liver lipid accumulation. Supplemental adenine also prevents fatty liver of rats fed arginine-free diets (7). This effect of adenine and allopurinol is virtually limited to orotate-induced fatty liver and probably related to their inhibition of the metabolism of orotate to uridine monophosphate. Thus, feeding of these compounds provides an experimental model for assessing the role of orotate in the development of fatty liver.

These experiments, employing lysine-, adenine- and allopurinol-supplemented diets, indicate that lysine-induced fatty liver results from increased de novo orotate synthesis caused by lysine-induced arginine deficiency.

MATERIALS AND METHODS

Two 2 x 2 factorial experiments employed weanling male Sprague-Dawley rats (ARS Sprague-Dawley, Madison, WI), housed individually in stainless steel, wire-mesh cages in a room with a 12-hr light, 12-hr dark lighting schedule and an average temperature of 22 ± 1 C. The basal diet contained vitamin-free casein (Teklad Test Diets, Madison, WI) 15%; L-methionine (Ajinomoto Co., Inc., Tokyo, Japan) 0.3%; sucrose 73.7%; corn oil (Mrs. Tucker's Pure Corn Oil, Anderson Clayton Foods, Dallas, TX) 5%; minerals (Rogers and Harper Mineral Mix, Catalog no. 70760, Teklad Test Diet, Madison, WI) 5% (this supplied the following in mg/kg diet: ammonium molybdate, 0.125; calcium carbonate, 1,464.5; calcium phosphate, 21.5; cupric sulfate, 6.05; potassium iodide 0.025; potassium phosphate, 1716.0; sodium chloride 1253.0; sodium selenite, 0.075; and, zinc chloride, 1.0); vitamins (Vitamin Fortification Mix, Catalog No. 40060, Teklad Test Diet, Madison, WI) 1% (this supplied the following in mg/kg diet: *p*-aminobenzoic acid, 110.2; ascorbic acid, 1017.5; biotin, 0.44; choline dihydrogen citrate, 3715.1; folic acid, 1.98; *i*-inositol, 119.2; menadione, 49.6; nicotinic acid, 99.21; pyridoxine HCl, 22.05; riboflavin, 22.15, thiamin HCl, 22.15; retinyl palmitate (50,000 U/g), 29.68; ergocalciferol (500,000 U/g), 4.41; and tocopheral-acetate, 121.3. Additions of lysine-HCl (Ajinomoto Co., Inc., Tokyo, Japan), adenine sulfate (Nutritional Biochemical Corp., Cleveland, OH), and allopurinol

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(Sigma Chemical Co., St. Louis, MO) were included at the expense of sucrose. Experiment 1 employed all combinations of 0 and 5% lysine-HCl and 0 and 0.25% adenine sulfate in the diet. The diets fed in experiment 2 included all combinations of 0 and 5% lysine-HCl and 0 and 0.1% allopurinol. Except for these dietary variables, both experiments were performed identically but not coincidentally.

The rats were fed the basal 15% casein diet for 3 days, then 5 were randomly assigned to each of the 4 treatments and the experimental diets were fed for 14 days. Feed and tap water were offered ad libitum and feed intake was recorded weekly. On days 1, 2, 7, 8, 13 and 14, all of the rats were weighed and transferred to metabolism cages for urine collection. Urine, collected for 24 hr in tubes containing 3 drops of 6 N H_2SO_4, was stored at -15 C until assayed for creatinine (8), ammonia (9), urea (10) and orotate (11). At the end of day 14, the rats were fasted for 4 hr before being weighed and decapitated. Their livers were rapidly removed, weighed, frozen in liquid nitrogen, and stored at -15 C until assayed for total lipids (12). Statistical analysis followed methods described by Steel and Torrie (13).

RESULTS

Averaged for both experiments, rats fed 15% casein with no additional lysine consumed 183 ± 3.4 g (X ± SEM, n = 19) of feed and gained an average of 94 ± 2.9 g in 14 days. Feeding 5% lysine-HCl with the basal diet decreased feed intake and weight gain to 153 ± 4.4 g and 65 ± 3.1 g, respectively. With no additional lysine, daily urinary ammonia-nitrogen, urea-nitrogen and creatinine averaged 7.5 ± 0.4 mg, 28.6 ± 1.3 and 28.6 ± 0.8 mg/kg BW, respectively. Additional lysine increased these values 4.8-, 1.9- and 1.2-fold, respectively. Feeding 0.25% adenine sulfate or 0.1% allopurinol with the control or lysine-supplemented diet caused no changes in growth or excretion of urea, ammonia or creatinine. (In experiment 1, one of the 5 rats assigned to each of the 0.25% adenine treatments failed to thrive and died prior to necropsy. No values from these rats are included in statistical analysis. Kidneys of the adenine, 0.0% lysine-HCl-fed rats showed white spots. These probably were uric acid crystals previously observed with high adenine feeding [14]. Similar kidney lesions were absent with adenine in the high-lysine diet and both allopurinol treatments in experiment 2.) However, adenine and allopurinol had significant influence on urinary orotate and total liver lipids (Tables I and II). High dietary lysine significantly raised urinary orotic acid and caused fatty livers (Tables I and II). Adding adenine or allopurinol to the basal diet increased urinary orotic excretion but caused no change in total liver lipids. Both adenine and allopurinol markedly increased lysine-induced orotic aciduria and completely prevented the lysine-induced fatty livers.

DISCUSSION

Numerous nutritional and pharmacologic factors cause fatty liver by a variety of mechanisms (15). In 1955, Standerfer and Handler (4) observed that high dietary orotate caused fatty liver which was not prevented by lipotropic agents such as folic acid, vitamin B_{12}, choline or methionine. Subsequent studies have shown that orotate-induced fatty liver results from a discrete hepatic lesion which prevents the glycosylation of otherwise normal apo-β-lipoprotein, thus leading to low export of β-lipoprotein and accumulation of liver triglyceride (16,17).

Blocking the phosphoribosyl pyrophosphate (PRPP)-dependent conversion of orotate to uridine monophosphate (UMP) with adenine (5) or allopurinol (6) prevents orotate-induced fatty liver, but feeding of pyrimidines, such as thymine or uracil formed beyond this critical step, does not cause fatty liver (4). Thus, though the link between increased conversion of orotate to UMP and altered lipoprotein glycosylation is unknown, it is probably related to orotate-induced PRPP depletion with a subsequent decrease in purine synthesis and the purine to pyrimidine ratio (18).

Elevated endogenous synthesis of orotate and orotic aciduria results from injections of ammonium salts (19). This causes shunting of mitochondrially generated carbamyl phosphate into cytoplasmic pyrimidine biosynthesis (20), with orotate accumulating presumably because the capacity for its conversion to UMP is exceeded. Similar ammonia-induced orotic aciduria also results when urea synthetic capacity is inappropriately low due to arginine deficiency. Arginine deficiency with orotic aciduria is induced by low dietary arginine (1) or by antagonism of arginine function by high dietary lysine (unpublished observation). The marked increase in lysine-induced orotic aciduria caused by both adenine and allopurinol, which inhibit the conversion of orotate to UMP, emphasizes the magnitude by which de novo orotate synthesis is enhanced by excess dietary lysine.

Feeding diets without arginine or with excess lysine to rats also causes fatty liver (2,3;

unpublished observations). Excess liver lipid in both cases, like that due to a high dietary orotate (21), is predominantly triglyceride (2,3). Recently, Milner (7) observed that adenine prevented fatty liver induced by feeding an arginine-free diet to rats. Our data in Tables I and II show that both adenine and allopurinol prevent fatty liver induced by excess dietary lysine. Because neither of these compounds reversed lysine-induced growth depression, it is unlikely that they act by correcting lysine-induced inhibition of protein synthesis. These observations support the conclusion that lysine-induced fatty liver results from high de novo orotate synthesis, acting through a mechanism identical to that respon-

TABLE I

Final Body Weight, Liver Weight, Total Liver Lipids and Urinary Orotate of Weanling Male Rats Fed 15% Casein Diets, Varying in Lysine and Adenine Concentration, for 14 Days

Dietary		Final body weight (g)	Wet liver weight (g)	Total liver lipids (% wet wt)	Urinary orotate[a] (mg/day)
Lysine-HCl (%)	Adenine sulfate (%)				
0.0	.00	149[b]	7.9	3.62	.40
	.25	150	7.6	4.47	1.56
5.0	.00	119	7.0	9.78	1.66
	.25	122	6.0	3.79	19.73
ANOVA[c]					
	Lys	S	S	S	S
	Ade	N	N	S	S
	LXA	N	N	S	S
	EMS	65.1	.84	3.20	3.15

[a]Average daily urinary orotate excretion determined on samples pooled from collections on days 1, 2, 7, 8, 13 and 14.

[b]Mean of 5 rats/treatment, except for both .25% adenine treatments which represent only 4 rats/treatment.

[c]ANOVA, abbreviated analysis of variance table, including single degree of freedom tests for significant difference due to lysine (Lys), adenine (Ade), their interaction (LXA) and the error mean square (EMS). An "S" indicates a significant difference, whereas "N" indicates nonsignificance at $p<0.05$.

TABLE II

Final Body Weight, Liver Weight, Total Liver Lipids and Urinary Orotate of Weanling Male Rats Fed 15% Casein Diets, Varying in Lysine and Allopurinol Concentration, for 14 Days

Dietary		Final body weight (g)	Wet liver weight (g)	Total liver lipids (% wet wt)	Urinary orotate[a] (mg/day)
Lysine-HCl (%)	Allopurinol (%)				
0.0					
0.0	0.0	167[b]	10.7	3.48	.38
	0.1	172	10.9	2.65	1.95
5.0	0.0	135	8.7	12.68	1.88
	0.1	147	8.3	3.45	8.63
ANOVA[c]					
	Lys	S	S	S	S
	Allo	S	N	S	S
	LXA	N	N	S	S
	EMS	66.4	.60	1.03	2.03

[a]Average daily urinary orotate excretion determined on samples pooled from collections on days 1, 2, 7, 8, 13 and 14.

[b]Mean of 5 rats/treatment.

[c]ANOVA, abbreviated analysis of variance table, including single degree of freedom tests for significant difference due to lysine (Lys), allopurinol (Allo), their interaction (LXA) and the error mean square (EMS). An "S" indicates a significant difference, whereas "N" indicates nonsignificance at $p<0.05$.

sible for fatty liver induced by high dietary orotate. Because inappropriately low ureagenic capacity with accompanying high orotate synthesis and orotic aciduria is a potential risk in numerous conditions (22), the induction of fatty liver in rats by orotate, previously considered a biochemical curiosity, may be produced more commonly than previously recognized.

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Regulation of 3-Hydroxy-3-methylglutaryl CoA Reductase by Analogs of Cholesterol and Bile Acids in Cultured Intestinal Mucosa

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ABSTRACT

Sodium fusidate and its glycine conjugate, which have the same detergent properties as bile acids, significantly ($p<0.05$) stimulate HMG-CoA reductase of cultured intestine below the critical micellar concentration (CMC) without affecting brush border enzymes. Above CMC, both amphiphiles are cytotoxic. At concentrations between 1 and 5 mM, sodium fusidate decreased cholesterol contents of cultured mucosa ($p<0.05$), the increase in synthesis only partially compensating for the sterol loss. Oxygenated sterols, 7-keto- and 25-hydroxycholesterol, also depleted mucosal cholesterol at 0.5 mM, exerting their effect differently by inhibiting HMG-CoA reductase ($p<0.01$). In contrast to their marked effect on total mucosal cholesterol contents, brush border cholesterol was unaffected by both cholesterol and bile acid analogs.

INTRODUCTION

Cholesterol synthesized in the small intestine contributes to the circulating plasma cholesterol pool (1,2), interferes with intrinsic brush border enzymes and transport processes by altering microvillus membrane fluidity (3,4) and probably is involved in cell proliferation (5,6).

The question of whether intestinal cholesterol synthesis is regulated by bile acids or cholesterol itself is still controversial (7,8). In short-term (6-hr) organ culture, both were suppressive (9); however, under long-term (24-hr) culture conditions, we found bile acids to stimulate HMG-CoA reductase at comparable concentrations (10). This response was interpreted as a compensation for the loss of cellular cholesterol mediated by the detergent bile acids, because their effect was blocked in the presence of cholesterol. Here, we report on a similar property of fusidic acid, a bile acid analog with comparable micellar characteristics (11,12). In addition, we studied the effect of oxygenated sterols on cholesterol synthesis and contents in cultured intestinal mucosa and its brush border.

MATERIALS AND METHODS

D,L-Hydroxymethyl-[3-^{14}C]glutaryl-CoA and [^{3}H]mevalonolactone was obtained from New England Nuclear. Nonradioactive substrates were from Sigma Chemical Co. 7-Ketocholesterol and 25-hydroxycholesterol were from Steraloids Inc. The fusidates (sodium salt and glycine conjugate of 3α,11α-dihydroxy-16-β-acetoxylfusida-[17,20-(16-21-*cis*)-24]-diene-21-oic acid) were a gift of Dr. W.O. Godtfredsen (Leo Pharmaceutical Products, Ballerup, Denmark). Culture media were from Gibco.

Male chinchilla rabbits were kept and fed as previously described (13). Ileum mucosal biopsies were taken and cultured for 24 hr as detailed elsewhere (14,15). Culture medium consisted of 90% Trowel's T8, 10% lipoprotein-free fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and the respective additions as specified. Viability was routinely checked by histologic examination; as a biochemical indicator, we used the brush border enzyme alkaline phosphatase, measured according to ref. 16. For sucrase assay, we used Dahlqvist's method (17). HMG-CoA reductase was determined according to Shapiro et al. (18) with minor modifications (15). The homogenization medium consisted of 0.1 M sucrose, 0.04 M KH_2PO_4, 0.01 M dithiothreitol, 0.03 M EDTA and 0.25% Kyro EOB at pH 7.2. The mucosal homogenate was preincubated at 37 C for 15 min, before 500 μg protein in 0.07 ml were added to the assay mixture (1 M potassium phosphate buffer, 1 M EDTA, 0.5 M glucose-6-phosphate, 0.1 M NADP, 0.24 M dithiothreitol, 10 mg/ml serum albumin, 3.5 U/ml glucose-6-phosphate dehydrogenase at pH 7.4).

In a total of 0.25 ml, the mixture contained 48 nmol D,L-[3-^{14}C]HMG-CoA (10 Ci/mol). Under these conditions, the enzyme was measured in the totally activated state (a detailed validation of the assay procedure was given in ref. 15). Cholesterol was measured after Folch extraction by the cholesterol oxidase method. The brush border fraction was prepared as detailed (19) from cultured mucosal specimens. Mean alkaline phosphatase

activity was 2988 mU/mg protein, representing a 10-fold enrichment. Statistical evaluation was calculated by Student's t-test.

RESULTS AND DISCUSSION

In agreement with previous results (10), intestinal HMG-CoA reductase increased 2.5-fold during 24 hr culture in lipoprotein-free medium (Fig. 1). This increase was further stimulated in a concentration-dependent fashion by adding sodium fusidate to the culture medium. No response was observed with alkaline phosphatase and sucrase at the lower concentrations tested. At 5 mM, a concentration exceeding the critical micellar concentration (11), HMG-CoA reductase and both brush border enzymes were clearly suppressed, indicating toxicity of the fusidate. Stimulation by the glycine conjugate required a 10-fold higher level compared to sodium fusidate; again, the toxic concentration was reached at 5 mM.

With respect to the common micellar properties shared by fusidates and bile acids (11,12), we hypothesized that both detergent amphiphiles (class III B polar lipid) deplete intestinal membrane cholesterol. Indeed, despite a 60% increase in HMG-CoA reductase, the mucosal cholesterol/protein ratio (nmol/mg) was slightly (15%) suppressed at 1 mM, but was significantly decreased with increasing fusidate levels from 227.6 (54.3, SD) to 85.3 (18.1) in the presence of 5 mM sodium fusidate. In accordance with these data, fusidates are active in solubilizing cholesterol (12,20) and enhance biliary cholesterol excretion in the rat (21) and the rhesus monkey (20). Furthermore, the increase in endogenous cholesterol synthesis partly compensating for the fusidate-induced loss in cellular cholesterol is analogous to lecithin-treated hepatocytes (22). Compared to bile acids, the enhancement of reductase is less pronounced (10). Therefore, also in cultured intestine, HMG-CoA reductase activity seems to be a function of tissue cholesterol homeostatis, balancing the detergent effects of bile acids, demonstrated previously (10), and similar substances to avoid changes in fluid secretion (23) and membrane fluidity (4). This is supported by the remarkably stable brush border cholesterol (Table I), when sodium and glycofusidate concentrations were varied between 1 and 5 mM. Interestingly, plasma high density lipoproteins also are potent stimulators of intestinal reductase, probably via tissue cholesterol uptake (15).

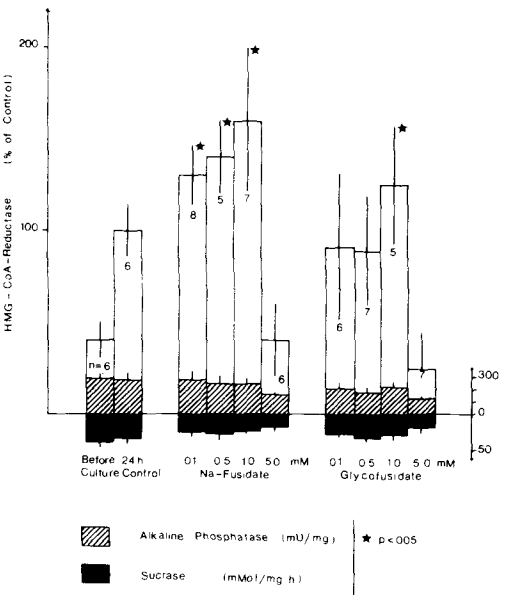

FIG. 1. Effect of sodium and glycofusidate on HMG-CoA reductase, alkaline phosphatase and sucrase of cultured intestine (means ± SD) $p<0.05$ alkaline phosphatase and sucrase at 5 mM vs control.

This mechanism of depleting intestinal cholesterol contents is opposed to the blockade of endogenous synthesis by oxygenated sterols (24). 7-Keto- and 25-hydroxycholesterol totally suppressed the increase of HMG-CoA reductase in cultured intestine (Table II). This enzyme inhibition is reflected in the 50% decrease in the mucosal cholesterol/protein ratio to 108.1 (24.6) and 111.2 (25.8) nmol/mg, respectively, at 0.5 mM. Again, brush border cholesterol was unaltered by both reductase inhibitors (Table I), probably indicating the enterocyte's capacity to shift cholesterol from intracellular compartments to the brush border. These results confirm and extend previous data in short-term culture of dog mucosa (9), although the degree of suppression was somewhat higher after long-term culture. Still, intestinal HMG-CoA reductase cannot be totally inhibited by oxygenated sterols even after long-term culture conditions, but the partial inhibition is sufficient to deplete tissue cholesterol. The reason for the incomplete effect of these sterols is unclear, but may be due to a rapid excretion and/or a longer half-life time of the intestinal enzyme (25). In accordance with our in vivo studies in the rabbit (26), bile acids and related detergents do not directly regulate intestinal cholesterol synthesis, but act via depleting mucosal cholesterol. Ultimately, the same effect can be achieved by blocking endogenous synthesis using oxygenated sterols. However, both techniques to induce cholesterol defi-

TABLE I

Effect of Fusidates and Oxygenated Sterols on Cholesterol Content of Brush Borders from Cultured Mucosa[a]

	mM	nmol/mg protein	n (rabbits)
Control		401.2 (102.6)	5
Na-fusidate	1	476.5 (106.6)	6
	2	413.7 (152.8)	6
	3	398.3 (18.8)	4
	5	414.7 (81.2)	6
Glycofusidate	1	435.5 (114.8)	4
	2	540.0 (167.3)	4
	3	385.3 (32.8)	4
	5	424.7 (114.2)	4
7-Ketocholesterol	0.1	361.8 (182.5)	6
	0.5	370.0 (152.4)	6
25-Hydroxycholesterol	0.1	358.2 (146.7)	6
	0.5	359.3 (140.5)	6

[a]Data are given as means (SD).

TABLE II

Effect of Oxygenated Sterols on HMG-CoA Reductase of Cultured Intestine[a]

		(nmol mg^{-1} hr^{-1})	n (rabbits)	p
Control		0.370 (0.076)	10	
7-Ketocholesterol	0.05	0.295 (0.155)	5	>0.05
	0.1	0.165 (0.107)	5	<0.001
	0.5	0.190 (0.086)	5	<0.01
25-Hydroxycholesterol	0.05	0.236 (0.086)	5	<0.01
	0.1	0.159 (0.077)	5	<0.001
	0.5	0.151 (0.046)	5	<0.001

[a]Data are given as means (SD).

ciency in cultured mucosa fail to interfere with brush border cholesterol, most likely due to an induction of HMG-CoA reductase and/or use of intracellular reserves in order to stabilize membrane fluidity (23).

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ERRATUM

In the paper by S.C. Goheen et al. entitled "The Prevention of Alcoholic Fatty Liver Using Dietary Supplements: Dihydroxyacetone, Pyruvate and Riboflavin Compared to Arachidonic Acid in Pair-Fed Rats" (*Lipids* vol. 16, no. 1, p. 43), line 3 of the abstract should read, "6-fold greater in these rats than in those fed an alcohol diet without the supplements (AA-). The liver . . . ."

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Long Chain Polyenoic Acid Levels in Viably Sorted, Highly Enriched Mouse Testis Cells[1]

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ABSTRACT

Twenty- and 22-carbon fatty acids of the linoleic (n-6) and linolenic (n-3) acid families were measured in murine spermatogonia and preleptotene spermatocytes (early), pachytene primary spermatocytes (1°), round spermatids (RS), condensing spermatids (CS) and Leydig cells enriched by staput velocity sedimentation at 1 G, followed by viable microflow sorting on the basis of light scatter and DNA content. 22:5(n-6) increased progressively from 2 to 20% of total fatty acid in the progression of germinal cell differentiation, early → 1° → RS → CS, but decreased in mature sperm. The precursor 20:4(n-6) showed a roughly reciprocal relationship. 22:6(n-3) showed no significant correlation with cell type. 22:5(n-6) was found highest in triglycerides of later differentiation stages whereas 20:4(n-6) and 22:6(n-3) were found primarily in phospholipid in all cell fractions.

INTRODUCTION

The testis is a very complex organ containing more than 30 distinguishable cell types, most of which are various stages of differentiation of germinal cells. The germinal cells are contained within the seminiferous tubules where they go through several mitotic and meiotic divisions in the process of differentiation from diploid spermatogonia (2C in DNA content) to spermatocytes (mostly 4C) to round spermatids (1C) to condensing spermatids (1C) to spermatozoa (1C) which are released into the lumen (1). These broad classifications can be further subdivided according to criteria of morphology and intercellular associations in the tubule. Between the tubules are found the diploid interstitial cells of Leydig responsible for androgen production.

Long chain polyenoic acids (LCPA) of 22 carbons, derived from the essential linoleic (n-6) and linolenic (n-3) acids are found in the testes of many species, and there is a great deal of evidence that they serve some specialized and obligate role in spermatogenesis (2). Studies using immature developing testes or mutants with defects in spermatogenesis have provided indirect evidence for association of LCPA with spermatids and later stages of differentiation.

The predominant LCPA found in testis differs among species but in mammals, 4,7,10,-13,16-docosapentaenoic acid (22:5n-6) or 4,7,-10,13,16,19-docosahexaenoic acid (22:6n-3) are usually found in highest concentration. These fatty acids are synthesized by successive elongations and desaturations from linoleic (18:2n-6) or linolenic (18:3n-3) acids; e.g., 18:2 → 18:3 → 20:3 → 20:4 → 22:4 → 22:5 (3). The rat testis, which has thus far been used in most of these metabolic studies, contains 22:5 as its predominant LCPA; the human testis contains 22:6. In the mouse testis, on the other hand, each of these fatty acids is found as 5-10% of total fatty acids. Knowledge of the distribution of these and other LCPA among the various cell types is important to understanding their role in spermatogenesis.

Beckman et al. (4) used centrifugal elutriation to obtain enriched fractions of spermatocytes and spermatids from adult rat testis for lipid analysis, in order to localize 22:5(n-6) and measure levels of other lipids in each of these cell types. While their study supported, in the rat, indirect evidence that increased levels of 22:5 are associated with spermatids in rats and mice (2,5,6), other cell types were not sufficiently enriched to determine the contribution of each to the 22:5 in the whole testis.

We have used a similar batch separation to study the location of LCPA in mouse testis cell types. We have, however, also used microflow cell sorting to prepare highly enriched early cells, pachytene spermatocytes and round spermatids in order to unambiguously locate the stage in the differentiation train in which 22:5 begins to increase in proportion to other fatty acids in spermatogenic precursor cells.

MATERIALS AND METHODS

Enrichment of Testis Cells

Testes from adult Swiss mice (Microbiological Associates) were decapsulated, minced

[1]Abbreviations: WT = whole testis; EC = early cells; 1° = primary spermatocytes; RS = round spermatids; CS = condensing spermatids; RB = residual bodies; PL = phospholipid; TG = triglyceride; CE = cholesteryl ester.

with scissors and digested with collagenase (Sigma Type II) and hyaluronidase (Sigma Type V) for 25 min according to a modification (7) of the procedure described by Collins (8) for liver. Cells were filtered through 74 μm nylon mesh and layered on top of a 2-4% gradient of bovine serum albumin (Sigma, Fraction V) in a Staput apparatus as described by Romrell et al. (9). After 4 hr, the gradient was collected from the bottom in 40-mL fractions.

Cell Sorting

Cells were pelleted and resuspended in Eagle's minimum essential media for suspension cultures (Flow Laboratories) prior to sorting on a Coulter Epics IV flow microfluorometer/cell sorter. Cells were sorted at a cell throughput of 1-3,000/sec on the basis of light scatter alone or on the basis of DNA content with additional light scatter gates established to eliminate nonviable cells or artifactual multinucleates of the same DNA content as normal cell types. Cells were viably stained with Hoechst 33342 (Calbiochem), which binds electrostatically to DNA, as described for cultured cells by Arndt-Jovin and Jovin (10). Cells were maintained at 5 C during and subsequent to sorting. This procedure is described in detail elsewhere (7).

Cell Identification

Aliquots of pure or enriched cells were centrifuged onto microscope slides in a Shandon cytocentrifuge, air-dried, fixed in methanol, stained with Wright's stain and counterstained with Giemsa. Cells were identified on the basis of size, nuclear size and shape, degree of cytoplasmic staining, chromatin clumping, nuclear-to-cytoplasmic ratio, and in the case of sorted cells, DNA content. Approximately 100 cells were counted from each slide using a random search pattern. Details of cell identification and documentation of homogeneity of cell fractions may be found in Grogan et al. (7).

Fatty Acid Analysis

Cells were washed twice with phosphate buffered saline and hydrolyzed in 10% KOH containing 50% MeOH as described in Bridges and Coniglio (11). Digests were acidified (pH 2) with concentrated HCl and extracted 3X with petroleum ether. Combined extracts were taken to dryness under a stream of N_2 and methylated overnight at room temperature with methanolic HCl (Supelco). Methyl esters were extracted with petroleum ether and quantitated on a Packard 427 gas chromatograph equipped with dual flame ionization detectors and a 10% SP-2340 (Supelco) column, temperature-programmed from 165-240 C at 4 C/min. A duplicate column was used in the balance mode to cancel out baseline drift due to column bleed. Recovery was checked at every stage by internal standardization with methyl pentadecanoate (Supelco) and with actual samples of known composition. Methyl esters were identified by comparisons of retention times with those of commercial standards and biological samples of previously determined composition. Total fatty acid was calculated by careful calibration of mass to peak areas and use of appropriate dilution factors.

Statistical Analysis

A least squares multiple linear regression analysis (12) statistics package (Tektronix Plot 50, Volume 3) was used with a Tektronix 4051 minicomputer to analyze % fatty acid composition and % cell composition data for correlations between specific fatty acids and cell types.

These data included mean differential counts and fatty acid compositions for 27 sorted cell preparations and 44 gradient fractions each from 5 different gradients. Equations of the form shown in Table III (footnote a) were constructed and tested to calculated % fatty acid or the ratios of various fatty acids based on the cell composition.

Fatty Acid Analysis of Lipid Classes

Total lipids were extracted from tissues or pelleted cells by the modified Folch procedure of Bridges and Coniglio (13). Extracts were dried under a stream of N_2, redissolved in $CHCl_3$ and separated on Whatman LHP-K silica gel thin layer plates developed with petroleum ether/ethyl ether/HOAc (80:20:1). Bands of phospholipid, triglyceride and cholesterol esters were visualized with Rhodamine 6G spray (Supelco) under ultraviolet light and scraped into screw-capped vials for methylation by transesterification with methanolic sodium methoxide (Supelco). Samples were sealed under N_2 and allowed to stand overnight at room temperature. Three vol of water were added and methyl esters were extracted at 4 C with petroleum ether for analysis by gas chromatography as already described. Percentages of component lipid classes were calculated from values of fatty acid/lipid class using appropriate stochiometric factors corrected for fatty acid composition.

Phospholipid Class Analysis

Phospholipid classes were quantitated by a modification of the procedure used by Beck-

man et al. (4). Phospholipid classes were separated by 2-dimensional thin layer chromatography on Whatman LHP-K silica gel plates using tetrahydrofuran/MeOH/NH_4OH (10:10:1) in the first development and $CHCl_3$/MeOH/acetone/HOAc/H_2O (6:8:2:2:1) in the second dimension. Plates were charred lightly with H_2SO_4 and spots were scraped from the plates for total phosphorous analysis as described by Bartlett (14).

RESULTS

Fatty Acid Analysis of Staput Fractions

Figure 1 shows the distribution of early cells (EC), primary spermatocytes (1°), round spermatids (RS), condensing spermatids (CS), sperm heads (SH), residual bodies (RB) and Leydig cells (LC) in the 44 fractions of a Staput velocity sedimentation density gradient as determined by differential counting of stained cytocentrifuge preparations. This procedure enriches 1°, RS and RB to 70-80% homogeneity but fails to separate these cell types from other cell types or artifacts of similar size. Other cell types are not enriched beyond 30% (see ref. 7 for details of cell separation).

Figures 2a-b show average % composition of fatty acids from cells taken from these same gradient fractions. Cursory examination suggests that 22:5(n-6) is increased with increasing concentration of RS, CS, RB and SH in the gradient fractions and that 20:4 shows a reciprocal relationship. Multiple linear regression analysis of % fatty acid vs % cell type in each gradient fraction showed only CS to have a correlation coefficient with 22:5 ($r = 0.77$) greater than 0.5 ($r = 1$ is exact). 20:4, on the other hand, correlates only with 1° ($r = .82$) with a correlation coefficient greater than 0.5. The ratio 22:5/20:4, which may reflect the precursor product relationship between these fatty acids, is also best correlated with CS ($r = 0.84$), reaching its highest values concomitant with highest percentages of CS (see Fig. 2c), even though CS never exceeds ∿30% of total cells.

Table I contains mean % fatty acid compositions for Staput fractions of highest enrichment in 1°, RS, CS and RB, respectively. There are numerous highly significant differences in fatty acid composition among the cell fractions enriched in various cell types. While most of these differences are of low magnitude, 20:4 is 27% lower in the RS enriched fractions and 47% lower in CS enriched fractions than in 1° enriched fractions. 22:5, on the other hand, is 50% higher in RS enriched and 88% higher in CS enriched than in 1° enriched fractions. In the same progression of enriched fractions, 22:5/20:4 is 100% and 217% higher, respectively. Comparison of fatty acid compositions of CS-enriched and RB-enriched fractions suggest that CS-enriched fractions strongly reflect contributions of CS to the fatty acid composition, although contribution of contaminating RB must be substantial. Estimates of total fatty acid from sorted cells, obtained from quantitative gas liquid chromatography (GLC) support this observation. Each CS, which has ∿4 pg fatty acid/cell, should have twice the impact on fatty acid composition as a RB which has ∿2 pg/cell. Round spermatids, on the other hand, have ∿8 pg/cell and may be expected to influence accordingly fatty acid composition of fractions in which they are present. Values for fatty acid/cell are not yet available for other testis cell types.

Fatty Acid Composition of Sorted Cells

Fatty acids were measured in total lipids of testis cells enriched by microflow sorting of cells from Staput fractions. This enrichment is illustrated in Figures 3a-d. Figure 3a shows the DNA distribution of a whole testis cell suspension stained nonviably with propidium iodide. Gaussian distributions are seen for cells with haploid (1C), diploid (2C) and tetraploid (4C) DNA content, corresponding to late, early and intermediate stages of spermatogenesis, respectively. Also seen is evidence of multinucleated artifacts (symplasts) of the dispersal procedure having 2-8 nuclei in a single cell body (3C distribution). Figure 3b shows the DNA distribution for gradient fraction 15 enriched in spermatocytes, as indicated by the large 4C Gaussian. The large number of 1C particles are due to spermatid symplasts which have been ruptured by the staining procedure to yield single nuclei. Figure 3c shows the degree of enrichment achieved by electronically sorting fraction 15 on the basis of light scatter intensities characteristic of pachytene spermatocytes. These cells are ca. 90% pachytene primary spermatocytes with ca. 10% contamination with spermatid symplasts. Figure 3d shows the Hoechst dye-DNA fluorescence of fraction 15 stained viably and analyzed with light scatter gates which limit the analysis to viable cells of size similar to spermatocytes. By sorting the cells from the 4C peak, a homogenous preparation of pachytene spermatocytes (99+%) was obtained. Similar procedures resulted in preparations of 100% round spermatids, 90% early cells and 90% Leydig cells.

Table II shows the average fatty acid distributions for progressive stages of spermatogenesis, early cells (EC) → pachytene primary spermatocytes (1°) → round spermatids (RS)

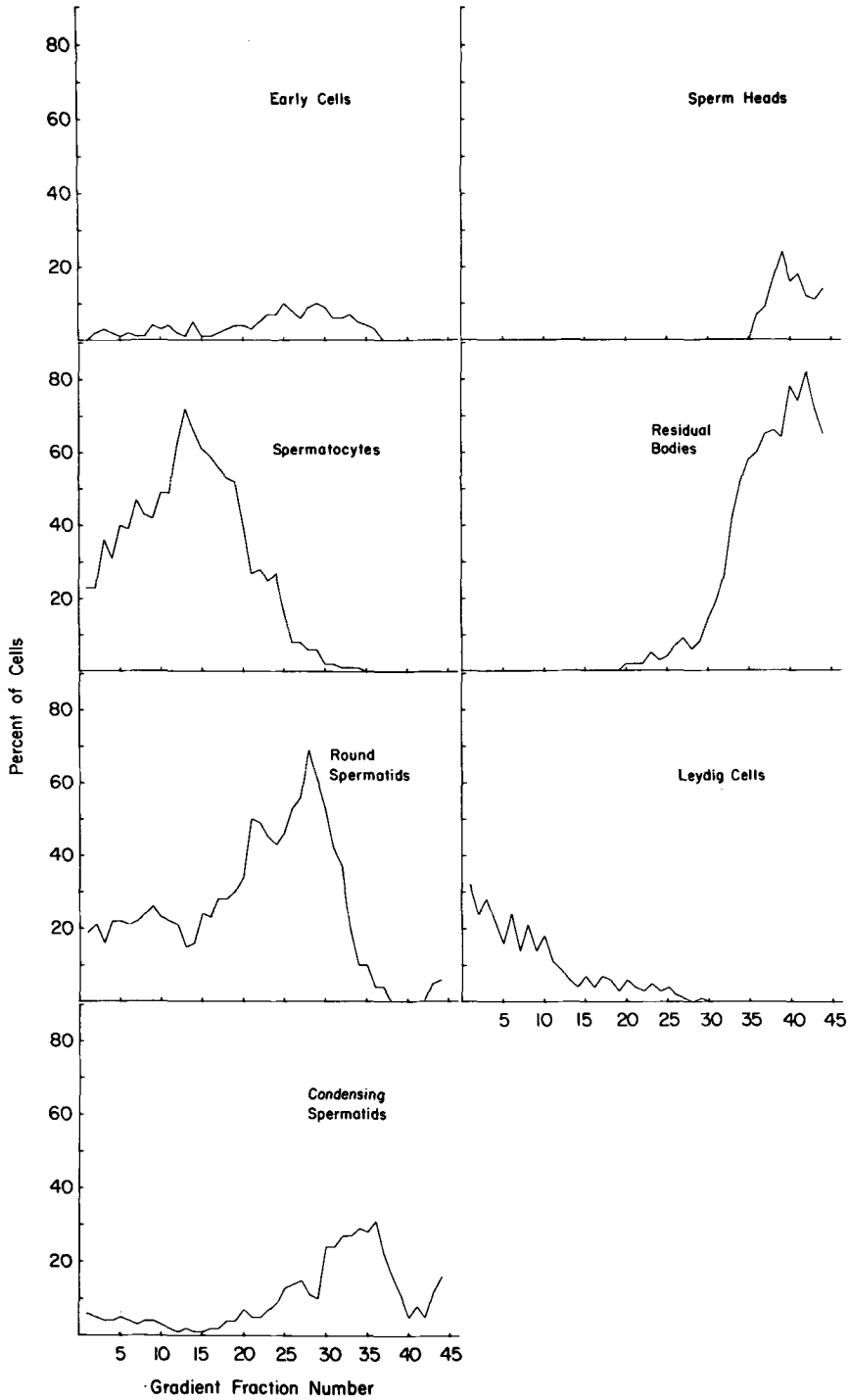

FIG. 1. Distributions of testis cell types through 44 fractions of a Staput 1 G sedimentation. (Fraction 1 is the bottom of the density gradient. Cell types comprising less than 5% in all gradient fractions are not shown but are included in calculations. Early cells = spermatogonia + preleptotene spermatocytes.)

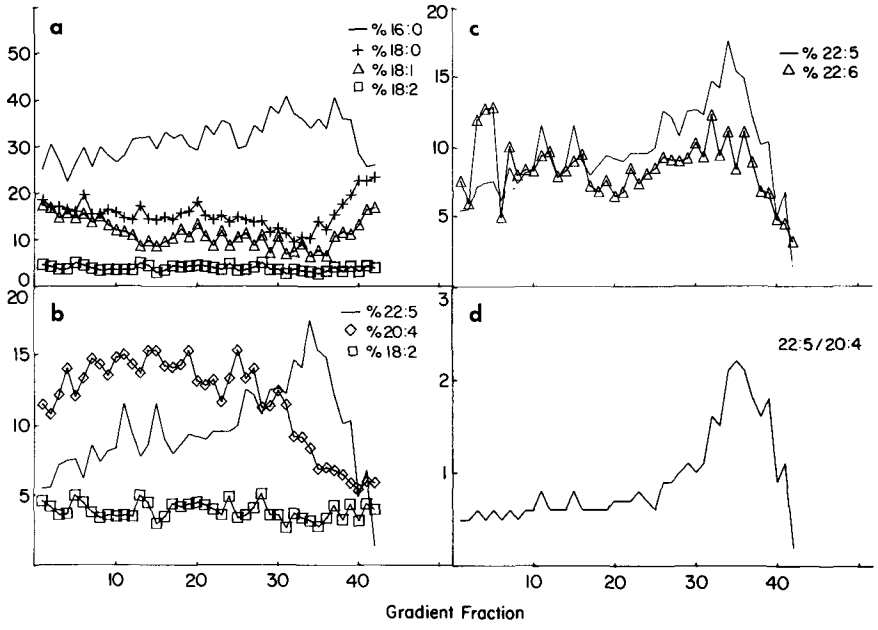

FIG. 2. Distributions of various fatty acids through Staput gradient fractions enriched in various testis cell types. (a) 16- and 18-carbon fatty acids. (b) All fatty acids of linoleic acid (n-6) family. (c) 22 carbon fatty acids of linoleic (n-6) and linolenic (n-3) acid families. (d) Ratio of 22:5(n-6) to the precursor 20:4(n-6).

→ condensing spermatids (CS) enriched by microflow sorting of Staput fractions. Included for comparison are epididymal sperm, anuclear residual bodies, which separate from spermatogonia during latter stages of differentiation and the nongerminal Leydig cells.

As with the Staput fractions, there are numerous small but significant differences in fatty acid composition between different enriched cell types. Here, too, the greatest variations are in 20:4 and 22:5. The 20:4 drops progressively 40% in the progression of differentiation EC → CS and 22:5 rises progressively 350% in the interval EC → RS, and apparently continues to rise in CS although variability of the cell composition in these samples prevented statistical significance. One sort containing 85% CS did, in fact, contain 20% 22:5 (see Table III), 10X the percentage of 22:5 in EC. We have not yet obtained a substantially homogeneous sort of testicular sperm, however, the fatty acid composition of epididymal sperm is included in Table II for comparison.

As in the case of Staput fractions, 22:5/20:4 values generally reflect the aforementioned changes in these fatty acids and tend to sharpen the difference between cell types in the sequence EC → 1° → RS → CS. Highly enriched (92%) Leydig cells have a fatty acid composition which is unique and different from the other cell types, but quite similar to EC with respect to 20:4, 22:5 and 22:5/20:4.

Fatty Acid Compositions of Pure Cell Types

Since there are highly significant differences in fatty acid compositions of several of the testis cell types and fatty acid compositional data are available from mixtures and highly enriched preparations of EC, 1°, RS, CS, RB, spermatozoa and Leydigs, multiple linear regression equations of the form shown in Table III have been constructed which predict the fatty acid compositions of various mixtures of cell types. The equations can also predict the fatty acid composition of a population 100% homogeneous for a given cell type. All equations used had an R-square greater than 0.99 and were quite accurate in predicting the fatty acid compositions of mixtures of cells from Staput gradients or sorts. Fatty acid compositions calculated for 100% homogeneous cell types are shown in Table III in comparison with values obtained from the most highly enriched sorted preparations. Predicted values are generally quite close to values actually observed in homogeneous sorts. Not surprisingly, the equations predict the progressive decrease in 20:4 and increase in 22:5 and 22:5/20:4 with

progressive stages of differentiation. Additionally, however, the contribution of 22:5 by RB to a mixture of cells is negligible and the contribution of CS is quite large, in agreement with observations made earlier regarding contributions of CS to 22:5 and 22:5/20:4 values in Staput fractions and sorted cells. Although testicular sperm have not been highly enriched, equations were able to predict accurately the fatty acid composition of Staput fractions enriched in sperm heads.

Fatty Acids of Lipid Classes

Table IV gives the fatty acid compositions of phospholipids (PL), triglycerides (TG) and cholesteryl esters (CE) from whole testis (WT), and pooled Staput fractions enriched in spermatocytes (1°), spermatids (RS) and late stages of differentiation, respectively. Percentages of 16:0 were higher in PL than in TG in all cases, significantly so in the case of WT ($p<.005$), 1° ($p<.02$) and RS ($p<.02$). Differences in 16:0 between cell populations were not significant. A trend toward higher percentage of 18:1 in TG than in PL and still higher percentages in CE was seen in all cell populations and was statistically significant or highly significant in all comparisons except CE vs TG of RS. The 20:4 percentages were 2-4 times higher in PL than in TG in WT ($p<.005$), 1° ($p<.001$), RS ($p<.001$) and late stages ($p<.01$).

In general, similar significant differences in fatty acid composition were observed between lipid classes but not between different cell populations, except in the cases of 20:4 and 22:5. The 20:4 percentages in total lipid and phospholipid follow the expected trend of decreasing with increasing stage of differentiation of the predominant cell types present ($p<.02$ for 1° vs RS and RS vs late stages). As expected, 22:5 levels were higher in total lipid and PL of RS and late stages than in PL of 1°, but differences were only significant at $p<.05$ in total lipid. The 22:5/20:4, on the other hand, was 50% higher in PL of RS than in PL of 1° ($p<.05$) and 78% higher in PL of late stages than PL of RS ($p<.005$).

The most notable differences, however, were found in TG. 20:4 was 60% lower in TG of late stages than in TG of RS ($p<.02$), whereas 22:5 was 200% higher in TG of late stages ($p<.02$). The corresponding increase in 22:5/20:4 of 1.4 in TG of RS to 10.6 in TG of late stages is 650% ($p<.02$).

Estimates of lipid class composition from quantitative GLC of component fatty acids are 84% PL, 12% TG, and 4% CE (duplicates) for both 1° spermatocytes and RS, and 70% PL, 21% TG, and 9% CE for late stages.

TABLE I

Fatty Acid Composition of Staput Fractions Enriched in Various Testis Cell Types

Fractions inclusive[a]	Enriched in	% Fatty acid[b]							
		16:0	18:0	18:1	18:2	20:4	22:5	22:6	22:5/20:4
12-18	1° Spermatocytes (61%)	32 ± 0.7	10 ± 0.5	15 ± 0.6	4 ± 0.2	15 ± 0.4	8 ± 0.4	8 ± 0.6	0.6 ± 0.02
23-30	Round spermatids (58%)	37 ± 1.2[c]	8 ± 0.8[e]	12 ± 0.8[d]	4 ± 0.5	11 ± 0.5[c]	12 ± 0.6[c]	10 ± 0.6[e]	1.2 ± 0.1[c]
31-36	Condensing spermatids (28%) + residuals (42%)	35 ± 0.8[g]	8 ± 0.7	12 ± 1.4	3 ± 0.3[g]	8 ± 0.4[c]	15 ± 1.1[e]	10 ± 0.9	1.9 ± 0.2[d]
39-42	Residual bodies (75%)	30 ± 3.3	14 ± 1.7[d]	22 ± 4.7	4 ± 1.0	6 ± 0.5[f]	6 ± 1.6[c]	5 ± 1.1[d]	1.2 ± 0.3

[a]See Fig. 1. for cell composition of gradient fractions.
[b]Mean values for fractions inclusive from 4 different gradients (% of total fatty acid ± SEM).
[c]Different from value immediately above ($p<.001$).
[d]Different from value immediately above ($p<.005$).
[e]Different from value immediately above ($p<.05$).
[f]Different from value for fractions 12-18 ($p<.001$).
[g]Different from value for fractions 12-18 ($p<.01$).

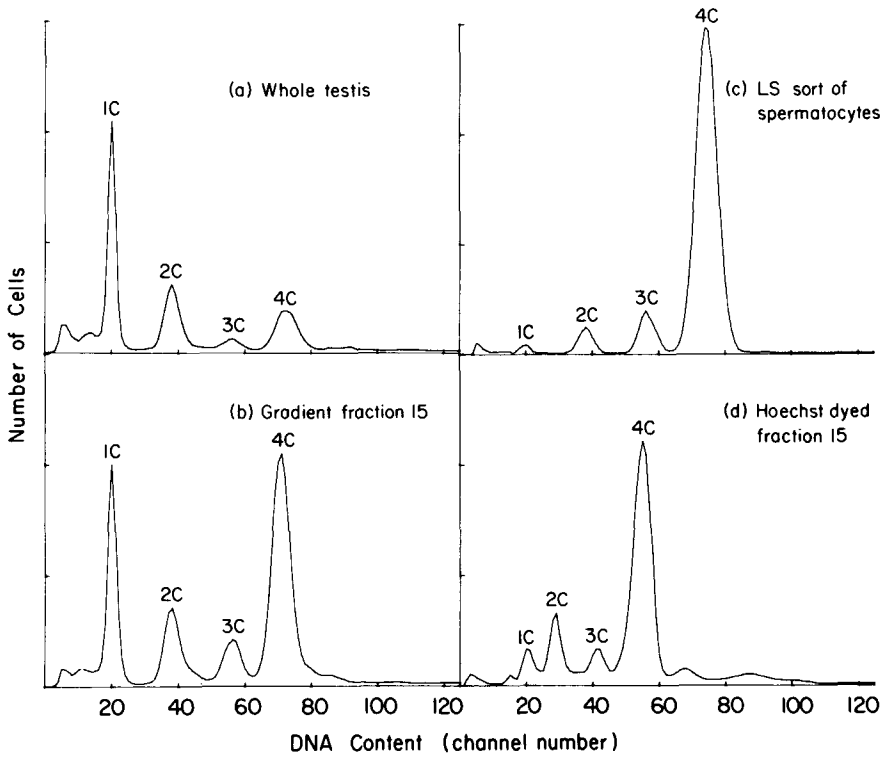

FIG. 3. Illustration of representative isolation of ultrapure testis cell types by Staput gradient and 2-parameter cell sorting. (a) Propidium-DNA fluorescence of whole testis cell suspension. (b) Propidium-DNA fluorescence of gradient fraction enriched to 70% in pachytene spermatocytes. (c) Propidium-DNA fluorescence of same gradient fraction sorted to 90% pachytene spermatocytes on the basis of light scatter. (d) Hoechst dye-DNA fluorescence with light scatter gate–used to sort 99+% pachytene spermatocytes. Cell compositions determined by light microscopy. 1C = 1/2 DNA content of diploid cell.

Phospholipid Classes

Phospholipid class composition was measured in pooled Staput fractions enriched in spermatocytes, spermatids and late stages. Percentage of component phospholipids did not differ significantly with respect to Staput fractions analyzed, nor was any enriched pool significantly different from whole testis in phospholipid composition (see Table V).

DISCUSSION

These studies with highly enriched testis cell types clearly establish a distribution of LCPA within the differentiation train of the adult mouse testis, which is highly cell specific and unique among the fatty acids found in testis. Measurement of fatty acids in 90% early spermatogenic stages represents the first reported study utilizing highly enriched early cells (EC) from normal adult testis. The level of 22:5 (2%) in these cells is not different from that of nongerminal Leydig cells (Table II). The 22:5 is 3-fold, 4-fold and 10-fold higher, respectively, in highly enriched pachytene spermatocytes (1°), round spermatids (RS), and condensing spermatids (CS), the successive stages of spermatogenesis. These findings are supported by fatty acid compositions of less highly enriched Staput gradient fractions (Table I), and by extrapolations to 100% cell homogeneity with multiple linear regression analysis (Table III). 22:6, which has been associated with maturation of human testis (15), showed no statistically significant differences with respect to any cell type in sorted cells or Staput fractions, although 22:6 percentages were quite high in the CS and spermatozoa of highest homogeneity. These findings suggest separate pathways and regulatory mechanisms for metabolism, and/or transport of these fatty acids, as well as the possibility of separate functions, despite the observation that biosynthesis of 22:6(n-3) could probably proceed with the same sequence of reactions used in

TABLE II

Fatty Acid Composition of Sorted Testis Cell Types[a]

Cell type (N)	% Fatty acid composition[b]								
	16:0	18:0	18:1	18:2	20:4	22:5	22:6	22:5/20:4	Homogeneity (% cells)
Early (4)	32 ± 3	20 ± 1 d,l g,i	20 ± 3	4 ± 0.3 e,i f,l g,l h,i	10 ± 0.6 e,j f,l	2 ± 0.5 d,l e,k g,m	4 ± 0.3	0.2 ± .05 d,l e,m f,i	92 ± 4
1° Spermatocytes (8)	39 ± 3 h,k	14 ± 1 c,l e,j h,j	17 ± 2 h,i	3 ± 0.4 f,i g,i	9 ± 1.0 f,j	6 ± 0.8 c,l e,i h,l	4 ± 1.2	0.7 ± .10 c,l e,l h,l	82 ± 4
Round spermatids (6)	34 ± 2 h,i	20 ± 2 d,j	18 ± 2	3 ± 0.2 c,i f,l g,l	7 ± 0.8 c,j	9 ± 1.0 c,k d,i h,m	5 ± 0.4 g,i	1.2 ± .08 c,m d,l h,m	80 ± 5
Condensing spermatids (3)	33 ± 4	16 ± 4	14 ± 2 h,i	2 ± 0 c,l d,i e,l h,m	6 ± 0.3 c,l d,j	12 ± 4.1	8 ± 2.8	2.0 ± .60 c,i h,i	58 ± 13
Epididymal sperm (1)	24	15	24	11	7	8	9	1.2	95
Residual bodies (3)	34 ± 3	16 ± 1 c,i	18 ± 2	2 ± 0.1 c,l d,i e,l h,m	7 ± 1.9	7 ± 0.4 c,m h,l g,l	3 ± 0.6 e,i	1.0 ± .40	57 ± 1
Leydig cells (2)	22 ± 4 d,k e,i	26 ± 4 d,j	23 ± 1 d,i f,i	3 ± 0 c,i f,m g,m	12 ± 2.0	2 ± 0 d,l e,m	4 ± 0.5	0.2 ± .05 d,l e,m f,i	92 ± 0

[a]All values are ± SEM. Pairs of superscripts refer to footnotes giving statistical differences and confidence levels.

[b]Balance of 100% composed of fatty acids present in amounts less than 2%.

[c]Different from early cells (spermatogonia + preleptotene spermatocytes).

[d]Different from primary spermatocytes.

[e]Different from round spermatids.

[f]Different from condensing spermatids.

[g]Different from residual bodies.

[h]Different from Leydig cells.

[i]$p<.05$.

[j]$p<.02$.

[k]$p<.01$.

[l]$p<.005$.

[m]$p<.001$.

TABLE III

Fatty Acid Composition for Testis Cell Types Calculated by Multiple Linear Regression Analysis of Data from Sorted Cells[a]

Cell type	% Fatty acid composition							Highest homogeneity (%)
	16:0	18:0	18:1	18:2	20:4	22:5	22:6	22:5/20:4	
E	32 (32)	20 (20)	20 (20)	4 (4)	10 (10)	2 (2)	4 (4)	0.1 (0.2)	92
1°	40 (54)	12 (13)	17 (12)	3 (2)	9 (8)	5 (4)	4 (2)	0.6 (0.5)	100
RS	33 (41)	21 (17)	19 (15)	3 (3)	7 (7)	9 (10)	5 (4)	1.1 (1.4)	100
CS	33 (26)	15 (9)	9 (10)	1 (2)	5 (6)	17 (20)	12 (14)	3.0 (3.3)	85
Sperm	24 (24)	15 (15)	24 (24)	11 (11)	7 (7)	8 (8)	9 (9)	1.2 (1.2)	95
RB[b]	35	15	22	2	8	2	0	0.2	
Leydig	21 (22)	27 (26)	24 (23)	3 (3)	12 (12)	1 (2)	4 (4)	.05 (0.2)	92

[a]Number in parentheses is value for sample or samples having highest homogeneity for the cell type in question as given in the last column. See Table II for homogeneity and actual fatty acid composition of these samples. Form of regression equations was $y = AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 + GX_7 + K$ where y = % fatty acid or 22:5/20:4, $X_1, X_2, \ldots X_7$ = % of each cell type, A, B, . . . G = calculated coefficients, and K = a constant. R-square was greater than 0.99 in every case.

[b]Highest homogeneity was only 60%.

biosynthesis of 22:5(n-6) (3). These pathways may, in fact, share enzymatic activities but still be localized to specific cell types (16) by substrate gradients.

A decrease in 20:4 concomitant with increasing 22:5, which may reflect conversion of 20:4 to 22:5, results in a 10- to 15-fold increase in 22:5/20:4 in the sequence of stages, EC → 1° → RS → CS. However, Beckman and Coniglio have suggested that 22:5 is synthesized in Sertoli cells in the rat and that triglycerides may serve as a vehicle for transport of 22:5 from Sertoli cells to germinal cells where this fatty acid is then incorporated into phospholipid (16). Consistent with this suggestion is our finding of 22:5 predominantly located in triglycerides in later stages of spermatogenesis whereas 20:4 and 22:6 were found mainly in phospholipid in all cell types. Accumulation of 22:5-rich triglyceride in CS may be the result of cessation of net incorporation into phospholipid at the RS stage (Table IV). On the other hand, our estimates of 4 pg fatty acid/cell for CS and 8 pg fatty acid/cell for RS, which are consistent with their respective cellular volumes, suggest net loss of lipid, which could take place in the form of triglyceride.

Testing of these alternative hypotheses will require metabolic and other dynamic studies with the various enriched testis cell types, including Sertoli cells, which so far have not survived cell sorting with cytoplasm intact. Such studies will necessitate highly sensitive, specific, and sometimes unconventional analytical approaches, since the standard techniques of lipid biochemistry are often inadequate for dealing with the small cell masses available from the cell sorter.

ACKNOWLEDGMENTS

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TABLE IV

Fatty Acid Composition of Lipid Classes from Cells Enriched by Density Gradient Sedimentation

	% Fatty acid (± standard error of mean)							
% Total lipid	16:0	18:0	18:1	18:2	20:4	22:5	22:6	22:5/ 20:4
Whole testis (N)	30 ± 1	11 ± 0.2	17 ± 1	4 ± 1	11 ± 0.4	9 ± 0.4	9 ± 0.3	0.8
PL (6)	39 ± 2	12 ± 2	16 ± 2	3 ± 0.3	13 ± 2	9 ± 0.6	4 ± 0.5	0.7
TG (4)	29 ± 1	9 ± 0.4	26 ± 2	5 ± 0.5	4 ± 0.2	8 ± 0.9	4 ± 0.7	2.0
CE (5)	18 ± 2	11 ± 0.5	41 ± 2	2 ± 0.3	6 ± 2	–	2 ± 0.6	–
Spermatocytes[a]	32 ± 0.4	10 ± 0.4	15 ± 0.4	4 ± 0.2	15 ± 0.2	9 ± 0.4	8 ± 0.4	0.6
PL (4)	37 ± 3	10 ± 0.7	14 ± 2	4 ± 0.4	17 ± 1	9 ± 2	6 ± 1	0.5
TG (4)	26 ± 2	9 ± 0.8	25 ± 0.8	4 ± 0.5	6 ± 0.8	5 ± 0.8	9 ± 2	0.8
CE (5)	25 ± 3	16 ± 1.0	32 ± 3	3 ± 1	2 ± 1	1 ± 0.6	1 ± 1	0.5
Spermatids[b]	36 ± 1	9 ± 0.7	12 ± 0.7	4 ± 0.3	12 ± 0.6	13 ± 0.4	10 ± 0.4	1.0
PL (4)	40 ± 3	9 ± 1	11 ± 1	4 ± 0.2	13 ± 0.6	11 ± 1	8 ± 2	0.8
TG (4)	28 ± 2	7 ± 0.5	23 ± 2	4 ± 0.5	5 ± 0.3	7 ± 3	6 ± 2	1.4
CE (5)	27 ± 6	17 ± 4	29 ± 3	2 ± 1	6 ± 5	trace	1 ± 1	–
Late stages[c]	36 ± 1	9 ± 1	15 ± 1	4 ± 0.3	7 ± 0.3	13 ± 1	9 ± 0.8	1.9
PL (3)	45 ± 5	10 ± 1	11 ± 1	3 ± 0.4	8 ± 1	12 ± 1	6 ± 3	1.5
TG (3)	31 ± 2	6 ± 0.4	20 ± 1	3 ± 0.5	2 ± 0.6	21 ± 2	3 ± 1	10.6
CE (2)	31 ± 3	17 ± 0.2	30 ± 0.6	2 ± 2	8 ± 4	1 ± 1	1 ± 1	0.1

[a]60% Pachytene spermatocytes, 23% round spermatids, 6% Leydigs (Staput fractions 12-19).

[b]53% Round spermatids, 18% condensing spermatids, 13% residual bodies, 8% early cells (Staput fractions 26-32).

[c]60% Residual bodies, 23% condensing spermatids, 10% sperm heads (Staput fractions 34-39).

TABLE V

Phospholipid Classes of Mouse Testis Cells[a]

	%P
Phosphatidylcholine	49.6 ± 1.5
Phosphatidylethanolamine	23.6 ± 1.8
Phosphatidylinositol	7.2 ± 1.0
Phosphatidylserine	5.2 ± 1.4
Sphingomyelin	7.8 ± 1.1
Cardiolipin	7.2 ± 1.4

[a]Phospholipid class compositions of Staput gradient fractions enriched in 1° spermatocytes, round spermatids and residual bodies (see Table IV) were analyzed by Student t-test and found not to differ from each other or composition of whole mouse testis. Values are ± SEM.

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Increased Proportion of Medium Chain Fatty Acids in Nystatin-Resistant Yeast Mutants

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ABSTRACT

Fatty acid composition of phospholipids and steryl esters from four nystatin-resistant mutants of *Saccharomyces cerevisiae* was compared to that from the wild strain. All the mutant strains which produce several ergosterol intermediates incorporated two- to three-fold as much medium chain fatty acids, especially 14:0 and 14:1 in phospholipids, and 12:0, 14:0 and 14:1 in steryl esters as the wild strain did. The increase in the relative amount of medium chain fatty acids in these mutants was found at all the growth temperatures and the growth phases examined, and in all the phospholipid species.

INTRODUCTION

When one of the enzyme activities in the ergosterol biosynthesis in yeasts or fungi is deleted by mutation, the mutants produce ergosterol intermediates and incorporate them into membranes. They are usually more resistant to nystatin, a polyene antibiotic, which makes a complex with ergosterol in membranes with stronger affinity than to other sterols (1-3). Such yeast mutants have been isolated in several laboratories and used for elucidation of the biosynthetic pathway of ergosterol (4-7). Since sterol is believed to play its role in biomembranes of eukaryotes in contact with fatty acid residues of phospholipids, it seems reasonable to suppose that the fatty acid composition of complex lipids in such mutants might be different from that of the wild strain to maintain the physicochemical properties of the membrane with altered sterol structure.

We have isolated several nystatin-resistant yeast mutants and characterized the point of enzyme defect in some of the mutants (8). In this paper, we report the observed increase in the proportion of medium chain fatty acids in these mutants.

MATERIALS AND METHODS

Growth of Cells

Nystatin-resistant mutants were isolated after UV-irradiation of the haploid type *Saccharomyces cerevisiae* strain M10 (mating type a, methionineless, petite) by selecting for resistant mutants on nystatin-containing agar plates (8). Probable enzyme lesions of the four mutants used in this experiment are as listed in Table I. They were characterized by the UV-absorption spectra, gas liquid chromatograms of their sterols and by the incorporation of the radioactivity of [methyl-^{14}C]methionine into sterols as reported before (8). The stock cultures were recultured at least every 3 months. Nystatin resistance and sterol patterns of the mutants were sometimes checked. No contamination or reversion was observed for more than 3 yr.

Cells were grown on 1 L of a synthetic medium composed of 3% glucose, 0.2% NH_4Cl, 0.3% KH_2PO_4, 0.1% $MgCl_2 \cdot 6H_2O$, 0.025% $CaCl_2 \cdot 2H_2O$, 0.003% methionine, vitamins and trace metals (9) in 2-L Erlenmeyer flasks at 30 C (if not specified) without shaking. The cell growth was monitored by measuring absorbance at 660 nm and the cells were harvested at late exponential or early stationary growth phase.

Preparation of Membranes

The harvested cells were washed twice with water and once with an ice-cold solution of osmotic stabilizer (0.4 M HCl and 20 mM triethanolamine, pH 7.0). The yeast cell pellet was mixed and ground with 2 times of its weight of glass beads (0.25-0.30 mm diameter, Braun Melsunger, G.F.R.) in a morter on ice for 10 min. After grinding, 2 vol of ice-cold osmotic stabilizer solution was added and the crude plasma membrane fraction was prepared by a series of differential centrifugations according to the method of Fuhrmann et al. (10). Since all the mutants, as well as the wild strain, are petite and we observed only a small protein peak in the mitochondrial fraction compared to the major protein peak of the plasma membrane in a density gradient centrifugation, we omitted the last step. The supernatant of the first centrigugation at 5,000 G for 10 min to precipitate plasma membrane was centrifuged again at 10,000 G for 10 min. Then, microsomes were precipitated from the resultant supernatant at 105,000 G for 1 hr.

Analytical Methods

Lipids from membrane fractions or, in some experiments, from the crude cell homogenates

TABLE I

Nystatin-Resistant Mutants Used in the Experiments[a]

Strain	Nystatin resistance	Conjugated double bonds		Probable enzyme lesion
		$\Delta^{5,7}$	$\Delta^{22,24}$	
M10	<20 U/ml	+	-	wild strain
N3	20	-	-	demethylation?
N15	80	-	-	$\Delta 8 \rightarrow \Delta 7$ isomerization
N22	40	+	-	22(23) desaturation
N26	40	+	+	C-24 transmethylation

[a]Results are taken from ref. 8.

were extracted with chloroform/methanol. Suspended membrane preparation was stirred with 2.5 vol of methanol and 1.5 vol of chloroform for 10 min at room temperature. Then, 1.25 vol of chloroform and 2 min later, 1.25 vol of H_2O was added and stirred for another 2 min. After centrifugation, the chloroform phase was separated, brought to dryness in vacuo and the lipids were separated on a silicic acid column (Wakogel C200) into sterol esters (eluted with 5% ether in hexane), free sterol and free fatty acids (eluted with ether) and total phospholipids (eluted with methanol). In some cases, individual phospholipids were separated on thin layer plates (Merck, Kieselgel 60 F_{254}) with a mixture of chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v) as a developing solvent. Sterol esters were hydrolyzed in alkaline methanol and fatty acids were extracted from the acidified hydrolyzate in ether, dried and then converted to their methyl esters by heating at 60 C for 20 min in the presence of 14% BF_3 in methanol. Fatty acids in the phospholipid fraction were transmethylated by treating phospholipids for 2 hr at 70 C with 5% H_2SO_4 in methanol. The fatty acid methyl esters were extracted into hexane and analyzed routinely by a Shimadzu GC-6A gas liquid chromatograph equipped with a flame ionization detector. A 1.5-m glass column (4 mm diameter) packed with 15% DEGS absorbed on 60-80 mesh Neopakia (Nishio Industry Co., Tokyo) was used isothermally at 170 C with flow rate of nitrogen carrier gas at 50 mL/min. The chromatograms were quantitated by triangulation, and the fatty acid concentrations expressed as percentages of the fatty acid methyl esters by weight. Some samples were applied to a 2 m x 3 mm glass column containing 1.5% DV-1 on Uniport HP (60-80 mesh) and were chromatographed at 155 or 270 C with helium as a carrier gas at a flow rate of 30 mL/min. Mass spectra were taken every 5.0 S with a combined gas chromatography-mass spectrometry (GC-MS) instrument, GC-MS 9000 S (Shimadzu-LKB, Kyoto) with an ionization current of 60 μA, electron accelerating voltage of 70 eV and an ion-source temperature of 270 C.

RESULTS

Fatty Acid Composition of Phospholipids and Steryl Esters in Plasma Membrane

Fatty acid compositions of total phospholipids and of steryl esters in the crude plasma membranes from the nystatin-resistant and the wild strains were determined as shown in Table II. There was no dramatic change in long chain fatty acids, but increasing tendency of the relative amount of medium chain fatty acids (12:0, 14:0 and 14:1) both in phospholipids and in steryl esters from all of the nystatin-resistant mutants can be seen. The relative amount of medium chain fatty acids in steryl esters from some of the mutant was as high as 25%. The relative amount of 14:1 obtained from phospholipids was four- to five-fold higher in N3 and N15 than in the wild strain. In the mutants which produce sterols with conjugated double bonds (N22 and N26), the increase of 14:1 was less prominent than in N3 and N15. According to the increase of medium chain fatty acids, relative amounts of 16:1 and 18:1 decreased in the mutants. On the other hand, the increases in the relative amounts of 18:0 in phospholipids and 16:0 in steryl esters in some of the mutants were observed. Therefore, the SFA/USFA ratios in phospholipids and steryl esters were higher in most of the mutants than those in the wild strain. Confirmation of the peaks in gas chromatography as fatty acid methyl esters from 12:0 through 18:1 was done by GC-MS (data not shown).

Since the increase in the proportion of medium chain fatty acids in nystatin-resistant mutants was similarly observed in phospholipids and steryl esters from crude cell homogenates, further experiments were carried out

TABLE II

Fatty Acid Composition of Phospholipids and Steryl Esters in Plasma Membrane[a]

Fatty acid	Composition in phospholipids (%)					Composition in steryl esters (%)				
	M10	N3	N15	N22	N26	M10	N3	N15	N22	N26
12:0	0.7	0.8	1.4	1.4	1.2	4.2	10.0	7.9	8.7	14.5
14:0	1.8	3.8	3.5	2.1	2.7	3.2	5.1	6.4	3.6	6.1
14:1	0.5	2.2	2.1	1.2	1.1	2.0	8.6	7.6	3.9	4.6
16:0	24.6	32.9	27.1	24.0	22.2	9.9	9.4	10.8	13.0	16.5
16:1	43.6	41.2	37.4	48.0	41.0	56.6	48.3	49.5	49.3	40.2
18:0	5.9	6.4	9.1	5.8	11.1	5.4	4.0	5.2	4.4	5.3
18:1	23.0	21.6	19.5	17.4	20.8	18.8	14.6	12.6	17.2	12.9
≤14:1	3.0	6.8	7.0	4.7	5.0	9.4	23.7	21.9	16.2	25.2
SFA/USFA	0.490	0.538	0.695	0.502	0.590	0.292	0.399	0.435	0.420	0.733

[a]Results are the mean of 2 determinations by GLC. The experimental error in the fatty acid analyses was within 10%. In this experiment, 10:0 and 10:1 were not determined.

with microsomes or crude cell homogenates. As shown in the next section, however, the relative amounts of medium chain fatty acids in the lipids from crude cell homogenates and microsomes were higher even in the wild strain than those observed in plasma membranes.

Changes of Fatty Acid Composition with Cell Growth

The mutant cells proliferated almost as fast as the wild strain, but maximal cell growth attained by the mutant strains was less than that of the wild strain by about 30% (data not shown). Fatty acid compositions of phospholipids and steryl esters from crude cell homogenates of N15 and of the wild strain were determined at several stages of cell growth.

The relative amounts of total medium chain fatty acids in phospholipids from the wild and the mutant strains were higher during late exponential growth phase, and then decreased with time (Fig. 1). Among the medium chain fatty acids, only the levels of 10:0 and 14:0 decreased with culture time and those of 12:0 and 14:1 remained constant. The level of total medium chain fatty acids and of 14:1 in the mutant N15 was about two- and three-fold higher than that in the wild strain throughout the growth periods, respectively. In spite of the decreasing proportion of total medium chain fatty acids during stationary phase, the percentages of 16:1 and 18:1 in phospholipids remained almost constant except that the level of 16:1 in the wild strain at the late stationary growth phase was slightly higher.

On the other hand, the proportion of the total medium chain fatty acids in steryl esters remained constant in the wild strain whereas it increased in the mutant strain with culture period (Fig. 2). Only 14:0 decreased as it did in phospholipids when the mutant cells entered into stationary phase. In the stationary phase cells, 10:1 was observed in the steryl esters from both strains. The confirmation of shorter chain fatty acids (10:0 and 10:1) was impossible by GC-MS as they were overlapped by the solvent peak, and they were tentatively assigned

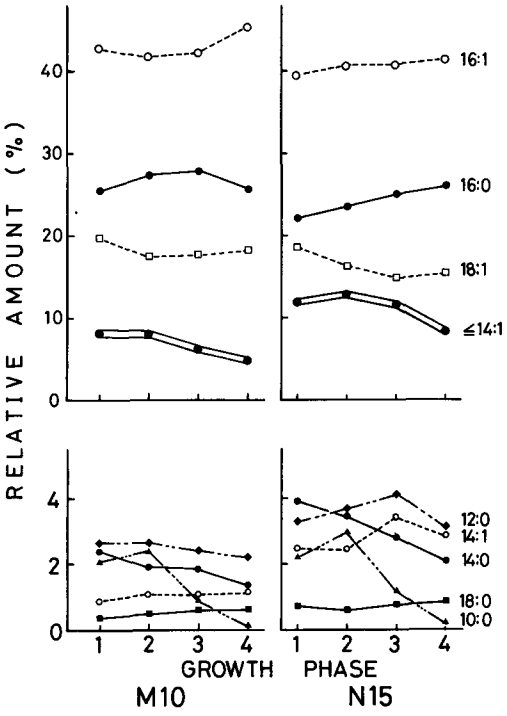

FIG. 1. Change in fatty acid compositions in phospholipids during the cultivation. Growth phase 1: late exponential, after 24 hr incubation; phase 2: early stationary, 31-34 hr; phase 3: middle stationary, 47-51 hr; phase 4: late stationary, 73-78 hr.

by their relative retention times on a DEGS column. The relative amounts of 16:1 and 18:1 in the mutant were less than those in the wild strain. The increase of 14:1 with time shows a contrast to the decrease of 14:0 and seems to suggest the increasing desaturation of fatty acids in the mutant. However, the percentage of 16:1 in the mutant was somewhat lower during the stationary phase than at the late exponential growth phase and the relative amounts of 16:0, 18:0 and 18:1 remained unchanged (Fig. 2).

Fatty Acid Composition and Growth Temperature

It is well known that the degree of unsaturation of fatty acids in phospholipids in the membrane from *Escherichia coli* grown at lower temperature is high (11). Similar results have been reported for *S. cerevisiae* and *Candida lipolytica* (12). It is also reported that medium chain fatty acids are incorporated into the position 2 of glycerophospholipids in place of unsaturated fatty acids when yeast cells are grown under unsaturated fatty acid deficient conditions (13).

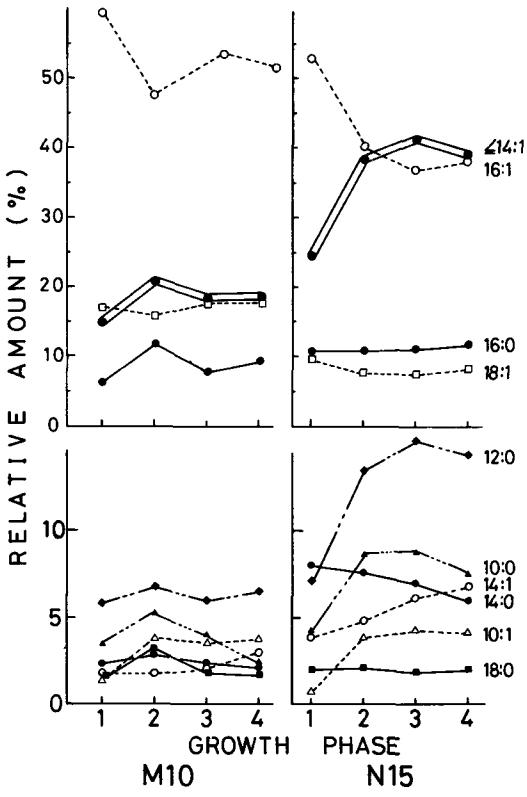

FIG. 2. Change in fatty acid compositions in steryl esters during the cultivation.

The nystatin-resistant mutants and the wild strain were, therefore, grown at 20, 30 and 40 C and the fatty acid compositions of phospholipids and steryl esters in microsomal fractions from the cells harvested at early stationary phase were determined to see if there is any correlation between the relative amount of medium chain fatty acids and the degree of unsaturation of C_{16} and C_{18} acids, the main fatty acids of the yeast.

The degree of unsaturation of C_{16} plus C_{18} (16:1 + 18:1/16:0 + 18:0) in phospholipids from the wild strain decreased with increasing growth temperature (3.6 at 20 C, 3.1 at 30 C and 1.8 at 40 C). Those in phospholipids from the mutants decreased likewise with increasing growth temperature. In the case of steryl esters, the ratio was higher at 20 C than at 30 C in all strains examined except N3. At 40 C, the fatty acids in the wild strain were more unsaturated whereas the mutant N3 and N22 showed lower degrees of unsaturation than at 30 C. A similar increase in total unsaturated fatty acids at temperatures above the growh optimum has been reported by Daum et al. (14). The proportion of medium chain fatty acids, on the other hand, was highest at 30 C in phospholipids and in steryl esters from all the strains as shown in Figure 3. The proportion was higher in the mutants than in th ewild strain except in th e case of N3 grown at 20 and 40 C.

Fatty acids in phospholipids and steryl esters from the mutants were less unsaturated and contained more medium chain fatty acids than those from the wild strain at all the growth temperatures examined, though there were some exceptions. Since the calculated correlation coefficients are r = -0.608 for fatty acids in steryl esters and r = -0.506 for those in phospholipids, the percentage of the medium chain fatty acids can be said to have a slight tendency to decrease with increasing degree of unsaturation of fatty acids. There was no correlation between the proportion of medium chain fatty acids and the C_{18}/C_{16} ratio (data not shown).

Fatty Acid Composition of Separated Phospholipids

Individual phospholipids from crude homogenates of the wild strain and of nystatin-resistant mutant N3 were separated on thin layer plates and their fatty acid compositions were analyzed (Table III). Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were well separated, but phosphatidylserine (PS) and phosphatidylinositol (PI) were not separated from each other in the system.

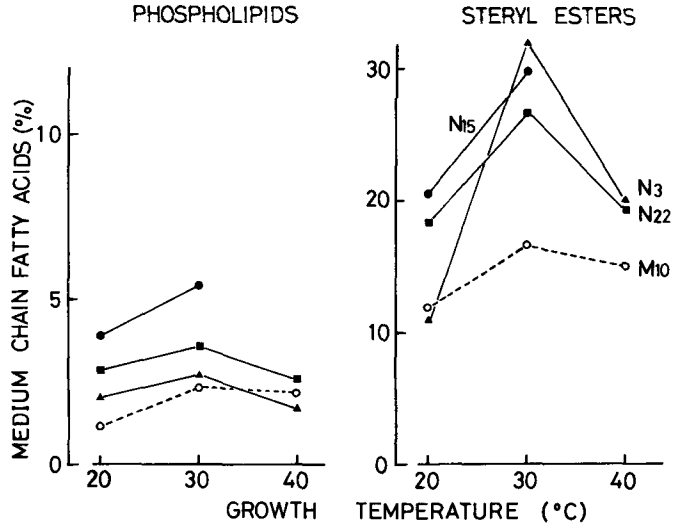

FIG. 3. Relative amount of medium chain fatty acids (≤14:1) from the wild and mutant cells grown at different temperatures.

In both strains, the fatty acids in PS + PI were less unsaturated and were composed of slightly higher level of medium chain fatty acids—especially with 10 and 12 carbon chains—than those in PE and in PC. In the mutant, increased levels of 14:0 and 14:1 acids which were already shown in Table II in total phospholipids, were seen in all the three phospholipid fractions. The percentages of C_{10} and C_{12} acids were nearly equal to those in the wild strain. Among the long chain fatty acids, the levels of 16:0 and 18:0 in PE and PC fractions were less than those of the wild strain, and the percentages of total unsaturated fatty acids increased slightly. In PC fraction, the relative amount of 16:1 was considerably higher in the mutant than that in the wild strain, but 18:1 was lower.

DISCUSSION

It has been reported that sterol depletion is accompanied by alteration in fatty acid composition of membrane phospholipids. Freter et al. reported an increase in the amount of 18:1 and a decrease in the amount of 16:0, especially in PC in the plasma membrane of sterol-depleted mutants of LM cells (15). On the other hand, Rottem et al. (16) found that the reduction in sterol content of *Mycoplasma mycoides* was accompanied by an increase in the saturated fatty acid content of the membrane phospholipids. Some nystatin-resistant strains of *Candida* become resistant against nystatin by producing less sterol (17), but it was also found that the total sterol concentration of most of nystatin-resistant yeast mutants previously reported was about the same as that of their wild-type sensitive progenitors. We have found as well that the sterol content in plasma membrane of the resistant yeast mutants examined was almost the same as that found in the wild strain (unpublished observations).

Mainly because of the difficulties in obtaining authentic standards, our study on the chemical structure of sterols in the nystatin-resistant mutants has not been completed. Nevertheless, we are certain that the mutant N26 cannot methylate sterol at C-24 and that the mutants N3 and N15 do not produce any sterol with conjugated double bonds (8). Methylation at C-24 in the isooctyl side chain of sterol can be regarded as having the same effect as unsaturation since branched chain iso- and anteiso-long chain fatty acids are present in place of unsaturated fatty acids in some microorganisms (18). Assuming that the chemical structure of the sterol is somewhat complementary with fatty acids in membrane phospholipids, one can expect more unsaturated fatty acids in these mutants than in the wild strain. However, we have found that the fatty acid composition in these sterol mutants showed an increase in the proportion of medium chain fatty acids in all the mutants examined, though there were some differences among the mutants in the relative degree of increase in 14:1. Lees et al. have mentioned without data that fatty acid compositions of nystatin-resistant mutants involving B-ring

TABLE III

Fatty Acid Compositions of Separated Phospholipids

Phospholipid	Strain	Fatty acid composition (%)									
		10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1	≤14:1	Total USFA
PE	M10	0.2	2.0	2.7	0.6	14.5	65.5	6.7	13.9	5.5	80.0
	N3	0.2	1.9	4.8	1.7	8.5	65.1	1.6	16.3	8.6	83.1
PC	M10	0.4	3.2	2.7	1.8	22.3	39.3	4.4	25.6	8.1	66.7
	N3	0.4	3.3	4.8	3.3	17.2	51.5	2.9	16.7	11.8	71.5
PS + PI	M10	1.1	5.6	1.2	1.6	32.0	31.0	12.0	15.4	9.5	48.0
	N3	1.1	6.1	2.2	2.8	30.3	26.4	14.7	16.4	12.2	45.6

$\Delta 8 \rightarrow \Delta 7$ isomerization and C-24 methylation were identical to that of the wild strain (19). We have no explanation for this discrepancy.

It has been reported that medium chain fatty acids are incorporated into glycerophospholipids when yeast cells are grown anaerobically on a medium without exogenous unsaturated fatty acids (13). It is also known that the relative amount of shorter chain fatty acid increases in in vitro experiments when the acetyl-CoA/malonyl-CoA ratio is high (20,21). We therefore tried to see if there is any correlation between the relative amount of medium chain fatty acids and the degree of unsaturation of fatty acids or the C_{18}/C_{16} ratio by growing the cells at various temperatures. However, the relative amount of total medium chain fatty acids correlated with the degree of unsaturation only slightly and did not correlate with the C_{18}/C_{16} ratio.

Recently, Buttke et al. reported (22) that a yeast mutant which requires exogenous sterol and unsaturated fatty acids for growth incorportaed less saturated fatty acids into phospholipids when the cells were grown in the presence of cholesterol or 7-dehydrocholesterol than when ergosterol or β-sitosterol was used as the sterol source, and that the lower saturated fatty acid content was most pronounced in phosphatidylethanolamine. Our data (Table III) indicated that such a phospholipid specificity was not evident for the increase in 14:0 and 14:1 content in the nystatin-resistant mutant. Furthermore, the higher content of medium chain fatty acids in the resistant mutants was more pronounced in steryl esters. The mole ratio of steryl ester to phospholipid in crude plasma membrane of the wild strain was 0.48 (unpublished data). Bailey and Parks also observed that 66% of the sterol in the cell wall (membranes) of yeast is esterified (23). However, the physiological significance of steryl esters in yeast cells is still obscure.

It is very interesting that Sobus et al. have already reported (24) a three-fold increase in the relative amount of medium chain fatty acids in steryl esters and in phospholipids from *S. cerevisiae* grown in the presence of trifluperidol which they found to inhibit the ergosterol biosynthesis at the step of sterol $\Delta 8 \rightarrow \Delta 7$ isomerization. They postulated that the drug might be interfering with fatty acid elongation by promoting the substrate to dissociate from the fatty acid synthetase complex before the longer chain acids are synthesized. One of our mutants, N15, is supposed to be genetically blocked at the $\Delta 8 \rightarrow \Delta 7$ isomerization step. Considering their finding together with ours, it might be probable that altered sterols in the mutants affect the properties of some membrane-bound enzymes which catalyze the synthesis of sterol ester and the transfer of fatty acid into phospholipids. In this regard, it is worthwhile to note that the change in fatty acid composition of *Tetrahymena*, caused by replacement of tetrahymanol with exogenously added ergosterol (25), has been partly explained by the loss of an enzyme activity which catalyzes fatty acid Δ^{12} desaturation by ergosterol (26).

Our results reported here clearly indicated that alteration of membrane sterol in nystatin-resistant yeast mutants is accompanied with changes in fatty acid composition of complex lipids. But further studies are necessary for understanding the physiological meaning of observed increase in medium chain fatty acids.

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Incorporation of [1-^{14}C] Acetate into Fatty Acids of the Crustaceans *Daphnia magna* and *Cyclops strenus* in Relation to Temperature

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ABSTRACT

Daphnia magna and *Cyclops strenus* were maintained in aquaria containing sodium [1-^{14}C] acetate and the effect of temperature on labeling of their lipids was investigated. Incorporation of radioactivity in total lipids was slowed by a factor of 4 in cold-exposed (5 C) specimens compared to those incubated at 25 C. There was no significant difference in the distribution of label in the lipid classes of animals incubated at the two extreme temperatures. Decrease of the temperature from 25 to 5 C brought about a considerable reduction in the formation of palmitic and stearic acids and an increase in labeling of monounsaturated (18:1) fatty acids in *D. magna*. Docosapolyenoic acids were absent from lipids of this crustacean. *C. strenus* directed a higher proportion of radioactivity into both oleic and docosahexaenoic acids upon cold exposure. In response to decrease of the temperature, *D. magna* formed a less unsaturated fatty acid population, as judged from dpm ratios of total saturated to total unsaturated fatty acids, than *C. strenus*. Inability to form and accumulate docosapolyenoic fatty acids by *D. magna* might be related to their poor survival at reduced temperatures.

INTRODUCTION

Comparison of the fatty acid compositions of a number of fresh-water planktonic crustaceans revealed that none of the lipids of the investigated cladocerans contained appreciable amounts of docosapolyenoic acid (1, 2), the presence of which characterizes the fats of aquatic animals (3). In contrast, all of the investigated copepoda species proved to be rather rich in this type of fatty acid (1, 2). Moreover, the content of long chain polyunsaturated fatty acids in their phospholipids could be related to their abilities to survive at reduced temperatures (2). Cladocerans form resting eggs when the temperature of water decreases to ca. 10 C, whereas some of the copepods reach their maximal population densities below this temperature. It is tempting to speculate that these differences in surviving at reduced temperatures are at least partially related to differences in fatty acid metabolism. Long chain polyunsaturated fatty acids have extremely low melting points and, therefore, are suitable to increase membrane fluidity when the temperature decreases. Species unable to form or to accumulate these fatty acids might be handicapped under these circumstances. An inability of cladocerans to accumulate docosapolyenoic fatty acids has already been shown in feeding experiments (2, 4). In this paper, the problem was approached by administering radiolabeled acetate in vivo to *Daphnia magna* and *Cyclops strenus*. *D. magna* was selected to represent fresh-water cladocerans and *C. strenus* represents the fresh-water copepods.

MATERIALS AND METHODS

Animals

D. magna and *C. strenus* were collected from experimental ponds of the Fisheries Research Institute, Szarvas, Hungary, on April 25 and April 15, 1980, respectively. The animals were brought into the laboratory live and maintained in 500-ml beakers. Labeling of lipids was done as described for marine copepods (5). Briefly, 1–1.5 g of the animals were placed in beakers containing 100 ml filtered and autoclaved lake water and also containing sodium [1-^{14}C] acetate (sp. act. 5 μCi/μmol^{-1}) at a concentration of 1 μmol/ml^{-1}. Some of the beakers were precooled to 5 C in a cold room whereas the others remained at room temperature (25 C). The beakers were covered with aluminum foil to keep them in the dark, thus preventing photosynthetic activity by any epibiotic algae possibly present. At selected intervals, 200–300 mg of the animals were removed from the beakers and placed in ice-cold tap water containing unlabeled sodium acetate at a concentration of 0.1 M. After several rinses, they were blotted and weighed, then homogenized in the presence of chloroform/methanol (2:1, v/v). Extraction and purification of lipids proceeded according to Folch et al. (6). The volume of the properly washed chloroform phase was adjusted to 5 ml and aliquots of this served for deter-

mination of the radioactivity as well as for further analyses.

Analytical Procedures

Lipid class separation was performed according to Salle and Adams (7). Unlabeled, 1,3- and 1,2-diacylglycerols, cholesterol, oleic acid, trioleate and cholesteryl oleate (Nu-Chek-Prep, Elysian, MN) served as reference standards. Spots were visualized by short exposure of the plates to iodine vapors. After evaporation of the iodine, the spots were transferred into scintillation vials and counted in toluene cocktail using a Tri-Carb Liquid Scintillation Spectrometer. The counts were corrected for quenching and counting efficiency.

Gas chromatographic separation of fatty acid methyl esters, obtained by transesterification of total lipids in the presence of absolute methanol containing 5% HCl at 80 C, was performed using a JEOL JGC 1100 gas chromatograph equipped with dual flame ionization detectors. The coiled, stainless steel columns (6 ft × 3 mm id) were filled with 10% SP 2340 on Chromosorb W AW, 100–120 mesh (Supelco, Bellefonte, PA). Radioactivity was trapped by placing scintillation vials, lined with "soft absorbant paper" wet with Hyamine hydroxide, onto the outlet of the detector. Seventy-two percent of the radioactivity present in the peaks, as calibrated with [1-^{14}C] palmitic acid methyl ester, could be collected under these conditions. Quantitation was made with the aid of an electronic integrator (Packard Model 603) connected to the gas chromatograph.

RESULTS

D. magna is abundant in natural waters when the temperature is above ca. 10 C. Maximal population densities are reached around 20–25 C. Conversely, *C. strenus* exhibits maximal population densities in early spring and disappears from water bodies with increasing temperatures. It was completely absent from the zooplankton on April 25, 1980, when a new collection was attempted.

Figure 1 demonstrates that these crustaceans can incorporate radioactivity from labeled acetate into lipids at both temperatures. The ability of the marine copepod *Calanus pacificus* to take up [^{14}C] acetate (5) and of *Paracalanus parvus* to take up [^{14}C] acetate and ^{14}C-fatty acids (8) from water has already been reported. The rate of formation of fatty acids by *D. magna,* under these experimental conditions, was ca. 28 pmol/hr/animal^{-1}. The average weight of these animals was roughly 1.0 mg and our unpublished observations show that their

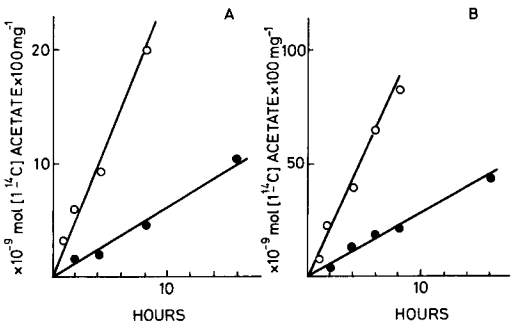

FIG. 1. Incorporation in vivo of [1-^{14}C] acetate into total lipids of *Daphnia magna* and *Cyclops strenus.* Animals (1–1.5 g) were incubated in 100 ml of sterile lake water containing sodium [1-^{14}C] acetate (sp act. 5 μCi × μmol^{-1}) in a concentration of 1 μmol × ml^{-1} at 5 C and 25 C. Animals (200–300 mg) were removed from the beakers at selected intervals to extract lipids. A = *D. magna,* B = *C. strenus.* Incubation temperatures: open circles = 25 C; closed circles = 5 C.

fat content is 2% by wet weight. Taking an average molecular weight of glycerolipids as 700, their total lipids can be calculated to be ca. 30 nmol. It can also be calculated that 0.9% of the total fatty acids were renewed in 1 hr, provided all of the lipid classes have equal turnover times. Similar calculations for *C. strenus* (0.1 mg wet weight) show that about one-third of its total fatty acids is turned over in 1 hr. Decreasing the temperature from 25 to 5 C slowed the formation of fatty acids by a factor of 4 in both species.

Table I indicates that the distribution of radioactivity among the different lipid classes is almost identical at both temperatures in the crustaceans investigated. Interestingly, *C. strenus* directed an overwhelming proportion of labeled fatty acids into the phospholipid fraction and the formation of triglycerides was less pronounced than in *D. magna.* Some radioactivity also was present in sterol fraction. GLC analysis of the isolated spot showed that cholesterol was the only sterol present in these crustaceans.

The average fatty acid compositions of total lipids and the effect of temperature on the distribution of radioactivity among total lipid fatty acids are given in Tables II and III, respectively. Four samples of *D. magna* and three samples of *C. strenus,* collected at different locations and times, were used to evaluate average fatty acid compositions. Lipids of *D. magna,* in contrast to *C. strenus,* did not contain docosapolyenoic fatty acids and thus this species is very suitable for studying the possible involvement of these fatty acids in

TABLE I

Incorporation of [1-^{14}C]Acetate into Lipid Classes of *Daphnia magna* and *Cyclops strenus* at Various Temperatures

Species	*D. magna*		*C. strenus*	
Incubation temperature (C)	5	25	5	25
Incubation time (hr)	8	8	6	6
	Distribution of radioactivity (%)			
Phospholipids	66.1	67.0	86.3	87.4
Monoglycerides	ND	ND	3.5	2.7
1,3-Diacylglycerols	3.6	4.1	1.2	0.9
1,2-Diacylglycerols	2.7	2.9		
Cholesterol	0.6	0.3	0.5	0.4
Free fatty acids	4.5	4.1	1.2	0.9
Triacylglycerols	21.4	20.8	7.2	7.6

The values are the means of duplicate determinations. For experimental details, see legend to Fig. 1. ND = not determined.

temperature adaptation processes (Table II).

The major products of fatty acid biosynthesis in both species were saturated and monounsaturated fatty acids at 25 C (Table III). However, *D. magna* formed almost twice as much saturated fatty acids as did *C. strenus* under identical experimental conditions. Decreasing the environmental temperature from 25 to 5 C resulted in a reduction of labeling of saturated and an increase in labeling of monounsaturated fatty acids. Besides this, *C. strenus* directed roughly twice as much label into its total long chain polyunsaturated fatty acids when exposed to cold. The increase in labeling of 20:4ω6 and 20:5ω3 in cold-exposed *D. magna* is not significant. Repeating the experiments with two other lots of *D. magna* gave identical results (data not presented). There was some activity in the peaks for octadecapolyenoic acids (18:2ω6 and 18:3ω3), which are not synthesized by the animals. It is possible that this activity comes from some overlapped fatty acids or from dimethylacetals obtained from plasmalogens.

TABLE II

Total Lipid Fatty Acid Composition of *Dephania magna* and *Cyclops strenus* Collected in Their Natural Environments

Species	*D. magna*	*C. strenus*
Fatty acids	(wt%)	
16:0	17.93 ± 1.93	11.29 ± 3.82
16:1	12.70 ± 2.51	11.61 ± 1.43
18:0	4.54 ± 1.51	2.72 ± 0.48
18:1ω9	21.50 ± 3.98	9.75 ± 2.22
18:2ω6	8.51 ± 3.41	5.43 ± 1.31
18:3ω6	0.79 ± 0.33	0.69 ± 0.08
18:3ω3	12.29 ± 3.28	8.90 ± 0.11
20:1ω9	1.64 ± 0.42	9.18 ± 2.95
20:2ω6	1.78 ± 0.59	1.36 ± 0.24
20:3ω6	0.91 ± 0.12	1.21 ± 0.42
20:4ω6	3.01 ± 0.41	3.00 ± 0.87
20:4ω3	1.09 ± 0.04	1.65 ± 0.98
20:5ω3	13.89 ± 5.64	9.43 ± 3.11
22:4ω6		2.44 ± 1.00
22:5ω6		1.95 ± 0.34
22:5ω3		1.74 ± 0.43
22:6ω3		17.49 ± 2.87

D. magna was collected from 4 and *C. strenus* from 3 different locations. Results are the means ± SD of 8 *D. magna* or 6 *C. strenus* determinations. Fatty acids eluted before palmitic and after docosahexaenoic acid were neglected.

DISCUSSION

Although the animals were not sterilized in our experiments, it is highly unlikely that microbial contamination significantly contributed to the label present in fatty acid fraction. Gut bacteria is the most probable source of label in lipids. However, their mass is negligible compared to that of animals and their fatty acid composition is probably more simple (consisting largely of C_{16} and C_{18} acids) than that occurring in their hosts. On the other hand, the presence of label in cholesterol might indicate some microbial activity. Crustaceans were reported to be unable to form cholesterol (9).

The major difference between fatty acid compositions of *D. magna* and *C. strenus* was the lack of docosapolyenoic fatty acids from the *D. magna*. Long chain polyunsaturated fatty acids are formed in animals by desaturation and chain elongation of octadecapolyenoic

TABLE III

Labeling of [^{14}C] Acetate of Total Lipid Fatty Acids at Different Temperatures

Species	*D. magna*		*C. strenus*	
Temperature (C)	5	25	5	25
Fatty acids	Distribution of radioactivity (%)			
16:0	30.86 ± 5.01	43.81 ± 3.95	13.14 ± 0.33	21.85 ± 0.94
16:1	12.74 ± 3.06	11.49 ± 3.21	29.60 ± 0.97	31.89 ± 0.98
18:0	7.86 ± 0.24	13.14 ± 4.73	1.51 ± 0.13	2.30 ± 0.11
18:1ω9	15.60 ± 0.03	9.98 ± 4.97	34.91 ± 1.48	26.33 ± 1.74
18:2ω6	1.95 ± 0.04	1.88 ± 0.45	1.79 ± 0.30	3.67 ± 1.15
18:3ω3	2.49 ± 0.13	2.19 ± 0.16	2.01 ± 0.07	2.44 ± 0.16
20:1ω9	–	–	1.11 ± 0.12	1.11 ± 0.37
20:2ω6	3.19 ± 0.12	1.68 ± 0.49	1.92 ± 0.56	0.93 ± 0.06
20:3ω6	6.90 ± 0.43	5.47 ± 1.99	1.00 ± 0.09	–
20:4ω6	7.77 ± 0.90	6.28 ± 3.01	2.61 ± 0.05	2.17 ± 0.24
20:4ω3	4.49 ± 1.53	1.69 ± 0.55	1.42 ± 0.11	1.90 ± 0.07
20:5ω3	5.58 ± 1.33	3.38 ± 1.26	1.61 ± 0.03	1.10 ± 0.05
22:4ω6	–	–	1.82 ± 0.46	0.93 ± 0.06
22:5ω6	–	–	1.25 ± 0.32	0.83 ± 0.27
22:5ω3	–	–	2.18 ± 0.83	1.74 ± 0.12
22:6ω3	–	–	2.04 ± 0.31	0.74 ± 0.09
Saturated/ unsaturated	0.63	1.27	0.17	0.31
16:1/16:0	0.41	0.26	2.25	1.45
18:1/18:0	1.92	0.75	23.11	11.44

Each value is the mean ± SD of triplicate determinations from a single experiment. For experimental details, see legend to Fig. 1 and Table 1.

acids taken up with the food (10, 11). High levels of linoleic and linolenic acids in total lipids of both species indicate their presence in the natural diet. The absence of docosapolyenoic fatty acids in lipids of *D. magna* suggests that the metabolism of polyunsaturated fatty acids is qualitatively different from that in *C. strenus*. It is probable that the *D. magna* fails to carry out some steps of the reaction $20{:}5\omega3 \overset{+2C}{\rightarrow} 22{:}5\omega3 \overset{-2H}{\rightarrow} 22{:}6\omega3$. The label in 20:5ω3 (Table III) suggests the endogenous origin of this fatty acid.

The temperatures of water bodies from which *D. magna* and *C. strenus* were sampled from were 16.5 and 15 C, respectively, and the population of the *D. magna* was growing rapidly whereas that of the *C. strenus* was starting to decline. For *D. magna*, incubation at 5 C, and for *C. strenus*, incubation at 25 C represented temperature stress to which their fatty acid composition had to be adjusted. Considering the dpm ratios of palmitoleic to palmitic, oleic to stearic, and total saturated to total unsaturated fatty acids (Table III), it can be stated that both species formed more unsaturated fatty acids when exposed to cold. An important difference that exists between both crustaceans, and perhaps between crustaceans overwintering in passive or in active form, is that the *C. strenus* are able to synthesize and accumulate long chain polyunsaturated fatty acids when the temperature decreases. Results of experiments in which several other crustaceans were exposed to reduced temperatures (2) fit well into this concept.

Eicosa- and docosapolyenoic fatty acids are often used by aquatic animals to increase lipid unsaturation with decreasing temperatures (12–20). However, this reaction also exhibits tissue specificity (21), and may depend on dietary factors, as well (22). Certain marine copepods (23), as well as fresh water cladocerans (2) and decapods (24), failed to elevate the level of these fatty acids under comparable experimental conditions. The present experiments, confirming our earlier observations made on crustaceans (2) and fish (15, 22), show that this adaptation process is rather rapid in certain aquatic animals and permits the suggestion that the type of fatty acid population formed in response to decrease of the temperature significantly contributes to their abilities to survive in cold.

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Effect of Candicidin on Cholesterol and Bile Acid Metabolism in the Rat

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ABSTRACT

Sterol metabolism studies were carried out in rats maintained on a diet containing a polyene antibiotic, candicidin, (30 mg/kg/day) for 2-1/2 months. Compared to the controls, the candicidin-treated animals had a smaller food intake and weight gain during this period. There was no difference between the 2 groups in serum cholesterol levels, biliary cholesterol or bile acid concentrations. However, in the experimental group, liver cholesterol content decreased by 27% and hepatic HMG-CoA reductase increased by 36%. Candicidin administration produced an 84% increase in neutral sterol output without change in bile acid output. Cholesterol absorption was reduced 80% by candicidin feeding. The weight of ventral prostate was reduced 33% by candicidin administration. Prostatic HMG-CoA reductase levels were 3 times higher than those of the liver, but enzyme activity was unchanged by candicidin treatment.

INTRODUCTION

Candicidin, a polyene macrolide, has long been recognized as a potent antifungal antibiotic (1,2). The activity appears to be dependent on its ability to bind sterols in the membrane. Candicidin, fed at low dosages for 3 wk, was found to lower the serum cholesterol levels in dogs (3) and chicks (4). In chicks maintained on either a cholesterol-free or cholesterol-containing diet, it was also found to increase significantly the fecal output of bile acids (5). An increased amount of cholesterol was found in feces of candicidin-fed rats (6). A marked reduction in size of the prostate gland was noted in young dogs after 30 days of oral treatment with 20 mg/kg of candicidin (7); this was later confirmed in genetically defective B10 87.20 hamsters that develop spontaneous prostatic hypertrophy (8).

In this paper, we report the effect of candicidin on cholesterol and bile acid metabolism in rats. The effect of candicidin on prostate size and histopathology was also studied.

METHODS

Animal Maintenance

Adult male Wistar rats weighing 225-310 g were maintained on a semisynthetic diet containing 0.15% cholesterol (Bio-Serv, Inc., Frenchtown, NJ, Cat. #1441); water was available ad libitum. The test animals received ca. 30 mg/kg/day of candicidin which was premixed in the basal diet before the food was pelleted. The food intake was measured daily. After 2 months, the animals were transferred to metabolic cages for 16 days and maintained on the same diet. During the whole experiment, the animals were kept under an alternating 12-hr light and 12-hr dark schedule.

On the first day of the 16-day period of the cholesterol balance study, the animals were injected intraperitoneally with 10 μCi of DL-[2-^{14}C] mevalonolactone (Amersham Corp.)/mL of saline. Blood was withdrawn from the tail after 4, 6, 8, 10, 12 and 14 days. Two-day pools of fecal samples were collected on days 12, 14 and 16 after isotopic labeling. At the end of 16 days, the animals were anesthetized with pentobarbital (Fort Dodge Labs., Fort Dodge, IA). The common bile ducts of the animals were cannulated and bile was collected for 1 hr. The animals were killed by exsanguination and the liver and ventral prostate were excised and weighed. Aliquots of these tissues were used for the determination of cholesterol concentration and specific activity (sp act) of cholesterol, and another section for histopathology. The rest of the two tissues was used for the preparation of microsomes.

Preparation of Microsomes

Microsomes were prepared in the cold room at 4 C. The tissues were passed through a garlic press and homogenized in a loose-fitting homogenizer in a medium containing sucrose, 300 mM; nicotinamide, 75 mM; neutralized EDTA, 2.5 mM; and mercaptoethanol, 25 mM. The homogenate was centrifuged at 10,000 G and the supernatant was further centrifuged at 100,000 G. The resulting pellet was homogenized in the same medium, in a tight-fitting homogenizer, and immediately frozen.

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Enzyme Assays

Hydroxymethylglutaryl-CoA reductase (HMGR) was assayed in duplicate in liver and prostatic microsomes by the procedure of Shefer et al. (9). Hepatic cholesterol 7α-hydroxylase activity was measured in duplicate as described previously (10).

Determination of Specific Activity of Cholesterol in Liver, Prostate, Plasma and Bile

These analyses were done as previously described (11).

Quantitation of Neutral and Acidic Steroids in Feces

These were carried out by the method of Cohen et al. (12).

Calculations

Various parameters of cholesterol metabolism were determined in the following manner:

Amount of endogenous cholesterol (mg/day) = Total radioactivity in fecal neutral sterol fraction (dpm)/ sp act of plasma cholesterol 1-1/2 days earlier (dpm/mg)

Amount of endogenous bile acids (mg/day) = Total radioactivity in fecal bile acid fraction (dpm)/ sp act of plasma cholesterol 5-1/2 days earlier (dpm/mg)

Daily unabsorbed dietary cholesterol (mg/day) = Total fecal neutral sterols (analyzed by gas chromatography) – daily endogenous neutral sterols (determined by radioactivity)

Daily exogenous cholesterol absorbed = Daily cholesterol intake – daily unabsorbed dietary cholesterol

RESULTS

As shown in Table I, the food intake of the candicidin group was less than that of the controls and the weight gain of that group during the first 2 months (42%) was less than that of the controls (61%). However, there was no significant weight change in the final 2 wk, during the cholesterol-balance study and no corrections for weight changes were applied to the data. Although 5% corn oil and 0.15% cholesterol were added to the semisynthetic diet, the serum cholesterol concentrations were low and were not affected by the antibiotic (Table II). Liver cholesterol levels were reduced 27% by candicidin treatment, whereas biliary cholesterol and bile acids remained unchanged.

Table III shows the effect of candicidin on hepatic regulatory enzymes. There was a 36% increase in HMG-CoA reductase activity on candicidin treatment. Cholesterol 7α-hydroxylase was not increased significantly. The increase in HMG-CoA reductase activity is to be expected if this activity is feedback regulated via hepatic cholesterol.

The effect of candicidin on fecal steroid output and cholesterol absorption is summarized in Table IV. While there was no significant change in bile acid excretion, there was an 84% increase in fecal neutral sterol output, but no significant increase in endogenous neutral sterol output. However, cholesterol absorption was reduced 81% by candicidin feeding and ca. 95% of dietary cholesterol was not absorbed.

During candicidin administration, the ventral prostate of the rats was significantly decreased in weight (33%) but the cholesterol concentration of the gland remained unchanged. As a result, total cholesterol per gland decreased by 35% (Table V). There were no significant histopathological changes in the prostate. Prostatic microsomal HMG-CoA reductase activity was ca. 3 times higher than that of liver, but this activity remained unchanged during candicidin feeding. It may be of interest that, although the cholesterol pools of all animals were labeled by the injection of [2-^{14}C] mevalonolactone 10 days before start of the balance study, no label was detectable in the prostatic cholesterol pool.

DISCUSSION

In order to obtain the maximal effect of candicidin on sterol metabolism, a large dose of candicidin, i.e., 30 mg/kg body wt/day, was selected. As a result, the animals on the candicidin diet gained weight at a slower rate than controls. However, during the period of the sterol balance study, i.e., the final 2 wk, the weight of the animals was reasonably constant and no correction for weight changes was applied to the data.

Candicidin has been found to lower serum cholesterol levels in dogs (3), chicks (4) and hamsters (13). There has been no published report thus far indicating a hypocholesterolemic effect of candicidin in rats. Another polyene antibiotic, neomycin, which is structurally similar to candicidin, was found to lack a hypocholesterolemic effect in rats (14) whereas hamycin lowered the rat serum cholesterol levels by ca. 20% (15). In this study, candicidin did not have an effect on serum cholesterol levels. Since the initial serum cholesterol levels on the semisynthetic diet were quite low, a striking effect of candicidin probably cannot be expected.

There was a striking decrease in liver cholesterol levels at the end of 2-1/2 months of candicidin feeding. Low liver cholesterol levels can be explained on the basis that candicidin prevented the absorption of cholesterol. While there is similarity in the action of sitosterol

TABLE I

Weight, Food Intake and Fecal Output of Rats Fed Candicidin[a,b]

Diet (no. animals)	Weight[c] Initial (g)	2 Months (g)	Final (g)	Food intake (g/day)	Fecal[d] output (g/day)
Control (4)[e]	266 ± 18	430 ± 21	429 ± 21	20 ± 1	2.50 ± 0.18
Candicidin (6)[e]	255 ± 11	388 ± 7	362 ± 7	16 ± 1	2.32 ± 0.11

[a]Candicidin was fed in a semisynthetic diet at 30 mg/kg/day.

[b]All values are expressed as mean ± SE.

[c]The balance study was initiated at the end of 2 months and the final weight refers to the same animals when sacrificed after an additional 16 days.

[d]Values represent the average daily fecal output during the final 6 days.

[e]The number of animals used in each group.

TABLE II

Serum, Hepatic and Biliary Sterol Levels in Rats Fed Candicidin[a]

Diet	Serum cholesterol (mg/dL)	Liver cholesterol (mg/g)	Biliary cholesterol (mg/dL)	Biliary bile acids (mg/mL)
Control (4)[b]	46.3 ± 3.1	2.58 ± 0.14	0.74 ± 0.05	8.96 ± 1.55
Candicidin (6)[b]	44.1 ± 2.5	1.87 ± 0.05[c]	0.82 ± 0.05	9.03 ± 1.26

[a]All results are expressed as mean ± SE.

[b]The number of animals used in each group.

[c]Differs from controls ($p<.01$).

TABLE III

Effect of Candicidin on Rate-Limiting Hepatic Enzymes[a]

Diet	HMG-CoA reductase (pmol/min/mg protein)	Cholesterol 7α-hydroxylase (pmol/min/mg protein)
Control (4)[b]	43.9 ± 4.4	7.8 ± 0.7
Candicidin (6)[b]	59.5 ± 6.8[c]	12.7 ± 3.2

[a]The values represent mean ± SE.

[b]The number of animals in each group.

[c]Differs from control ($p<.05$).

(16) and candicidin on cholesterol metabolism, the effects are not strictly comparable. Although both compounds stimulate hepatic HMG-CoA reductase activity (16) and lead to increased output of fecal neutral sterols, candicidin did not produce a significant increase in the endogenous neutral sterol output and did not increase biliary sterol concentration. Since there was no increase in endogenous neutral sterols on candicidin feeding, the increase in fecal neutral sterols represents a large amount of unabsorbed dietary cholesterol. Cholesterol 7α-hydroxylase, the rate limiting enzyme for bile acid production, did not change significantly upon candicidin treatment and there was no change in fecal acidic steroid output. This is in contrast to the results reported for chicks that respond to candicidin with increased fecal bile acid output (5).

Hypocholesterolemic drugs, such as the polyene macrolides (7) and colestipol (8), administered orally, have been shown to decrease the size of the enlarged prostate in dogs and B10 87.20 hamsters. After 10 wk of clofibrate treatment in rats, there was a 30% reduction in prostatic cholesterol concentration but no significant decrease in prostate weight was noted (17). In this study with candicidin, the total weight of the prostate decreased but there was no change in cholesterol concentration. As a result, total prostate cholesterol was decreased. The sp act of prostatic microsomal

TABLE IV

Effect of Candicidin on Fecal Steroid Output and Cholesterol Absorption in the Rat[a]

Diet	Daily acidic steroid output (mg/day)	Daily neutral steroid output (mg/day)	Daily endogenous neutral sterols (mg/day)	Daily cholesterol absorption (mg/day)	Daily dietary unabsorbed cholesterol (mg/day)
Control (4)[b]	11.77 ± 0.78	19.34 ± 1.88	4.89 ± 0.86	15.55 ± 2.14	14.45 ± 2.44
Candicidin (6)[b]	9.94 ± 1.32	35.06 ± 5.28[c]	5.44 ± 0.67	2.96 ± 1.68[d]	29.63 ± 4.80[e]

[a]All values are expressed as mean ± SE.

[b]The number of animals in each group.

[c,d,e]Differs from controls ($p<.01$).

TABLE V

Prostatic Cholesterol Metabolism in Rats Fed Candicidin[a]

Diet	Wet prostate weight (mg)	Total prostate cholesterol (mg)	Cholesterol conc. (mg/g)	Prostatic HMG-CoA reductase (pmol/min/mg protein)
Control (4)[b]	762.5 ± 23.9	1.65 ± 0.06	2.17 ± 0.06	128.5 ± 28.1
Candicidin (6)[b]	511.7 ± 47.6[c]	1.04 ± 0.08[d]	2.09 ± 0.20	134.4 ± 17.5

[a]All values are expressed as mean ± SE.

[b]The number of animals in each group.

[c,d]Differs from controls ($p<.01$).

HMG-CoA reductase activity was higher than in liver but did not change in response to candicidin treatment. Cholesterol synthesis was found to not have any correlation with DNA synthesis in liver and intestine (18). However, Kandutsch et al. (19) have shown that de novo sterol synthesis is required for DNA synthesis and cell division by some cultured cells. Inhibition of cholesterol biosynthesis was found to interfere with the testosterone-dependent proliferation of the prostate gland (17). Since candicidin is not absorbed, the action of candicidin on ventral prostate may be due to the inhibition of cell proliferation caused by the decrease in total prostate cholesterol.

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Structural Analysis of Hydroperoxides Formed by Oxidation of Phosphatidylcholine with Singlet Oxygen

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ABSTRACT

Soybean phosphatidylcholine (PC) and dilinoleoyl PC (di-18:2 PC) were oxidized with singlet molecular oxygen using methylene blue as the photosensitizer. The oxidation products, PC monohydroperoxides (PC-MHP) and PC dihydroperoxides (PC-DHP), were isolated by reverse phase liquid chromatography, and their structures were analyzed by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS). Signals for the hydroperoxy proton appeared downfield in NMR spectra of PC-MHP and PC-DHP. Soybean PC-MHP and di-18:2 PC-MHP were converted to trimethylsilyl (TMS) derivatives of hydrogenated diglycerides when treated with phospholipase C and hydrogenated. The *tert*-butyldimethylsilyl (TBDMS) derivatives of hydrogenated diglycerides were also prepared from di-18:2 PC-MHP. Fragmentation of the TMS and TBDMS derivatives was obtained in electron impact mass spectra. The isomeric composition of hydroperoxylinoleate component in di-18:2 PC-MHP was determined by methanolysis of the hydrogenated diglyceride and mass chromatographic analysis of the resulting isomeric hydroxy octadecanoates.

INTRODUCTION

Peroxidation of membrane phospholipids has been suggested as causing physiological damage in living organisms (1,2). Polyunsaturated fatty acids (PUFA) constituting phospholipids are susceptible to oxidation and produce hydroperoxides as the primary oxidation products (3). Hydroperoxides formed by free radical oxidation of PUFA have been analyzed using high performance liquid chromatography (HPLC) (4-6) and gas chromatography-mass spectrometry (GC-MS) (7-10). Recently, HPLC was applied to the separation of oxidized and unoxidized phosphatidylcholine (PC) molecular species (11). Porter et al. (12) succeeded in isolating PC hydroperoxides produced by free radical oxidation of unsaturated PC and used HPLC to characterize the oxygenated fatty acid constituents.

On the other hand, the role of singlet molecular oxygen ($^1O_2$) in lipid peroxidation has been widely discussed (13-17). This active oxygen molecule reacts with unsaturated fatty acid producing isomeric hydroperoxides (18-21). The isomeric compositions of monohydroperoxides formed by oxidation of PUFA with $^1O_2$ have already been determined by GC-MS analysis (22-24).

This paper reports on the oxidation of soybean PC and dilinoleoyl PC (di-18:2 PC) by $^1O_2$ using a methylene blue-sensitized photooxidation system (20), and the oxidation products, PC monohydroperoxides (PC-MHP) and PC dihydroperoxides (PC-DHP). PC-MHP were converted to saturated diglyceride derivatives when treated with phospholipase C and hydrogenated. GC-MS analysis of the saturated diglycerides was performed after trimethylsilylation or *tert*-butyldimethylsilylation. Isomeric composition of the hydroperoxy fatty acid component of di-18:2 PC-MHP was determined by mass chromatographic analysis. The reaction mechanism of unsaturated PC with $^1O_2$ is discussed.

EXPERIMENTAL PROCEDURE

Materials

Soybean PC purchased from Nakarai Chem. Co. Ltd., Kyoto, Japan, was washed before use with acetone and purified by silica gel column chromatography (25). Linoleic anhydride was synthesized from the reaction of linoleic acid (99%, Nakarai Chem. Co. Ltd.) with dicyclohexycarbodiimide in dry carbon tetrachloride (26). Glycerophosphorylcholine-cadmium complex (GPC-$CdCl_2$) was prepared from purified soybean PC according to the method of Chadha (27). Dilinoleoyl PC (di-18:2 PC) was synthesized by 1,2-diacylation of the GPC-$CdCl_2$ complex with linoleic anhydride according to the method of Patel et al. (28). Phospholipase C (EC 3.1.4.3) from *Clostridium perfringens* was obtained from P-L Biochemicals, Milwaukee, WI. Trimethylsilyl (TMS)/pyridine reagent and *tert*-butyldimethylsilyl (TBDMS)/imidazole reagent were purchased from Tokyo Kasei Kogyo, Tokyo, Japan, and Applied Science Labs, State College, PA, respectively.

Photooxidation Procedure

PC (100 mg) was dissolved in 5 ml of meth-

anol containing 0.1 mM of methylene blue. A reaction vessel holding the solution was placed in a water bath (25 C) and shaken continuously with illumination of a 30-W tungsten projection lamp for 12 hr (intensity at the sample; 10 mW/cm^2).

Isolation of PC Hydroperoxides

After photooxidation was completed, the reaction mixture was concentrated and applied to a reverse phase glass column (240 × 10 mm) prepacked with Lichloprep RP-8 (Merck, Darmstadt, silica gel powder binding octane, 40-63 μm size) and eluted with chloroform/methanol/water (1:10:0.5, v/v). Solvent flow was maintained at 1.8 ml/min and 1.0-ml fractions collected. Phosphorus content (29) and absorbance at 235 nm were determined for each fraction. The fractions with oxidation products were collected and concentrated in vacuo. Nuclear magnetic resonance (NMR) spectra were obtained in carbon tetrachloride with a Hitachi-Perkin Elmer Model 90 (90 MHz).

Derivatization

PC-MHP (30 mg) was dissolved in 3 ml of ethyl ether/ethanol (98:2). To this solution was added 0.5 ml of tris buffer (10 mM, pH 7.4) containing $CaCl_2$ (20 mM) and 5 mg of phospholipase C (1-2 unit/mg protein). The solution was shaken during the reaction for 15 min at 30 C. After the reaction was completed, the ethyl ether layer was evaporated in vacuo. The residue was dissolved in ethanol and hydrogenated with palladium on carbon in a stream of hydrogen. Trimethylsilylation and *tert*-butyldimethylsilylation of the hydrogenated derivatives were performed by heating with TMS reagent at 60 C for 5 min, and with TBDMS reagent at 160 C for 10 min, respectively.

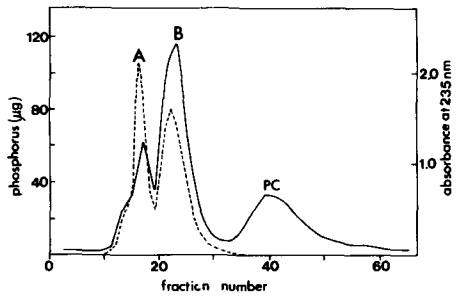

FIG. 1. Reverse phase liquid chromatography of oxidized soybean PC. ——: phosphorus content;-----: absorbance at 235 nm after dilution with ethanol.

GC-MS

A system of GC-MS PAC 300, consisting of a Shimdzu LKB-9000 spectrometer and OKITAC 4300S minicomputer, was used. The column was a glass spiral tube (0.35 m × 3 mm), packed with 2% OV-1 on Neopack 1A, 60/80 mesh. Helium gas was used at 30 ml/min. Temperature of the oven was programmed from 260 to 290 C (6 C/min). Operation conditions for mass spectrometer were: ion source temperature, 310 C; separator temperature, 300 C; ionizing electron energy, 22 eV, trap current, 60 μA; and accelerator voltage, 3.5 kV.

Analysis of Fatty Acid Composition and Molecular Species of Soybean PC

Fatty acid composition of soybean PC was determined by gas liquid chromatography (GLC) after methanolysis using sodium methoxide in methanol solution. For GLC, a Shimdzu GC-5A was used with a glass column (2.5 m × 3 mm) packed with 15% DEGS on Neopack AS, 60/80 mesh. The flow rate of nitrogen gas was 60 ml/min, and column oven temperature was 187 C. Molecular species of soybean PC were analyzed according to the method of Nishihara and Kito (30).

Determination of Isomeric Composition of Hydroperoxylinoleate Component in Di-18:2 PC-MHP

Hydrogenated diglyceride prepared from di-18:2 PC was subjected to methanolysis. Isomeric composition of methyl hydroxyoctadecanoate derived from hydroperoxylinoleate component of PC-MHP was determined by mass chromatographic analysis according to the method of Frankel et al. (22). The conditions for mass chromatography were the same as described previously (23).

RESULTS

Fatty acid composition of soybean PC was determined as follows: palmitic acid (16:0), 14.4%; stearic acid (18:0), 3.2%; oleic acid (18:1), 11.3%; linoleic acid (18:2), 64.8%; and linolenic acid (18:3), 6.3%. Principal molecular species of soybean PC were 16:0-18:2 (25.2%), 18:1-18:2 (13.2%), and di-18:2 (31.3%). The percentages of other molecular species were each less than 10%.

Figure 1 shows the result of reverse phase liquid chromatography of photooxidized soybean PC. Two peaks, A and B, appeared as the oxidation products and were separated from nonoxidized PC. Ultraviolet spectra of the fractions, A and B, gave their λ_{max}^{EtOH} at 235 nm due to conjugated diene. In the NMR spectra

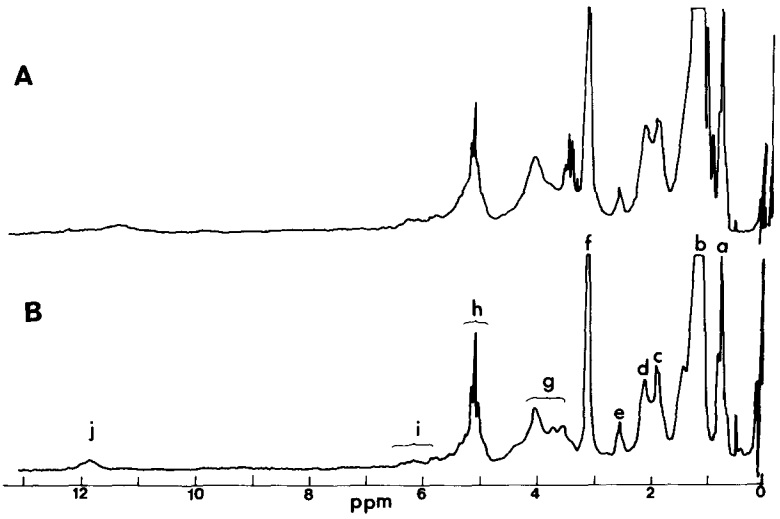

FIG. 2. NMR spectra of fractions A and B. Signals were assigned to each proton as follows: a, -CH_3; b, -CH_2-; c, =CH-CH_2-; d, CH_2CO; e, =CH-CH_2-CH=; f, $N(CH_3)_3$; g, CH_2OCO, $CH_2N(CH_3)_3$, CH_2OPO, and CHOOH; h, CHOCO-, CH=CH-CH_2; i, -CH=CH-CH=CH-; J, -HCOOH.

of the two fractions (Fig. 2), broad signals downfield in the region of 10-13 ppm were assigned to hydroperoxy protons. After addition of D_2O to the NMR tube, the signals for hydroperoxy protons diminished to the base line. The number of protons in the region of hydroperoxy protons were 1.6 for fraction A and 0.5 for fraction B. GLC analysis of the fatty acid components of the two fractions after hydrogenation and methanolysis showed that fraction A consisted of only monohydroxyoctadecanoate and fraction B consisted of three components, i.e., monohydroxyoctadecanoate, octadecanoate and hexadecanoate. From these data, fraction A was identified as PC-DHP consisting of two hydroperoxy fatty acid components and fraction B, PC-MHP consisting of one hydroperoxy fatty acid and one unoxidized fatty acid component. Two oxidation products were also obtained from di-18:2 PC using the same procedure as for soybean PC, and were identified as PC-DHP consisting of two hydroperoxylinoleate components, and PC-MHP consisting of one hydroperoxylinoleate and one unoxidized linoleate component.

Figure 3 shows the gas chromatogram of the TMS derivatives of hydrogenated diglycerides derived from soybean PC-MHP and di-18-2 PC-MHP. Mass spectra of the three peaks (Fig. 4) show fragment ions characteristic for TMS derivatives of diglycerides at m/z 129 and 145 (31,32), although the molecular ions, [M], were not detected. In the spectrum of peak 1,

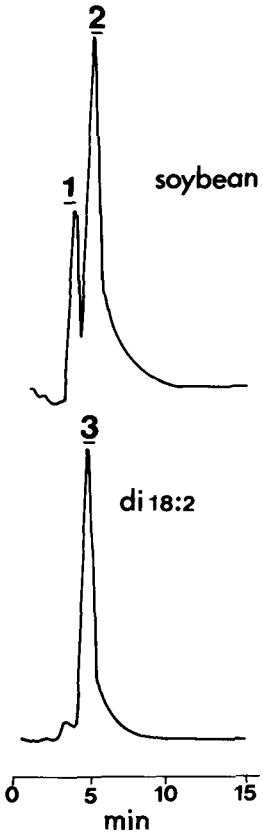

FIG. 3. Gas chromatograms of the TMS derivatives of hydrogenated diglycerides obtained from PC-MHP.

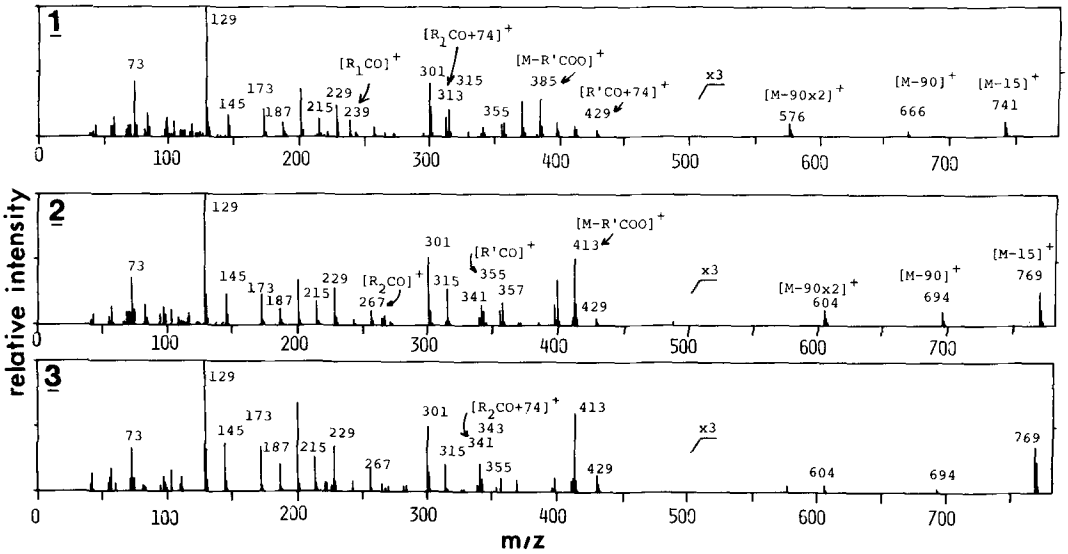

FIG. 4. Mass spectra of peaks 1, 2 and 3. $R_1 = C_{15}H_{31}$-, $R_2 = C_{17}H_{35}$-, $R_2' = C_{17}H_{34}OSi(CH_3)_3$-.

characteristic ions for the TMS derivative of hexadecanoyl-hydroxyoctadecanoyl diglyceride were present at m/z 741 [M-15, loss of CH_3], 666 [M-90, loss of trimethylsilanol], 576 [666-90], 429 [$C_{17}H_{34}OSi(CH_3)_3CO$ + 74], 385 [M-371, loss of $C_{17}H_{34}OSi(CH_3)_3COO$], 355 [$C_{17}H_{34}OSi(CH_3)_3CO$], 313 [$C_{15}H_{31}CO$ + 74], and 239 [$C_{15}H_{31}CO$]. The spectra of peaks 2 and 3 show characteristic ions for the TMS derivative of octadecanoyl-hydroxyoctadecanoyl diglyceride at m/z 769 [M-15], 694 [M-90], 604 [694-90], 429, 413 [M-371], 355, 341 [$C_{17}H_{35}CO$ + 74], and 267 [$C_{17}H_{35}CO$]. These fragmentation patterns are analogous to those of TMS derivatives of diglycerides (31,32). Thus, peak 1 was identified as the TMS derivatives of hexadecanoyl-hydroxyoctadecanoyl diglyceride and peaks 2 and 3, the TMS derivatives of octadecanoyl-hydroxyoctadecanoyl diglyceride. Peaks 1 and 2 are presumably derived from different PC molecular species, i.e., peak 1 from 16:0-18:2 and peak 2 from 18:1-18:2 and di-18:2. Fragment ions formed by elimination of the acyloxy group from molecular ion, [M-R_1COO] or [M-R_2COO] m/z 501, did not appear in the three spectra, although fragment ions formed by elimination of the trimethylsilyloxyacyloxy group from molecular ion, [M-R_2'COO], were of significantly high intensity (Fig. 4).

In the mass spectra of the TMS derivatives of diglyceride from soybean PC-MHP and di-18:2 PC-MHP (Fig. 4), fragment ions indicating the position of the hydroxy group attached to the fatty acid component were present at m/z 229 [$(CH_3)_3SiOCH(CH_2)_8CH_3$], 215 [$(CH_3)_3SiOCH(CH_2)_7CH_3$], 187 [$(CH_3)_3SiOCH(CH_2)_5CH_3$], and 173 [$(CH_3)_3SiOCH(CH_2)_4CH_3$]. These fragment ions seem to be yielded by α-cleavage of the trimethylsilyloxy group producing a hydrocarbon fragment (20). The other series of fragment ions at m/z 301 [$C_2H_5OCOOC(CH_2)_7CHOSi(CH_3)_3$], 315 [$C_2H_5OCOOC(CH_2)_8CHOSi(CH_3)_3$], 343 [$C_2H_5OCOOC(CH_2)_{10}CHOSi(CH_3)_3$], and 357 [$C_2H_5OCOOC(CH_2)_{11}CHOSi(CH_3)_3$] is probably derived from the α-cleavage, producing an ester fragment. Fragmentation of these four ions can be explained by elimination of the acyl and trimethylsilyloxy groups or acyloxy and trimethylsilyl groups from the α-cleavage ions. The carbon numbers indicating the position of the hydroxy group attached to the fatty acid component are: m/z 229, 301 (9-), 215, 315 (10-), 187, 343 (12-) and 173, 357 (13-).

Figure 5 shows the mass spectrum of the TBDMS derivatives of hydrogenated diglyceride obtained from 18:2 PC-MHP. Fragment ions of high intensity appeared at m/z 811 [M-57, loss of $C(CH_3)_3$], 455 [M-413, loss of $C_{17}H_{34}OSi(CH_3)_2C(CH_3)_3$], and 171 [$CH_2CH$-$CHOSi(CH_3)_2C(CH_3)_3$]. This fragmentation pattern is very similar to that of TBDMS derivatives of diglycerides (33). The ions formed by elimination of the acyloxy group, [M-RCOO], m/z 575, were not detected in the spectrum as in the case of the TMS derivatives. It is probable that elimination of the acyloxy group containing a silylated hydroxy group occurs predominantly during fragmentation of the

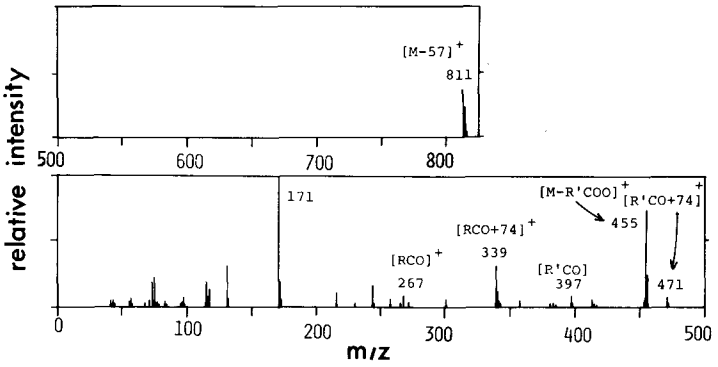

FIG. 5. Mass spectrum of the TBDMS derivatives of hydrogenated diglyceride obtained from di-18:2 PC-MHP. R = $C_{17}H_{35}$-, R' = $C_{17}H_{34}OSi(CH_3)_2C(CH_3)_3$-.

TMS and TBDMS derivatives of diglycerides from PC-MHP.

Mass chromatography of the hydroperoxylinoleate component of di-18:2 PC-MHP was done after enzymatic hydrolysis, hydrogenation, methanolysis and trimethylsilylation. Four positional isomers, the 9-, 10-, 12-, and 13-isomers, were present in the resulting TMS derivatives of methyl hydroxy octadecanoate (Fig. 6). Thus, it is apparent that the four positional isomers are present in the hydroperoxylinoleate component of di-18:2 PC-MHP. The quantitative ratio of the isomeric hydroperoxylinoleate component was determined from the peak areas in Figure 6 as follows: 9-:10-:12-:13- = 34:16:16:34.

DISCUSSION

Interaction of $^1O_2$ with olefins by an ene-type reaction results in allylic hydroperoxides (34). Unsaturated fatty acids (18-21) and unsaturated triacylglycerols (35) yield isomeric monohydroperoxides when $^1O_2$ attacks their double bonds. Thus, oxidation of unsaturated phospholipids with $^1O_2$ seems capable of forming hydroperoxides by an ene-type reaction. Soybean PC and di-18:2 PC were found to react with $^1O_2$ at the position of unsaturated fatty acid component to produce PC-MHP during photosensitized oxidation. It is also probable that the oxidation of 1,2-diunsaturated acyl PC with $^1O_2$ yields PC-DHP by incorporating two oxygen molecules into each unsaturated fatty acid component. Soybean PC-DHP seems to be composed of 1,2-diunsaturated molecular species, such as 18:1-18:2, di-18:2 PC.

It has been found that the distribution of the positional isomers of unsaturated fatty acid monohydroperoxides formed by oxidation with $^1O_2$ is different from that formed by free radical oxidation (20,22). Oxidation of methyl linoleate with $^1O_2$ produces the 9-, 10-, 12- and 13-monohydroperoxide isomers by attack of $^1O_2$ at both ends of Δ9 and 12 double bonds (20-23). On the other hand, free radical oxidation of methyl linoleate yields only the 9- and 13-isomers (7,9). The isomeric distribution of the hydroperoxylinoleoyl group of di-18:2 PC shown in Figure 6 agrees with that obtained

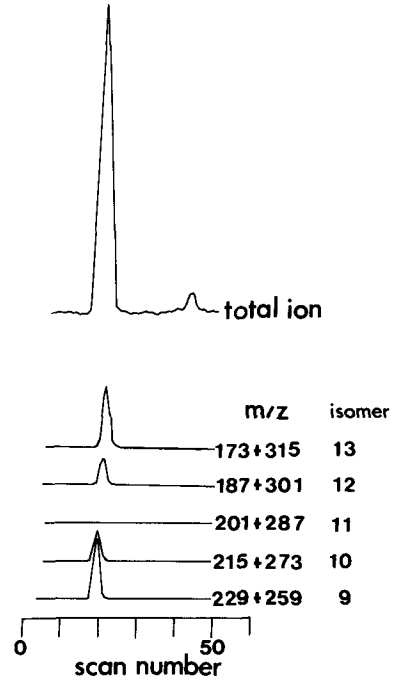

FIG. 6. Mass chromatography of isomeric methyl hydroxy octadecanoate obtained from hydroperoxylinoleate component of di-18:2 PC-MHP.

from oxidation of methyl linoleate with $^1O_2$. It is therefore concluded that the unsaturated acyl group of PC is subject to the attack of $^1O_2$ in the same manner as the corresponding fatty acid methyl ester.

In a study of free radical oxidation of 16:0-18:2 PC (12), it was found that the hydroperoxy linoleate component of PC-MHP consists of the 9- and 13-positional isomers. Therefore, analysis of the isomeric hydroperoxy fatty acid components in oxidized phospholipids may be a useful method for determining whether $^1O_2$ participates in peroxidation of membrane lipids. Furthermore, GC-MS analysis of the diglyceride derivatives from phospholipid hydroperoxides seems to be available for determination of oxidized molecular species in phospholipids.

The TMS derivatives and TBDMS derivatives of hydrogenated diglycerides gave useful information on the structure of PC-MHP. However, GC-MS analysis applied here could not distinguish the position of the hydroperoxy fatty acid component in the glycerol moiety when PC-MHP from 1,2-diunsaturated acyl PC was analyzed by GC-MS. We are now comparing the mass spectra of 1-octadecanoyl-2-hydroperoxyoctadecanoyl diglyceride and 1-hydroperoxyoctadecanoyl-2-octadecanoyl diglyceride and 1-hydroperoxyoctadecanoyl-2-octadecanoyl diglyceride. Our preliminary data suggest that there is no significant difference in their mass fragmentation.

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Alteration of Cholesterol Metabolism by 4-0-Methylascochlorin in Rats

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ABSTRACT

The effect of 4-0-methylascochlorin (MAC), an experimental hypocholesterolemic agent, on cholesterol metabolism was investigated in rats in two separate experiments. The administration of MAC for 2 and 6 consecutive weeks at daily doses of 100–135 mg/kg resulted in reduction in serum cholesterol levels of 16% after 2 weeks of treatment in the first experiment, and 13% after 6 weeks in the second experiment in comparison to the corresponding controls. MAC administered at a daily dose of 100 mg/kg for 2 weeks showed a significant increase in the biliary excretion of bile acids and cholesterol in bile-duct cannulated rats with or without the administration of taurocholate. In the second experiment, MAC treatment for 6 weeks produced a marked increase in the fecal output of acidic sterols during a 2 to 6-week period. MAC treatment also further enhanced hepatic cholesterol 7α-hydroxylase in the rats. Therefore, it appears that the mechanism of serum cholesterol lowering due to MAC is related to the enhancement of hepatic bile acid synthesis and the increase in biliary and fecal excretion of bile acids.

INTRODUCTION

A principal mechanism for reducing total body cholesterol is through an increase in fecal excretion of both neutral and acidic sterols (1). Lofland et al. (2) have shown that the conversion rate of cholesterol to bile acids controls the plasma cholesterol levels in squirrel monkeys. On the basis of what has been reported so far, it has been speculated that an increase in both hepatic bile acid synthesis and fecal excretion of acidic sterols contributes significantly to the reduction of total body cholesterol, unless a greater fecal loss induces a compensatory increase in cholesterogenesis.

Fungal prenylphenols, ascochlorin (3) and ascofuranone (4), were discovered by us and their hypolipidemic properties were reported (5, 6). In our preceding paper (7), we showed that a modified product, 4–0-methylascochlorin (MAC), lowered the serum total cholesterol by more than 30% in mice concomitant with an increased fecal loss of sterols. The hypocholesterolemic activity was not accompanied with hepatomegaly, which is an unavoidable side effect of the aryloxy compounds such as clofibrate. These results suggested that MAC reduced the serum cholesterol through increased fecal output of sterols. Therefore, this study was undertaken to examine the effect of MAC on cholesterol metabolism in rats.

This paper deals with the effect of MAC on (a) the serum cholesterol levels, (b) the activity of hepatic cholesterol 7α-hydroxylase, (c) the biliary secretion of sterols, and (d) the fecal excretion of acidic sterols.

MATERIALS AND METHODS

Experiment 1

Male Wistar rats weighing ca. 250 g were used. For two consecutive weeks, 100 mg/kg MAC suspended in a 2% gum arabic solution was administered orally once daily to the first group and only the vehicle was administered to the second group. Both groups were allowed free access to a standard laboratory pellet diet (Nihon CLEA, CE-2) and tap water.

On the 14th day, a bile fistula was made under light ether anesthesia immediately after the final dosing. At the time of cannulation, blood was withdrawn from the tail vein of the rats for the measurement of serum cholesterol levels. Prior to placing the rats in Bollman-type restraining cages, rats of both groups received either a single oral administration of taurocholate (25 mg/rat) or saline solution. This resulted in 4 different groups of animals. Bile samples were collected during 0–6 and 6–24 hr intervals. Biliary bile acids and cholesterol were determined by the method of Nakayama (8). Briefly, an aliquot of the bile was extracted with 25 volumes of ethanol and filtered. Equal volumes of water, ether and *n*-heptane were added to the filtrate and vigorously mixed. After centrifugation, the lower aqueous ethanol layer was evaporated in vacuo. The residue was hydrolyzed at 120 C for 5 hr in 5% aqueous sodium hydroxide. The hydrolyzate was acidified with hydrochloric acid and extracted with ether. The extract was evaporated to dry-

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ness and methylated with diazomethane. The bile acid methyl esters formed were trifluoroacetylated and analyzed by gas liquid chromatography (GLC). A Shimazu Model GC-1C with 1 m glass column containing 100–120 mesh Gas-Chrom Q coated with 1% QF-1 was used. For analysis of cholesterol, another aliquot of the bile was extracted with 20 volumes of chloroform/methanol (2:1). The mixture was filtered and a small amount of water was added. After centrifugation, the lower layer was evaporated in vacuo. The residue was dissolved in ethyl acetate and the cholesterol was determined by GLC. SE-30 (3%) was used for the liquid phase.

Experiment 2

A total of 32 male Wistar rats weighing 250 g were randomly allocated into 4 groups and each group was housed in a cage with free access to food and tap water. These groups received MAC orally once daily at doses of 15, 45 and 135 mg/kg, respectively, for 6 consecutive weeks. The control rats received the vehicle as described in experiment 1. The body weight of the 4 groups attained ca. 440 g/rat at the end of the study and there was no significant difference in weight gain of the rats of the four groups.

At weekly intervals, blood (0.3 ml) was withdrawn from the tail vein under light ether anesthesia and feces were collected in 24 hr pools. After the final dosage, the animals were killed, and their livers were quickly removed for determination of cholesterol 7α-hydroxylase (9).

The fecal sterols were twice extracted under reflux from the dried feces with ethanol. The ethanol extracts were filtered and evaporated in vacuo to dryness. Aqueous sodium hydroxide (1.2 N) was added to the residue and the mixture was hydrolyzed at 120 C for 4 hr. The neutral sterols were removed with *n*-heptane and the acidic sterols were extracted with ethyl acetate after acidifying the lower layer with hydrochloric acid.

Fecal acidic sterols were purified by thin layer chromatography (TLC) (10, 11). The acidic sterols were determined by GLC using a QF-1 column.

The hepatic microsomes were prepared by the method of Mitropoulos and Balasubramanian (12). One ml of the incubation mixture contained: the microsomal preparation, 3 μmol $NADP^+$, 10 μmol glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, and 25 μmol reduced glutathione in 0.01 M phosphate buffer (pH 7.4). [4-^{14}C]Cholesterol (0.05 μCi, 21 nmol) was added to each tube as a suspension in Tween 80. The mixture was incubated for 60 min at 37 C and the reaction terminated by adding 10 ml of chloroform/methanol (2:1). The chloroform layer was evaporated in vacuo to dryness and the residue applied to TLC plates (Kieselgel H). After developing the plates with ethyl acetate/benzene mixture (7:3, v/v), the areas corresponding to the bands of authentic standards were scraped off and put into scintillation vials. Radioactivity was determined by a liquid scintillation counter.

Serum cholesterol was determined by Zurkowski's method (13). Microsomal protein was determined by the method of Lowry et al. (14).

Statistical significance from each control value was calculated according to the unpaired Student's t-test. The analysis of variance was also performed to determine the significance of MAC treatment on fecal excretion of sterols.

RESULTS

The effect of the administration of MAC for two weeks on serum cholesterol levels, measured at the time of bile duct cannulation, is shown in Table I. The administration of MAC

TABLE I

Serum Total Cholesterol Levels in the Rats Immediately before the Bile Duct Cannulation

Groups	n	Serum total cholesterol (mg/dl)
Saline-control	6	73.7 ± 3.8
Saline-MAC, 100 mg/kg	6	62.4 ± 1.6[a] (−15%)
Taurocholate-control	7	72.1 ± 1.7
Taurocholate-MAC, 100 mg/kg	7	60.6 ± 1.3[b] (−16%)

The results are expressed as the mean ± SEM. [a]$p<0.05$ and [b]$p<0.01$, compared to each control by the unpaired t-test. MAC was given orally once daily for 14 days.

Bile-duct-cannulated rats received a single oral administration of solution of taurocholate (25 mg/rat) or saline.

Figures in parentheses indicate % change from each control.

TABLE II

Bile Flow Rates and Biliary Sterols of MAC-Treated Rats

	Loading before collection	Groups	Collection period 0–6 hr		Collection period 6–24 hr	
Bile flow (ml/rat/hr)	Saline	Control	1.57 ± 0.09		1.00 ± 0.07	
		MAC	1.99 ± 0.10[b]	(+27%)	1.21 ± 0.03[a]	(+21%)
	Taurocholate	Control	1.62 ± 0.15	(+3%)	1.24 ± 0.05[a]	(+24%)
		MAC	1.63 ± 0.20	(+4%)	1.20 ± 0.20	(+20%)
Cholic acid (mg/rat/hr)	Saline	Control	2.38 ± 0.03		0.74 ± 0.06	
		MAC	2.92 ± 0.16[a]	(+23%)	0.77 ± 0.04	(+4%)
	Taurocholate	Control	4.06 ± 0.51[b]	(+71%)	0.95 ± 0.07[a]	(+28%)
		MAC	4.09 ± 0.40[b]	(+72%)	1.49 ± 0.09[b]	(+101%)[d]
Chenodeoxycholic acid (mg/rat/hr)	Saline	Control	0.63 ± 0.03		0.13 ± 0.01	
		MAC	0.61 ± 0.05	(−3%)	0.12 ± 0.02	(−8%)
	Taurocholate	Control	0.59 ± 0.06	(−6%)	0.11 ± 0.01	(−15%)
		MAC	0.83 ± 0.07[a]	(+32%)[c]	0.19 ± 0.02[a]	(+46%)[d]
Cholesterol (μg/rat/h)	Saline	Control	198 ± 13		113 ± 3	
		MAC	334 ± 29[b]	(+69%)	140 ± 3[a]	(+24%)
	Taurocholate	Control	257 ± 22[a]	(+30%)	143 ± 5[a]	(+27%)
		MAC	331 ± 56[a]	(+67%)	191 ± 19[b]	(+69%)[c]

[a]$p<0.05$ and [b]$p<0.01$, compared to the each control receiving saline by the unpaired t-test. [c]$p<0.05$ and [d]$p<0.01$, compared to the control receiving taurocholate.

N = 6 in the saline groups and n = 7 in the taurocholate groups, MAC, 100 mg/kg, was given orally once daily for 14 days. The bile-fistulated rats received orally a single dose of either saline or taurocholate, 25 mg/rat, immediately after the bile duct cannulation. The results are presented as the mean ± SEM. Figures in parentheses indicate % change from the saline controls.

to the rats without taurocholate loading (saline-MAC) produced a 15% reduction in serum cholesterol levels as compared to the saline control. The administration of MAC to the rats with taurocholate loading (taurocholate-MAC) exhibited a 16% reduction in serum cholesterol levels compared to the taurocholate control.

Table II shows the effects of MAC on bile flow and secretion of bile lipids. A significantly greater bile flow for 24 hr above the corresponding control was observed in the MAC group receiving saline. On the other hand, no significant change in bile flow was observed during the 0–6 hr period in the 2 taurocholate groups compared to the control receiving saline.

During the subsequent 6 to 24-hr period, the taurocholate-loaded control rats increased bile flow over that of the saline control. The taurocholate-loaded MAC group increased the flow by 20%, although the difference in comparison to the saline control was not significant.

The saline-loaded MAC group produced a significant increase of biliary cholate by 23% above the corresponding control during the 0 to 6-hr period. It was expected that oral taurocholate would increase biliary secretion of cholate. Interestingly enough, when the secretion rates were compared between the 2 groups loaded with taurocholate, MAC caused more secretion of cholate during the 6 to 24-hr period than the control.

Biliary chenodeoxycholate was unaffected by MAC in the groups receiving saline. The taurocholate-loaded MAC group again increased the secretion above the controls with or without taurocholate.

It can be seen that the secretion rate of biliary cholesterol parallels, for the most part, the rates of bile flow and bile acid secretion. As expected, the rate of cholesterol secretion was increased either by MAC or by taurocholate. The taurocholate-loaded MAC group increased their output the most, by 30% in comparison to the corresponding control.

Figure 1 shows the effect of MAC on serum cholesterol levels in the experiment 2 study. MAC at a daily dose of 15 mg/kg produced no significant reduction in serum cholesterol throughout the entire period. The daily dose of 45 mg/kg was also ineffective on serum cholesterol levels except in the third week. Only the highest dose, 135 mg/kg, began to lower serum cholesterol on week 2 and the efficacy continued thereafter. At the end of this study, the highest dose group exhibited a 13% reduction compared to the control.

Figure 2 shows the effects of MAC on fecal excretion of acidic sterols. The major fecal

sterols were cholesterol and coprostanol in the present study (data are not shown). Fecal excretion of acidic sterols was increased in a dose-dependent manner and the effect of MAC treatment was highly significant ($p<0.001$) by the analysis of variance. The highest dose increased the secretion by 40–88% during a 2 to 6-week period over that of the corresponding control values.

The liver microsomal cholesterol 7α-hydroxylase was determined for each rat at the end of 6 weeks. The activity of the enzyme is expressed in terms of the specific activity (Table III). Rats in the high-dose group increased the

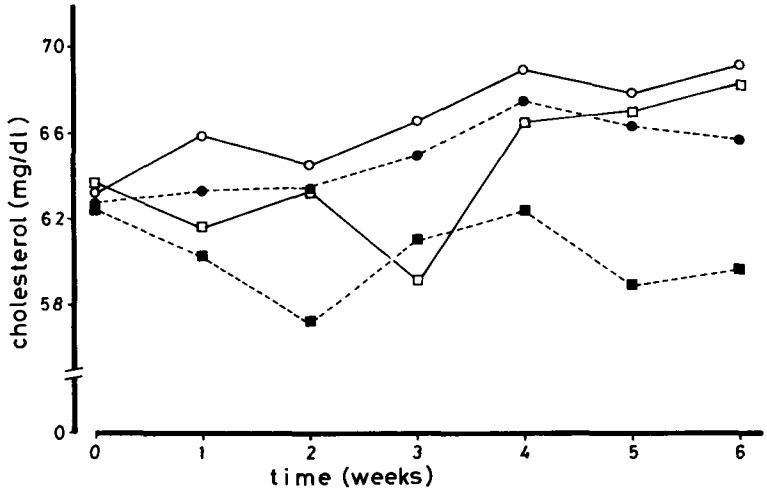

FIG. 1. Serum total cholesterol levels in MAC-treated rats. A total of 32 rats was randomly allocated into 4 groups (n = 8) and MAC was given orally once daily for 6 weeks. The statistical significance by the unpaired t-test for each week is: the control vs the MAC 135 mg/kg group, $p<0.05$ on weeks 2, 3, 4 and 5, $p<0.01$ on week 6; the control vs the MAC 45 mg/kg group, $p<0.05$ on week 3. o——o control; •——• MAC 15 mg/kg; □——□ MAC 45 mg/kg; ■——■ MAC 135 mg/kg.

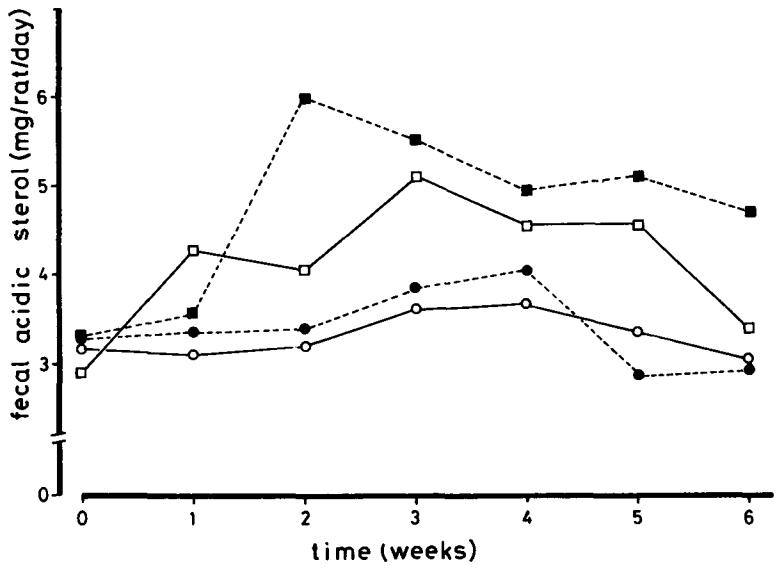

FIG. 2. Fecal excretion of acidic sterols in MAC-treated rats. The fecal acidic sterols were determined at each point using pooled feces collection of each group for 24 hr. the effect of MAC on the fecal excretion from 1 to 6 is statistically significant, $p<0.001$, by the analysis of variance. o——o control; •——• MAC 15 mg/kg; □——□ MAC 45 mg/kg; ■——■ MAC 135 mg/kg.

TABLE III

Effect of MAC on Hepatic Cholesterol 7α-Hydroxylase of Rats

Groups	Dose (mg/kg)	7α-Hydroxycholesterol formation (nmol/hr/mg protein)
Control	0	9.6 ± 0.9
MAC	15	10.3 ± 1.5 (+7%)
	45	12.6 ± 1.3 (+31%)
	135	12.0 ± 0.6[a] (+25%)

The results are expressed as the mean ± SEM. Figures in parentheses indicate % change from control.

[a]$p<0.01$, compared to the control by the unpaired t-test (n = 8).

specific activity by 25% over the control. No changes were noted with the MAC groups dosed with 45 and 15 mg/kg in comparison to the control.

DISCUSSION

Previous studies on MAC have shown that oral dosage reduces serum cholesterol in mice (7) and rats and dogs (unpublished studies of authors) fed standard laboratory diets. Unlike clofibrate and its derivatives (15, 16), MAC induced neither hepatomegaly nor retention of cholesterol and did not inhibit incorporation of acetate and mevalonate into cholesterol in these animals. In addition, it stimulated disappearance of cholesterol from the plasma and liver of mice given MAC parenterally (7). Therefore, MAC apparently decreased the total cholesterol pool whereas cholesterogenesis was unaltered. In fact, fecal excretion of parenterally given [^{14}C]cholesterol was accelerated by MAC in mice. For this reason, the possibility existed that MAC inhibits intestinal absorption. However, preceding studies demonstrated that sterol absorption was unaffected by MAC (7).

In this study, MAC enhanced biliary output of cholate but not chenodeoxycholate in rats receiving saline during the early period of bile collection, indicating that MAC enlarged the hepatic pool of cholate. This idea is supported by the enhancement of cholesterol 7α-hydroxylase activity, the key enzyme of bile acid synthesis (17, 18), caused by MAC. Both bile fistula and bile acid chelating ion exchange resins derepress the bile acid synthesizing system through interruption of the enterohepatic circulation (19, 20), so it is plausible that there was no difference in the bile acid output during the time that rats received saline. The administration of thyroid hormone increases fecal excretion of chenodeoxycholate accompanied by an increase in the activity of cholesterol 7α-hydroxylase (21, 22). However, the compound which specifically increases cholate output is unknown.

It is known that taurocholate decreases the activity of cholesterol 7α-hydroxylase and decreases bile acid synthesis (23, 24). The feedback inhibition of taurocholate is evident in this study, because the output of chenodeoxycholate was smaller in the taurocholate control than the saline control. However, when MAC-dosed rats were loaded with taurocholate, they increased chenodeoxycholate output by 32 and 41% during the early period, and by 46 and 73% during the later period over that of the saline and taurocholate control rats, respectively.

In addition, they secreted more cholate during the later period than the other 3 groups. This fact suggests that MAC releases the repression of bile acid synthesis caused by oral taurocholate. Thus, our speculation is that MAC enhances the specific activity of the key enzyme without affecting the enzyme production rate, or that MAC accelerates the rate of the enzyme production leading to bile acid synthesis, or both. The first possibility is most probable, because MAC increased the output of chenodeoxycholate in the rats loaded with taurocholate during the early period when 7α-hydroxycholesterol synthesis would be maximally suppressed by endogenous taurocholate. Thus, overproduced 7α-cholesterol would alternatively enter into the chenodeoxycholate synthesizing pathway resulting in overproduction. The second possibility is contradictory to the observation that hepatic cholesterogenesis is enhanced in parallel with an increase in bile acid production (25, 26). A significant increase of cholate output in the group loaded with taurocholate during the later period suggests that cholate production would still continue at a considerable rate even under endogenous bile acid repression. This assumption was corroborated by the fact that biliary output of cholesterol was larger in the MAC

groups than the corresponding controls. Fecal analysis also supported the enhanced production of bile acid caused by MAC.

Therefore, the mechanism of MAC in cholesterol reduction seems to occur through enhancement of hepatic synthesis of cholic acid followed by a larger drainage of fecal acidic sterols, as some portions of the overproduced cholate would escape from the enteric reabsorption. However, it is unclear why such a large loss does not induce a compensatory increase in cholesterogenesis in the MAC-treated animals.

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High Pressure Liquid Chromatography of Autoxidized Lipids: II. Hydroperoxy-Cyclic Peroxides and Other Secondary Products from Methyl Linolenate

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ABSTRACT

A previous study of autoxidation products by high pressure liquid chromatography (HPLC) of methyl oleate and linoleate was extended to methyl linolenate. Autoxidized methyl linolenate was fractionated by HPLC either after reduction to allylic alcohols on a reverse phase system, or directly on a micro silica column. Isolated oxidation products were characterized by thin layer and gas liquid chromatography and by ultraviolet, infrared, nuclear magnetic resonance and mass spectrometry. Secondary products from the autoxidation mixtures (containing 3.5-8.5% monohydroperoxides) included epoxy unsaturated compounds (0.2-0.3%), hydroxy or hydroperoxy-cyclic peroxides (3.8-7.7%), epoxy-hydroxy dienes (<0.1%), dihydroxy or dihydroperoxides with conjugated diene-triene and conjugated triene systems (0.9-2.9%). Cyclization of the 12- and 13-hydroperoxides of linolenate would account for their lower relative concentration than the 9- and 16-hydroperoxides. Dihydroperoxides may be derived from the 9- and 16-linolenate hydroperoxides. Cyclic peroxides and dihydroperoxides are suggested as important flavor precursors in oxidized fats.

INTRODUCTION

Previous high pressure liquid chromatography (HPLC) studies of oxidized fatty esters were primarily concerned with the separation of hydroperoxide isomers. Chan and Levett (1) separated the eight geometric and positional isomers of autoxidized linolenate hydroperoxides. Funk et al. (2) also resolved by HPLC the hydroperoxides produced by soybean lipoxygenase with α-linolenic acid as substrate. Less attention has been given to HPLC of secondary oxidation products.

Previously, we have used gas chromatography-mass spectrometry (GC-MS) in studies of autoxidized methyl linolenate and obtained indirect evidence of hydroperoxy-cyclic peroxides (3). Haverkamp Begemann et al. (4) obtained linolenate hydroperoxy-cyclic peroxides by partitioning autoxidized linolenate between 80% ethanol and light petroleum ether, followed by liquid-liquid partitioning on Celite coated with carbowax using isooctane-ether as mobile phase. On the basis of hydrogenation experiments, they designated the hydroperoxy-cyclic peroxides as a pair of positional isomers with a six-membered peroxide group, although five-membered cyclic peroxide compounds were not ruled out. Roza and Francke (5) reported positional isomers of five-membered hydroperoxy-cyclic peroxides in enzymatically oxidized linolenate, which was fractionated on a low-pressure silica gel column with a linear solvent gradient of light petroleum and diethyl ether.

A reverse-phase semipreparative HPLC system was previously described which allowed the characterization of secondary autoxidation products from methyl oleate and linoleate (6). This paper reports the extension of these studies to reverse-phase and micro silica HPLC separation of autoxidized methyl linolenate and the identification of secondary oxidation products. Major products identified included several isomeric hydroperoxy-cyclic peroxides. During the course of this study, preliminary reports were published on the preparation of a single 5-membered cyclic peroxide from the enzymatic oxidation of linolenate followed by autoxidation (7), and on the isolation of bicycloendoperoxides from the same oxidation mixtures (8).

EXPERIMENTAL PROCEDURES

Materials

Pure methyl linolenate (100% by GLC and TLC) was prepared by counter double current distribution (9) of linseed methyl esters followed by silicic acid (100 mesh, Mallinckrodt, Paris, KY) chromatography and vacuum distillation. GLC packing and silica gel plates were previously described (6). Reducing agents were triphenylphosphine (Ph_3P) and Ph_3P bonded

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on styrene-divinyl benzene copolymer (2% crosslinked) (Strem Chemicals, Inc., Newburyport, MA). A mixture of trimethylchlorosilane, hexamethyl disilazane, and pryridine (1:2:10) (Regis Chemical Co., Morton Grove, IL) was used as silylating reagent. This reagent was particularly effective for complete silylation of polyhydroxy compounds (10).

Oxidations

Methyl linolenate, 5 g, was stirred at room temperature in an oxygen atmosphere for 88 hr to a peroxide value (PV) of 1113 (sample I). This sample was reduced with Ph_3P (11) at 0 C in water-saturated diethyl ether with stirring for 1 hr, and then fractionated by reverse-phase HPLC. A second linolenate sample (6 g) was autoxidized at 40 C in an oxygen atmosphere for 21 hr to a PV of 904 and was not reduced (sample II) prior to silicic acid fractionation and HPLC on micro silica columns. Before GC-MS, hydroperoxy cyclic peroxides were reduced with Ph_3P bonded to styrene-divinylbenzene copolymer (100% molar excess Ph_3P/mol hydroperoxide) in diethyl ether at room temperature with stirring for 2-3 hr. The polymeric reducing agent was removed by filtration in ether through a Pasteur pipette packed with Celite under nitrogen pressure.

HPLC

Reverse-phase HPLC of the methyl linolenate sample autoxidized at room temperature (I) was done on a 122 x 0.78 cm column packed with C-18 hydrocarbon bonded to Porasil B (Waters Associates, Milford, MA) at room temperature and 5 mL/min flow with a Waters Model 6000A pumping system. The column eluant was monitored with a variable wavelength ultraviolet detector (Schoeffel Instruments, Westwood, NJ) set at 212 nm for ester functionality (12). A stepgradient of H_2O and CH_3CN mixtures was used for elution. The column was cleaned between runs with $CHCl_3$. Samples (100 μL neat) were introduced with the Waters U-6K injector.

The reverse-phase HPLC fractions were concentrated by partial removal of CH_3CN on a rotating evaporator at 40 C, and they were extracted with diethyl ether after addition of brine. The fractions were then combined on the basis of functional group purity as determined by TLC and stored in diethyl ether or CH_3CN at -20 C.

The linolenate sample autoxidized at 40 C (II) was fractionated first on silicic acid column with diethyl ether/hexane eluants by a procedure similar to that of Gardner (13). Most of the unoxidized linolenate was eluted with 200 mL 1:9 ether/hexane. The following oxidation products were then eluted with 100-mL portions of ether/hexane mixtures of the volume proportions indicated: (2:8) epoxy compounds; (3:7) hydroperoxide mixture; (4:6) mixture of hydroperoxides and hydroperoxy-cyclic peroxides; (1:1) hydroperoxy-cyclic peroxide mixture; (6:4) dihydroperoxides; and (7:3) unidentified polar compounds. The remaining oxidation products were eluted with 100% diethyl ether and methanol.

The mixture of hydroperoxy-cyclic peroxide was separated with a 50 x 0.94 cm column at room temperature packed with 10 μ silica (Magnum 9, Partisil 10, Whatman, Inc., Clifton, NJ), 5.0 mL/min (1000 psi, and 0.3% absolute ethanol in hexane. Typical sample sizes were 16-20 mg dissolved in mobile phase.

Methods

Infrared (IR), gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) methods used were as previously described (6). Except as noted, TLC was conducted with diethyl ether/hexane/acetic acid (50:50:1, v/v/v) on silica gel "60" plates (with fluorescent 254 nm indicator, E. Merck, Darmstadt, Germany). ^{1}H-NMR, except as noted, and ^{13}C-NMR spectra were obtained on a Bruker WH-90 Fourier transform spectrometer. The ^{13}C-NMR spectra were obtained at 22.63 MHz with proton noise decoupling. All spectra were taken in solutions of deuterochloroform, which also served as an internal deuterium lock. Chemical shifts are given as δ-values in ppm downfield from the internal tetramethylsilane signal.

RESULTS

Chromatographic Fractionation of Autoxidized Linolenate after Reduction (Fig. 1)

Reverse-phase HPLC separation of sample I (Fig. 1) yielded fractions containing dihydroxy, epoxy-hydroxy, hydroxy-cyclic peroxy, monohydroxy, epoxy esters and unoxidized starting material. The reverse phase HPLC system separated autoxidized linolenate as previously reported for the autoxidized oleate and linoleate (6) according to functional group with partial separation of positional and geometric isomers. The dihydroxy, hydroxy-cyclic peroxides and hydroxy trienes are apparently derived from the corresponding hydroperoxides. The hydroxy trienes are partially separated into *trans,cis* and *trans,trans* conjugated diene-triene isomers.

The reduced linolenate sample was analyzed by GLC after silylation. The GC chromatogram

showed the same products as obtained by HPLC (Fig. 2). However, the hydroxy-cyclic peroxides (TMS ethers) were not stable under our GC conditions, and only minor amounts were detected. Therefore, GC was not suitable for quantitation. However, approximate quantitative analyses of autoxidized linolenate were obtained below in the nonreduced sample by silicic acid column chromatography.

Epoxy unsaturated esters. GC of this fraction gave two partially resolved peaks with retentions 1.32 and 1.39 relative to linolenate. TLC showed one spot with R_f 0.89 relative to linolenate. IR (CS_2) (1734 cm^{-1}, ester carbonyl), (3002 cm^{-1}, *cis* unsaturation). ^{1}H-NMR supported *cis* unsaturation at 5.43 ppm (4H) and indicated the presence of a *cis* epoxide ring with absorptions at 2.79 and 2.98 ppm (2H) (14). These data support the presence of *cis* olefinic *cis* epoxy esters.

Hydroxy octadecatrienoates. GC of the silyl derivative of this fraction showed two major peaks (50.6 and 37.3%) followed in elution by two minor peaks (8.1 and 3.9%) with retentions 1.67, 1.77, 1.79 and 1.87, respectively, relative to linolenate. GC of this HPLC fraction after hydrogenation and silylation showed two peaks with retentions 1.51 and 1.72 relative to methyl stearate. TLC had two UV active spots of R_f 0.56 and 0.48 relative to linolenate. UV showed conjugated diene with a maximum at 232 nm. GC-MS m/e (rel intensity) of this fraction after silylation: 380 (M+,0.11); 365 (M-15,0.15) and 349 (M-31,0.15) and characteristic mass fragments for the 9-, 12-, 13- and 16-OTMS stearates (3). These data confirm GC-MS studies for autoxidized linolenate hydroperoxides (3).

Hydroxy-cyclic peroxy octadecadienoates. GC of this fraction after silylation gave two small peaks of retention 2.11 and 2.19 relative to methyl linolenate. After hydrogenation and silylation, GC showed two peaks of approximately equal area with the same retentions (2.46 and 2.88) relative to methyl stearate as methyl 9,10,12- and 13,15,16-trihydroxyoctadecanoate respectively (TMS ethers) (3). TLC gave one UV active spot of R_f 0.40 relative to linolenate. UV showed a maximum at 233 nm for conjugated diene. IR (CS_2) hydroxy absorption (3575 cm^{-1}, free C-OH), (3700-3220 cm^{-1}, H-bonded C-OH), and (3005 cm^{-1}, olefinic-H), (988 and 950 cm^{-1}, conjugated *cis,trans* unsaturation). ^{1}H-NMR supported the IR analysis with signals for the methine proton of the carbinol carbon 3.84 ppm (m) (1H), and the conjugated olefinic system with absorption centered at 6.49 and 5.39 ppm (4H). Additional absorptions were observed for cyclic

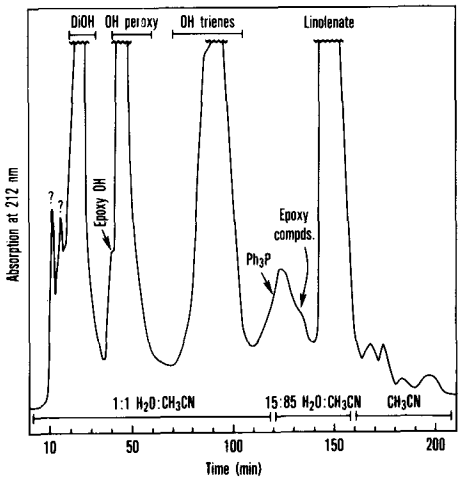


FIG. 1. Reverse phase (C-18 on Porasil B) HPLC chromatogram of Ph_3P-reduced linolenate, autoxidized at 27 C, PV 1113, (I) (flow 5 mL/min, detector set at 212 nm at 2 ABS units).

peroxide methine protons at 4.79 and 4.29 ppm (2H) and for cyclic peroxide methylene protons at 2.50 to 2.93 ppm (2H). A five-membered peroxide ring was assigned on the basis of the 360 MHz ^{1}H-NMR data of Chan et al. (7) for 16-hydroperoxy-13,15-peroxy-9,11-octadecadienoate and the ^{1}H-NMR data of Porter et al. (15) for methyl 6-hydroxy-7,9-peroxy-10,12-octadecadienoate. GC-MS m/e (rel intensity) after silylation: 396 (M-16,23) ion. After hydrogenation and silylation, GC-MS

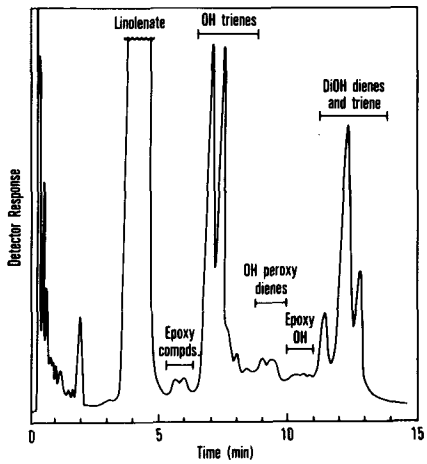


FIG. 2. Gas chromatogram of Ph_3P-reduced linolenate, autoxidized at 27 C, PV 1113, (I) (TMS ethers) (3% JXR packing in a 6 ft x 1/8 in. column, 180-250 C at 4 C/min temperature program).

indicated a mixture of 9,10,12- and 13,15,16-trihydroxystearates expected from methyl 9-hydroxy-10,12- and 16-hydroxy-13,15-cyclic peroxy octadecadienoates (3).

Epoxy-hydroxy octadecadienoates. GC showed three partially resolved peaks with retentions 2.41, 2.49 and 2.56 relative to linolenate. IR (CS_2) (3600 cm^{-1}, free C-OH), (3640-3140 cm^{-1}, H-bonded C-OH), and (3005 cm^{-1}, olefinic-H), (900 cm^{-1}, isolated *trans* unsaturation). ^{1}H-NMR supported the IR analysis with signals for the methine proton 4.18 ppm (1H), of the carbinol carbon and for isolated *trans* unsaturation 5.62 ppm. NMR also indicated the presence of a *cis* epoxide ring (14) with absorptions at 3.15 and 2.85 ppm (2H). GC-MS m/e (rel intensity) after Ph_3P reduction and silylation: 381 (M-15,11) ion corresponding to epoxy-hydroxy octadecadienoates and fragment ions, which indicated epoxy groups on carbon positions 9 and 10: 199 (5), 15 and 16: 71 (36), and 12 and 13: 111 (10). These data support the presence of epoxy-hydroxy or epoxy-hydroperoxy dienes in autoxidized linolenate. The epoxy-hydroxy compounds are apparently not artifacts of the Ph_3P reduction because MS of the silylated Ph_3P reduced hydroxy or hydroperoxy cyclic peroxides gave no evidence for these compounds (see below).

Dihydroxy octadecatrienoates. GC after silylation showed three peaks with retentions 2.69, 2.90 and 3.01 relative to linolenate. TLC showed two UV active spots of R_f 0.10 and 0.12 relative to linolenate. UV showed maxima at 229 and 267 nm for conjugated diene and triene, respectively. IR (CS_2) (3650-3120 cm^{-1}, bonded C-OH), (3005 cm^{-1}, olefinic) (998 and 950 cm^{-1}, conjugated *cis,trans*), (976 cm^{-1}, isolated *trans* unsaturation). ^{1}H-NMR supported the IR analysis with signals for the methine proton on the carbinol carbon 4.17 ppm and for the olefinic protons 5.61 ppm (center HC=CH). GC-MS m/e (rel. intensity) after silylation: 468 (M+,2) and 437 (M-31,5), for the TMS ethers of the dihydroxy triene. After hydrogenation and silylation, GC-MS m/e (rel intensity) showed evidence for dihydroxy stearate (OTMS ethers); 443 (M-31,18), with hydroxy on carbon-9:259 (96) and carbon-13:315 (39) on one end and on carbon-12:187 (36) and carbon-16:131 (63) on the other end. The spectral evidence supports a mixture of dihydroxy conjugated diene-triene and conjugated triene structures.

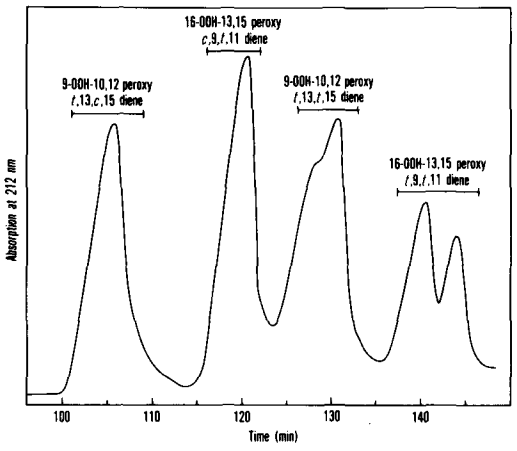


FIG. 3. 10-μ Silica HPLC chromatogram of hydroperoxy-cyclic peroxide mixture from linolenate, autoxidized at 40 C, PV 904, (II) (flow 5.0 mL/min, mobile 0.3% ethanol/hexane, detector set at 212 nm at 2 ABS units).

Chromatographic Fractionation of Nonreduced Autoxidized Linolenate (Fig. 3)

A hydroperoxy-cyclic peroxide mixture was first obtained by ordinary silicic acid column chromatography of linolenate autoxidized at 40 C (sample II). This mixture was then separated by HPLC on microsilica into positional and geometric isomers of the 9- and 16-hydroperoxy-cyclic peroxide dienes (Fig. 3). The *cis,trans* isomers of the hydroperoxy-cyclic peroxides were eluted before the *trans,trans* isomers in the same order as previously observed for the dienol isomers of linolenate hydroperoxides (1). The *trans,trans* isomers were also partially resolved apparently into their epimeric forms with respect to the hydroperoxy-bearing carbon, since the left- and right-side components of the partially resolved peaks in Figure 3 had similar ^{1}H-NMR characteristics, except for slightly different shifts for the proton at the hydroperoxy-bearing carbon.

Weights of oxidation products were estimated from fractions with the same functional group eluted by silicic acid column chromatography of autoxidized linolenate (Table I). Total recovery after silicic acid chromatography was ca. 98%. From peak areas in Figure 3, the relative composition is 27.8 and 24.5% for the respective 9-hydroperoxy *cis,trans* and *trans, trans* isomers, and 27.0 and 20.7% for the 16-hydroperoxy *trans,cis* and *trans,trans* isomers.

9-Hydroperoxy-10,12-peroxy-trans-13,cis-15-octadecadienoate. TLC (1:1 diethyl ether/hexane) showed one UV active spot of R_f 0.53 relative to linolenate. GC after reduction with Ph_3P and silylation gave a small peak of retention 2.11 relative to linolenate. GC of the silylated hydrogenated derivative gave one peak

TABLE I

Weight-Percent Composition of Silicic Acid Fractions from Two Samples of Linolenate Autoxidized at 40 C (II)

Fraction identification	PV 904	PV 1286
Linolenate	87.9	74.8
Epoxy unsaturated esters	0.2	0.3
Monohydroperoxides	3.5	8.4
Hydroperoxy-cyclic peroxides	3.8	7.7
Epoxy-hydroxy dienes	<0.1	<0.1
Dihydroperoxy compounds	0.9	2.9
Unidentified polar materials	3.7	5.9

with same retention relative to methyl stearate as methyl 9,10,12-triOTMS stearate. UV showed a maximum at 231 nm (E_m 24,200) for conjugated diene. IR (neat) (3700-3100 cm^{-1}, H-bonded OH or OOH), (3005 cm^{-1}, olefinic), (990-950 cm^{-1}, conjugated *cis,trans* unsaturation) and (900 cm^{-1}, peroxide) (16).

^{1}H-NMR data (Table II) are consistent with those of Chan (7) in establishing the cyclic 5-membered ring. All other assignments were corroborated with decoupling experiments. ^{13}C-NMR assignments (Table III) also confirm the identity of the title compound.

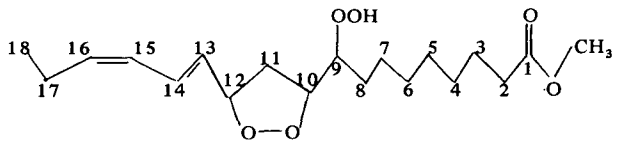
MS m/e (rel intensity) after reduction with Ph_3P and silylation: 396 (M-16,41) and 397 (M-15,10), for the hydroxy-cyclic peroxide as TMS ether, with OTMS on carbon-9: 259 (54). As suggested by GC retention data, MS after hydrogenation and silylation gave the expected methyl 9,10,12-triOTMS stearate derivative (3).

16-Hydroperoxy-13,15-peroxy-cis-9,trans-11-octadecadienoate. TLC (1:1 diethyl ether/hexane) showed one UV active spot of R_f 0.50 relative to linolenate. GC of the silylated Ph_3P-reduced derivative showed a peak of retention 2.19 relative to linolenate. GC of the silylated-hydrogenated derivative showed only one peak with the same retention as methyl 13,15,16-triOTMS stearate relative to methyl stearate. UV (methanol) showed a maximum at 234 nm (E_m 28,700) for conjugated diene. IR (CS_2) (3530 cm^{-1}, free OH or OOH), (3720-3200 cm^{-1}, bonded C-OH or C-OOH), and (3005 cm^{-1}, olefinic-H), and (982 and 950 cm^{-1}, conjugated *cis,trans* unsaturation). The ^{1}H-NMR (Table IV) and ^{13}C-NMR (Table V) assignments confirm the structure of the title compound. MS m/e (rel intensity) after reduction with Ph_3P and silylation: 396 (M-16,10) and 397 (M-15,3), for the hydroxy-cyclic peroxide as OTMS ether, with OTMS on carbon-16: 131 (76). MS after hydrogenation and silylation showed the expected derivative methyl 13,15,16-triOTMS stearate (3).

Mixture of 9-hydroperoxy-10,12-peroxy and 16-hydroperoxy-13,15-peroxy octadecadieno-

TABLE II

^{1}H-NMR of 9-Hydroperoxy-10,12-peroxy-*trans*-13,*cis*-15-octadecadienoate



δ ppm	Multiplicity[a]	J/Hz	Number of protons	Assignment
9.43	br, s		1	OOH
6.67	dd	15,11		H-14
6.01	br, t	11,10	4	H-15
5.62	dd	15,8		H-13
5.50	m			H-16
4.80	ddd	8,8,7.5	1	H-12
4.45	ddd	8,5.5,4	1	H-10
4.15	m	–	1	H-9
3.66	s	–	3	CH_3O
2.84	ddd	12,8,7.5	–	H-11β
2.47	ddd	12,8,5.5	–	H-11α
2.30	t	–	–	H-2
2.07	d	–	–	H-17
1.85-1.15	m	–	–	H-3-8
1.00	t		–	H-18

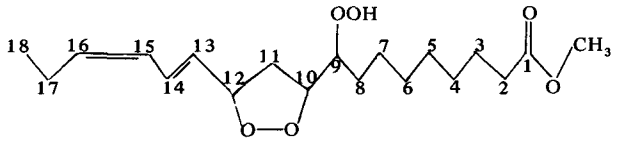
[a]Multiplicity: br = broad, s = singlet, d = doublet, m = multiplet, t = triplet.

ates. TLC (1:1 diethyl ether/hexane) showed a UV active major spot of R_f 0.46 relative to linolenate. GC of the silylated hydrogenated derivative showed two peaks with retentions 2.46 (70%) and 2.88 (30%) relative to methyl stearate for methyl 9,10,12- and 13,15,16-triOTMS stearates, respectively. Apparently, the second peak tailed into the third (Fig. 3). UV showed a maximum at 235 nm (E_m 27,900) for conjugated diene.

16-Hydroperoxy-13,15-peroxy-trans-9,trans-11-octadecadienoate. TLC (1:1 diethyl ether/hexane) gave one UV active spot of R_f 0.44 relative to linolenate. GC of the silylated hydro-

TABLE III

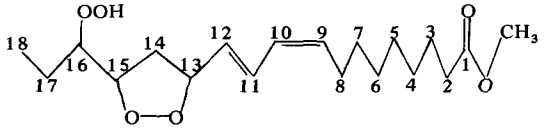
^{13}C-NMR 9-Hydroperoxy-10,12-Peroxy-*trans*-13,*cis*-15-octadecadienoate



δ ppm	Carbon assignment (17)
174.3	1
136.8,131.8,126.6,126.2	13-16
86.0	9
83.8	10,12
83.0	
51.3	OCH_3
41.3	11
34.1	2
29.6	4-8
29.0	
25.6	17
24.9	3
14.0	18

TABLE IV

^{1}H-NMR of 16-Hydroperoxy-13,15-Peroxy-*cis*-9,*trans*-11-octadecadienoate



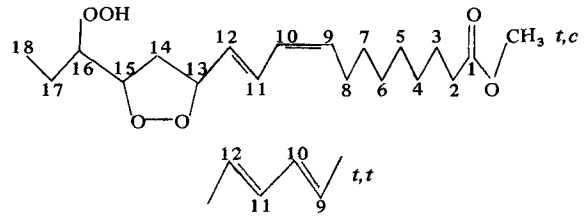
δ ppm	Multiplicity[a]	J/Hz	Number of protons	Assignment
9.38	br, s		1	OOH
6.67	dd	15,11		H-11
6.01	br, t	11,10	4	H-10
5.62	dd	15,8		H-12
5.55	m	10		H-9
4.80	ddd	8,8,8	1	H-13
4.49	ddd	9,7,5	1	H-15
4.15[b]	ddd	9,7,3	1	H-16
3.66	s		3	CH_3O
2.84	ddd	12,8,9	–	H-14β
2.47	ddd	12,8,5	–	H-14α
2.30	t	–	–	H-2
2.08	d	–	–	H-8
1.89-1.20	m	–	–	H-7-3
1.03	t		–	H-18

[a]See footnote a, Table II.

[b]The shift difference between protons for C-16 (Table VI) is apparently due to different epimeric forms.

TABLE V

^{13}C-NMR of 16-Hydroperoxy-13,15-peroxy-9,11-octadecadienoate and Isomers



δ *t,c* ppm	δ *t,t* ppm	C assignments (17)
174.3		1
135.2,131.8,127.3,126.3	127.3,126.6	9-12
87.4	87.2	16
83.5,82.9	83.8,82.6	13,15
51.4	51.4	OCH_3
41.3	43.7	14
34.1	34.2	2
29.4,29.1	29.1	4-7
27.8	–	8
25.0	25.0	3
22.8	22.1	17
10.2	10.2	18

genated derivative showed only one peak with the same retention relative to methyl stearate as methyl 13,15,16-triOTMS stearate. UV (methanol) showed the maximum at 233 nm (E_m 28,800) for conjugated diene. IR (CS_2) indicated hydroxy or hydroperoxy absorption (3530 cm^{-1}, free OH or C-OOH), (3700-3140 cm^{-1}, bonded C-OH or C-OOH) and (3005 cm^{-1}, olefinic-H), and (984 cm^{-1} conjugated *trans,trans* unsaturation).

^{1}H-NMR data (Table VI) confirm the identity of the title structure.

MS m/e (rel intensity) after reduction with Ph_3P and silylation showed OTMS on carbon-16: 131 (100); MS after hydrogenation and silylation showed the expected derivative for methyl 13,15,16-triOTMS stearate (3).

Dihydroperoxy octadecatrienoates. Silicic acid chromatography gave a dihydroperoxide fraction, more polar than the hydroperoxy-cyclic peroxide mixture, upon elution with 60:40 diethyl ether/hexane. TLC (1:1 diethyl ether/hexane) showed two UV active spots of R_f 0.31 and 0.27 relative to linolenate, that gave a strongly KI positive peroxide test (18). UV (methanol) showed maxima at 235 nm and 267 nm (triplet) for conjugated diene and triene, respectively. IR (CS_2): (3530 cm^{-1}, free OH or OOH), (3712-3210 cm^{-1}, bonded C-OH or C-OOH), (3005 cm^{-1}, olefinic-H), 988 and 950 cm^{-1}, conjugated *cis,trans*) and (968 cm^{-1}, isolated *trans*). ^{1}H-NMR ($CDCl_3$, 100 MHz) supported the IR analysis with signals for the methine protons on the hydroperoxy-containing carbons 4.40 ppm (2H), and for olefinic system 6.28-5.26 ppm. There was no indication of hydroxy-containing carbons (3.8-4.2 ppm). GC-MS after hydrogenation and silylation showed evidence for dihydroxystearate (TMS ethers): 443 (M-31,10) ion, with the hydroxy on carbon-9: 259 (29) and carbon-13: 315 (15) on one end of the molecule and on carbon-12: 187 (15) and carbon-16 131 (48) on the other end. The UV, IR, NMR and MS data support dihydroperoxy conjugated diene-triene and conjugated triene structures.

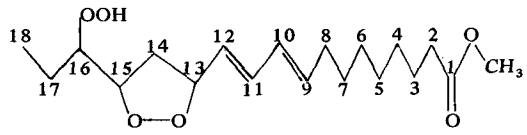
DISCUSSION

Secondary oxidation of hydroperoxides produces a complex mixture of volatile and nonvolatile compounds that may contribute either directly or as precursors to flavor deterioration in unsaturated fats (19-21). In this work, hydroperoxy-cyclic peroxides have been identified as major secondary products in autoxidized linolenate and dihydroperoxides as minor products. Other minor products identified after reduction (Ph_3P) include epoxy and epoxy-hydroxy esters.

Our previous studies (3,22,23) of autoxidized methyl linolenate showed the formation of significantly more 9- and 16-hydroperoxides (75-81%) than of 12- and 13-hydroperoxides (18-25%). The evidence of significant amounts of 9,10,12- and 13,15,16-trihydroxystearates in

TABLE VI

^{1}H-NMR of 16-Hydroperoxy-13,15-peroxy-*trans*-9,*trans*-11-octadecadienoate



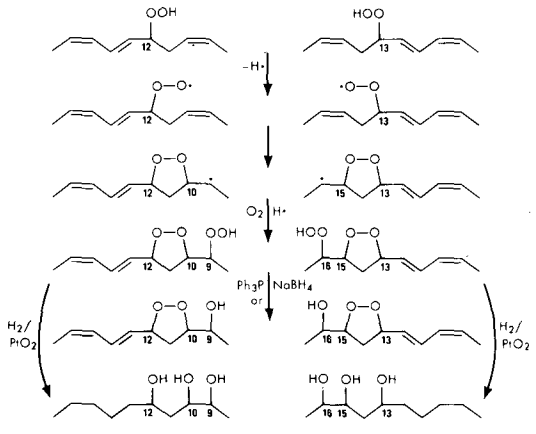
δ ppm	Multiplicity[a]	J/Hz	Number of protons	Assignment
8.98	br, s		1	OOH
6.65	dd	15,11		H-11
5.99	m	15,11	4	H-10
5.60	m	15,8		H-12
5.46	m	15,-		H-9
4.77	ddd	8,8,8	1	H-13
4.47	m	8,8	1	H-15
3.86[b]	m	8,5	1	H-16
3.66	s		3	CH_3O-
2.75	m	12,8,5	–	H-14β H-14α
2.30	t	–	–	H-2
2.09	d	–	–	H-8
1.84-1.17	m	–	–	H-7-4
0.87	t		–	H-18

[a]See footnote a, Table II.

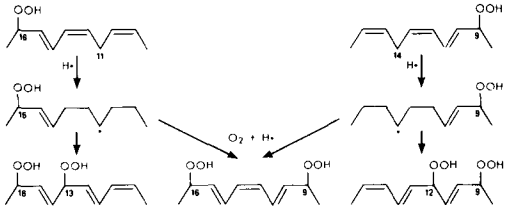
[b]The shift difference between protons for C-16 (Table VI) is apparently due to different epimeric forms.

the hydrogenated derivatives supported cyclization of the internal 12- and 13-hydroperoxides of linolenate. The results reported here provide more direct evidence for cyclization of the 12- and 13-hydroperoxides into hydroperoxy-cyclic peroxides. The 1,3-cyclization scheme previously formulated by Pryor et al. (24) can be advanced for the formation of 9- and 16-hydroperoxy-cyclic peroxides identified in this work and the 16-hydroperoxy-cyclic peroxide reported by Chan et al. (7) (Scheme I). This type of cyclization requires the presence of a *cis* double bond homo-allylic to the internal 12- or 13-hydroperoxides. Formation of 5-membered cyclic peroxides is consistent with the model studies of Porter et al. (15) and the rules of cyclization reported by Baldwin (25).

The minor dihydroperoxides identified in this study are those expected from the secondary oxidation of the terminal 9- and 16-hydroperoxides of methyl linolenate. According to Scheme II, abstraction of hydrogen from the doubly allylic methylene groups 11 and 14 produces pentadienyl radicals. Oxidation at either end of these pentadienyl radicals form the 9,12- and 13,16-dihydroperoxides with conjugated diene-triene systems and the 9,16-dihydroperoxides with conjugated triene system. The structures of these dihydroperoxides are consistent with those of the dihydroxy



SCHEME I.



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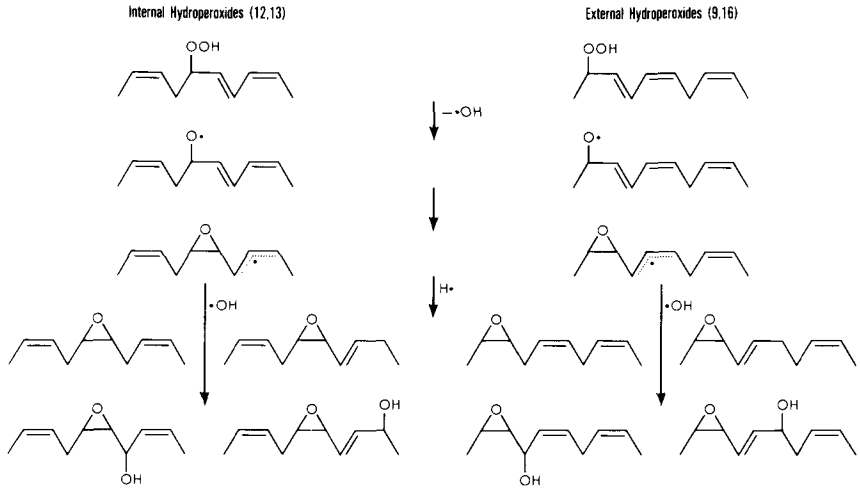

SCHEME III.

esters identified after reduction and of the dihydroperoxides identified directly. Because these dihydroperoxides are minor products, they would not be expected to affect significantly the relative proportion of the 9- and 16-hydroperoxides in autoxidized linolenate.

The formation of minor epoxy and epoxy-hydroxy esters can be explained by a mechanism similar to that previously advanced for autoxidized linoleate (19,20,26). 1,2-Cyclization of linolenate hydroperoxides would produce nonconjugated 12-epoxydienes from the internal isomers and a mixture of 9- and 16-epoxydienes from the external isomers (Scheme III). Further hydroxylation (or hydroperoxidation) would produce the corresponding epoxy-hydroxy (or epoxy-hydroperoxy) dienes. The structures of these epoxy esters are supported by the chromatographic and spectral data. Epoxy-hydroxy diene structure is further supported by MS data, but additional evidence is necessary to establish position of double bonds and epoxy groups.

The possible contribution of secondary oxidation products to flavor deterioration in unsaturated fats has been discussed previously (19-21). Allylic epoxy aldehydes recently identified in oxidized butterfat (27) and trilinolein (28) can be derived from epoxy-hydroperoxides identified by Gardner et al. (29) among the secondary oxidation products of methyl linoleate hydroperoxides. Similarly, the hydroperoxy-cyclic peroxides were suggested as precursors of volatile compounds produced by hydroperoxides from autoxidized and photosensitized-oxidized methyl linolenate (21). The dihydroperoxides identified in this study can also serve as precursors of volatile compounds by carbon-carbon cleavage on either side of the alkoxy radicals postulated as intermediates in the decomposition of monohydroperoxides (21,30). Current research is aimed at determining more directly the contribution of these secondary oxidation products of linolenate to flavor deterioration. The biological role of these secondary oxidation products may also become important if some of the symptoms of intracellular lipid oxidation can originate from the prostaglandin-related cyclic peroxides identified in this and other studies (4,5,7,24).

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Effect of Polyestradiol on Lecithin:Cholesterol Acyltransferase in Male and Female Rats

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ABSTRACT

The effects of two doses of polyestradiol phosphate on lecithin:cholesterol acyltransferase activity and on liver and plasma cholesterol levels have been studied on female and male rats. Both treatments increased the hepatic content of esterified cholesterol, but the LCAT activity expressed as a percentage of cholesterol esterification was unaltered. The progress of esterification was not affected by the administration of the hormone. The LCAT activity in terms of the initial rate of esterification was decreased by the high dose of estradiol. This decrease was associated with a reduction of free plasma cholesterol level, as there is a significant positive correlation between these two parameters. The findings suggest that the increased esterified cholesterol in liver of estradiol-treated rats is not mediated by an alteration in the LCAT activity.

INTRODUCTION

The liver plays an important role in the uptake and degradation of lipoproteins and, subsequently, in the clearance of plasma cholesterol (1). The remnants of very low density lipoproteins (VLDL) and chylomicrons are quantitatively the most important particles in the delivery of esterified cholesterol to the liver (2,3). This delivery usually has been attributed to high density lipoproteins (HDL) which plays a major role in the transport of cholesterol from peripheral tissues to the liver (4,5). Liver secretes a nascent lamellar HDL (6) which increases the content of its cholesteryl esters via the removal of free cholesterol from tissues and through the action of the lecithin:cholesterol acyltransferase (LCAT) (7). The cholesteryl esters as HDL components are then taken up by the liver and other tissues. Low density lipoproteins (LDL), a minor substrate for the LCAT, seem to be implicated mainly in the transport of esterified cholesterol to extrahepatic tissues (8).

It has been reported previously that the administration of pharmacological doses of estrogens increases the hepatic content of cholesteryl esters (9-11) and produces an altered lipoprotein pattern, resulting in a decrease of free and esterified cholesterol plasma levels and in hypertriglyceridemia (9,12-15). The mechanisms by which the hormone induces this massive hepatic cholesteryl ester deposition have been studied to some extent. The current experiments were designed to determine whether estradiol treatments on rats alter the activity of LCAT, the enzyme implicated in the turnover and catabolism of cholesterol-bearing lipoproteins.

MATERIALS AND METHODS

Animals and Experimental Design

Male (170-220 g) and female (140-190 g) Wistar rats were obtained from the University of Bilbao animal laboratory. The animals were maintained at a 12-hr light/12-hr dark cycle, and were fed a standard laboratory chow.

Rats were treated daily with different doses of polyestradiol phosphate by the subcutaneous route. One group received 0.3 mg/100 g body weight/day for five days (N+E). Another group received 1.6 mg/100 g body weight/day for three days (N+EE).

Animals were deprived of food for 18 hr overnight and killed by exsanguination under light ether anesthesia. The collected blood specimens were immediately placed on ice and centrifuged at 4 C for 15 min. Heparin was used as anticoagulant. Quantitation of LCAT activity was performed immediately. Aliquots of plasma were kept frozen at -20 C until assay of cholesterol.

Chemicals

Polyestradiol phosphate (Estradurin) was purchased from Abelló S.A. (Madrid, Spain). [7α-^{3}H] cholesterol, sp act 9.5 Ci/mmol from Radiochemical Centre (Amersham, U.K.); Ellman reagent, 5,5′dithiobis-2-nitrobenzoic acid (DNTB) was obtained from Boehringer Mannheim Gmbh (W. Germany); crystalline human albumin from Behring-Werke AG (Marburg-Lahn, W. Germany); naphthalene, 2-mercaptoethanol and 2,5-diphenyloxazole (PPO) from Merck AG (Darmstadt, W. Germany); 1,4-di-2,5-phenyloxazolylbenzene (POPOP) from Hopkin & Williams Ltd., Chadwell Heath (Essex, U.K.).

All chemicals used were reagent grade.

Analytical Procedures

Enzyme assay. LCAT activity was estimated as described by Stokke and Norum (16). Thirty μL of [^{3}H] cholesterol-albumin suspension and 20 μL of DTNB (dissolved in 0.1 M sodium phosphate buffer, pH=7.1, final concentration 1.4 mM) were added to 100 μL of plasma. During a preincubation period of 4 hr, the labeled cholesterol equilibrated with endogenous lipoprotein cholesterol. The enzyme was reactivated by the addition of 20 μL of 0.1 M 2-mercaptoethanol and the activated samples were then incubated for 20 min. At the end of the incubation period, the enzyme reaction was stopped by adding 20 vol of chloroform/methanol (2:1) and left overnight at room temperature. The precipitate was washed three times with chloroform/methanol (2:1) and the combined filtrates were evaporated under a stream of nitrogen. The dry residue was dissolved in a small amount of chloroform and dried again. Lipid classes were separated by thin layer chromatography (TLC) according Krell and Hashim (17). Cholesterol fractions were located by exposure to iodine vapors and labeled free and esterified cholesterol were scraped into liquid scintillation counting vials. The liquid scintillator contained 100 g of naphthalene, 7 g of PPO, 0.3 g of POPOP, 1,000 mL of dioxane and 200 mL of distilled water. Ten mL of this solution was used per counting vial. Radioactivity was measured in an automatic liquid scintillation counter.

Analysis of liver and plasma lipids. Lipids were extracted from liver according to the method of Folch et al. (18) and separated by TLC (17). Cholesterol determinations were done as described by Liebermann-Burchard (19).

Plasma cholesterol was measured by an enzymatic method (Carlo Erba, Milano, Italy).

Statistical calculations were made using Student's t-test and the method of least squares for the correlation of coefficient r.

RESULTS

The results of experiments showing the effects of polyestradiol phosphate on cholesterol concentrations in rat liver and plasma are expressed in Tables I and II. As expected, estradiol treatments tended to lower plasma cholesterol levels. The greatest effect was observed with the high dose of the hormone, which produced marked decreases in both sexes. The decreases were statistically significant in female free cholesterol and male esterified cholesterol. The basal values of cholesterol were greater in the plasma of female than male rats. In liver, both estradiol doses produced a strong rise in the cholesteryl ester content in female and male rats. The percentage of increase was ca. 65% with the low dose and 200% with the high dose. Free cholesterol levels were not different from those detected in the control rats, which is not in agreement with preliminary reports (13,20).

Tables I and II summarize the values of LCAT activity in female and male rats. LCAT activity was calculated as the percentage of radioactivity in the cholesteryl fraction. From this parameter and the basal concentration of unesterified cholesterol, the initial rate of esterification was calculated as μmol/min/mL. Compared to that of the control group, the rate of cholesterol esterification was significantly decreased in rats treated with the high dose of estradiol, whereas the percentage of esterification was similar in all groups. Female rats presented a higher basal rate of esterification than male rats according to previous data (21).

Figure 1 is a plot of the initial rates of esterification against the respective plasma free

TABLE I

Plasma and Liver Cholesterol Concentrations and LCAT Activity in Female Control Rats, Female Rats Treated with Low Dose of Estradiol (N+E) and High Dose of Estradiol (N+EE)[a]

Females	Liver		Plasma			
Group	Chol. free (mg/g tissue)	Chol. esters (mg/g tissue)	Chol. free (mg/100 mL)	Chol. esters (mg/100 mL)	LCAT (% esterif.)	LCAT (μmol/mL/min)
Control	1.93 ± 0.07[b]	0.176 ± 0.02	15.27 ± 0.9	39.16 ± 1.7	10.88 ± 0.49	0.215 ± 0.011
N+E	1.95 ± 0.04	0.292 ± 0.04	13.09 ± 1.1	37.12 ± 2.1	12.30 ± 0.73	0.202 ± 0.008
	N.S.	$p \leq 0.025$	N.S.	N.S.	N.S.	N.S.
N+EE	1.84 ± 0.02	0.554 ± 0.01	11.90 ± 0.98	33.79 ± 2.4	11.40 ± 0.59	0.161 ± 0.006
	N.S.	$p \leq 0.001$	$p \leq 0.01$	N.S.	N.S.	$p \leq 0.005$

[a]The LCAT activity was expressed as initial rate of esterification (μmol/mL/min) and as percentage of cholesterol esterification.

[b]Mean ± standard error (n=5); N.S. = not significant.

TABLE II

Plasma and Liver Cholesterol Concentrations and LCAT Activity in Male Control Rats, Male Rats Treated with Low Dose of Estradiol (N+E) and High Dose of Estradiol (N+EE)[a]

Males	Liver		Plasma			
Group	Chol. free (mg/g tissue)	Chol. esters (mg/g tissue)	Chol. free (mg/100 mL)	Chol. esters (mg/100 mL)	LCAT (% esterif.)	LCAT (μmol/mL/min)
Control	1.62 ± 0.15[b]	0.227 ± 0.05	9.34 ± 0.72	39.50 ± 2.32	10.78 ± 0.40	0.110 ± 0.016
N+E	1.64 ± 0.05	0.379 ± 0.04	10.93 ± 1.13	36.32 ± 3.55	10.58 ± 0.62	0.145 ± 0.009
	N.S.	$p \leqslant 0.05$	N.S.	N.S.	N.S.	N.S.
N+EE	1.69 ± 0.02	0.668 ± 0.09	7.85 ± 0.50	32.14 ± 2.25	10.22 ± 0.61	0.078 ± 0.005
	N.S.	$p \leqslant 0.001$	N.S.	$p \leqslant 0.05$	N.S.	$p \leqslant 0.05$

[a]The LCAT activity was expressed as initial rate of esterification (μmol/mL/min) and as percentage of cholesterol esterification.

[b]Mean ± standard error (n=5); N.S. = not significant.

cholesterol concentrations. The correlation between these two parameters has been calculated in normal and treated rats taken as a group. The pattern obtained showed a highly positive correlation in both sexes (female, r=0.78, male, r=0.93). LCAT activities of rats treated with high doses fall into the lower range in keeping with their cholesterol content. Similar positive correlations were found in early experiments (22-24). As we have used a method for determining the LCAT activity which utilizes autologous lipoproteins as substrate, it was not possible to exclude the dependence between initial rate of esterification and plasma free cholesterol levels. This fact suggests that the decreased LCAT activity observed in treated rats would be secondary to a reduced concentration of substrate lipoprotein cholesterol. The use of the percentage esterification is more suitable for comparative studies in which the mean values of plasma are altered (25). Therefore, the percentage values would indicate that LCAT was similar in control and treated rats, in spite of the different absolute rate of esterification. The rise of cholesterol esterification with time in control and treated female rats is indicated in Figure 2. Estradiol treatments did not produce significant differences in the LCAT activity during the course of incubation.

DISCUSSION

The level of hepatic cholesteryl esters is a complex function of the rate of several processes, including the uptake of cholesterol from HDL, LDL and "remnant particles," cholesterogenesis and the degradation of this lipid from the liver, which is, in part, hydrolyzed and subsequently degraded to bile acids (26). Female sex hormones seem to alter these mechanisms.

It has been postulated that the content of cholesteryl esters in the liver reflects net plasma cholesterol uptake by the hepatocyte (27). Plasma cholesteryl esters derive from two different sources. Those of triglyceride-rich

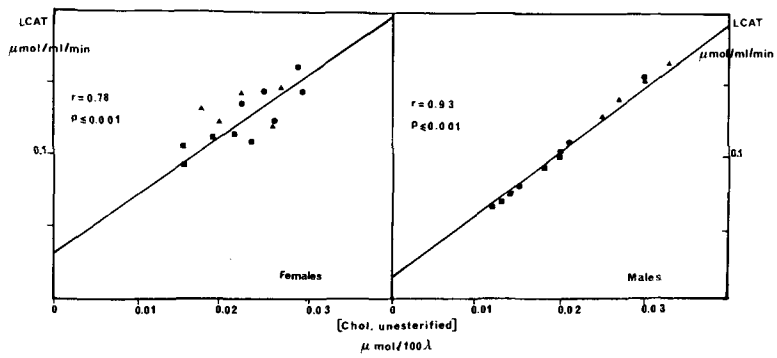

FIG. 1. Relationship between the concentration of unesterified cholesterol and the initial rate of cholesterol esterification in female (*left*) and male (*right*) rats. ●=control rats, ▲=rats treated with low dose of estradiol and ■=rats treated with high dose of estradiol.

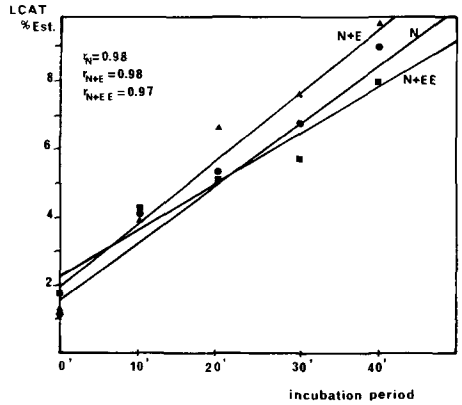

FIG. 2. Esterification of plasma cholesterol during a 40-min incubation period in control and treated female rats. For this study the same components were used as described elsewhere. From a total incubation volume of 50 μL, aliquots were removed at suitable intervals and pipetted into 20 vol of chloroform/methanol (2:1). Lipids were extracted and LCAT activity was determined as described in Materials and Methods. •=control rats, ▲=rats treated with low dose of estradiol (N+E) and ■=rats treated with high dose of estradiol (N+EE).

lipoproteins are of hepatic and intestinal origin and those of HDL and LDL are produced mainly by the action of LCAT. As stated earlier, our assumption that LCAT could be altered by the estrogen treatment was based on the well known ability of the liver to take up and degrade lipoproteins substrate of the enzyme; thus, esterified cholesterol might be accumulated because of a rise in the sterol uptake. In normal rats, only a small amount of plasma LDL is taken up by the liver (28), whereas it has been generally accepted that HDL retrieves cholesterol to the liver. However, the quantitative contribution of HDL in the delivery of esterified cholesterol to this organ seems to be controversial. Sigurdsson et al. (29) have recently reported that only ca. 7% of rat HDL is degraded directly by the liver, a new role arising for HDL in returning cholesterol mainly to extrahepatic tissues. On the other hand, Ose et al. (30) studied the distribution of ^{125}I-labeled HDL in various organs and found that steroidogenic tissues, i.e., adrenals, showed the highest uptake/mg weight; nevertheless, the liver removed the largest portion of the injected labeled lipoproteins (14, 6%). Although it is difficult to evaluate to what extent HDL transports cholesterol to the liver, it seems likely that the percentage of removal of the major cholesterol-bearing lipoproteins in rats is a factor to be considered.

Our data showed that both of the treatments used increased the hepatic cholesteryl esters but neither modified the LCAT activity in terms of percentage esterification (Tables I and II) nor affected the progress of the enzyme reaction with time (Fig. 2). However, the high dose significantly decreased the initial rate of cholesterol esterification in both sexes (Tables I and II). These results suggest that the augmented cholesteryl esters in liver of estradiol-treated rats is not mediated by an alteration in the LCAT activity and, in consequence, the turnover of HDL/LDL is not affected.

Recent studies showed that liver membranes of rats treated with pharmacologic doses of 17-α-ethinyl estradiol produced specific saturable binding sites for human ^{125}I-LDL (31). It has been also observed that, in the perfused liver, estrogens increase the uptake for rat ^{125}I-HDL rich in apo-E but not for human ^{125}I-HDL (32). The presence of apo-E seems to be a determining factor in the transfer of cholesterol into the liver (33). Since human LDL and rat HDL are particles containing apo-E, the commented effects produced by the estradiol (31,32) could be a function of their apo-E content. In rat HDL, only ca. 5% of the total protein mass is apo-E (32), whereas the bulk of HDL is rich in apo-A-I protein. Hence, a small fraction of plasma HDL would be affected by estrogens, which is not in disagreement with our results.

Chylomicrons and VLDL released by the liver and the intestine constitute an alternative pool of plasma cholesteryl esters. These lipids are formed through the action of the acylcoenzyme A:cholesterol acyltransferase, the main enzyme responsible for the cholesterol esterification in liver, which is reported to be stimulated by estrogens (34). Both lipoproteins are degraded by the enzyme lipoprotein lipase and the "remnant particles" are quickly cleared from the blood, mainly by the hepatocyte. If the changes produced in liver by estradiol are related to any alteration in this process, it is still to be resolved.

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Zinc Deficiency-Induced Changes in the Composition of Microsomal Membranes and in the Enzymatic Regulation of Glycerolipid Synthesis

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ABSTRACT

The effects of zinc deficiency and/or castration on the lipid composition of microsomal membranes of liver, small intestine and testes were studied in rats. The results showed that feeding a zinc-deficient diet to castrated rats decreased phospholipid content and consequently increased the cholesterol-to-phospholipid ratio in liver microsomes. An increase in cholesterol-to-phospholipid ratio occurred also in small intestine and testes microsomes from rats fed the zinc-deficient diet. It is postulated, therefore, that zinc deficiency alters the lipid composition and fluidity of microsomal membranes. Zinc deficiency also affected the activities of the enzymes involved in the formation of triglycerides and phospholipids. There was a large increase in total and specific activity of phosphatidate phosphatase and the changes in the total activity of choline phosphotransferase correlated well with the changes observed in serum or liver triglycerides and phospholipids. Stearoyl CoA desaturase, which is a control enzyme for hepatic lipogenesis, was also increased by more than 200% in zinc-deficient states, as was the diglyceride content of hepatic microsomes. These results indicate that the increased synthesis of triglycerides and phospholipids in zinc deficiency may be due to the increased availability of substrates as well as to increased activities of the enzymes involved in these processes.

INTRODUCTION

Zinc deficiency in experimental animals produces a number of biochemical abnormalities (1), and it is even more deleterious when accompanied by testosterone deficiency (2). Exogenous supply or endogenous production of testosterone is associated with increased zinc levels (3). Recently (4), we have demonstrated the effects of zinc deficiency with or without testosterone deficiency on lipid metabolism. Rats fed diets deficient in zinc for a period of 45 days showed a marked increase in serum and liver triglycerides and a smaller increase in cholesterol and phospholipids. Microsomal phospholipids, in addition to being structural and storage components, play a role in the regulation of enzyme activities and control of membrane permeability (5). Since zinc deficiency has been shown to change phospholipids of liver and serum (6), we studied first the effects of dietary zinc on the lipid composition of microsomal membranes in liver, testes and small intestine. Testis was chosen because of the relationship between zinc and testicular function (7) whereas the small intestine plays an important role in zinc transport (8). Cholesterol level was studied because of its known metabolic and structural functions and also as a regulator of membrane fluidity (5).

The second objective of this study was to understand the hepatic regulation of triglycerides and phospholipids in zinc-deficient rats. Diglycerides, as substrates, and the enzyme which catalyzes the conversion of phosphatidic acid into diglycerides, phosphatidate phosphatase (EC 3.1.3.4), may be important in triglyceride synthesis (9). However, diglycerides are also utilized as substrates for the synthesis of phosphatidylcholine and phosphatidylethanolamine. Thus, enzymes like *sn*-glycerol 3-phosphate acyltransferase (EC 2.3.1.15), diacylglycerol acyltransferase (EC 2.3.1.20), choline phosphotransferase (EC 2.7.8.2) and ethanolamine phosphotransferase (EC 2.7.8.1) may also be involved in the expression of the effects of zinc deficiency on lipid metabolism. This view is supported by results from experiments in vivo and in vitro with isolated rat hepatocytes (10) and liver slices (11). Jeffcoat et al. (12) have shown that hepatic lipogenesis is controlled by stearoyl-CoA desaturase which influences triglyceride production indirectly by regulating fatty acid synthesis. Therefore, activities of these enzymes were measured and changes in lipids and enzymes are interpreted in the context of zinc deficiency.

MATERIALS AND METHODS

Experimental Design

Forty-two male Sprague-Dawley rats, weighing 100 ± 18 g, were randomly assigned to six groups. Castration was performed by mid-line abdominal incision in 28 rats under anesthesia with 3.5% chloral hydrate injected intraperitoneally.

Group 1 consisted of seven rats which were fed a purified Zn-supplemented diet containing 100 mg/kg zinc as zinc chloride in addition to sucrose 15%, egg white 19.9%, soy flour 3%, RP vitamin mix 2%, RP mineral without zinc 5.0%, DL-methionine 0.15%, choline chloride 0.2%, corn oil 5.0%, lard 5.0%, dextrin 44.6%, and biotin 0.0005% (Ralston Purina Co., St. Louis, MO). Group 2 consisted of seven rats fed a Zn-deficient diet (1.2 ppm Zn) and deionized water ad libitum. Zn content was the only difference between the two diets. Group 3 consisted of five castrated rats on Zn-supplemented diet and group 4 contained seven castrated rats on Zn-deficient diet. Groups 5 and 6 consisted of eight castrated rats each, which were also fed Zn-supplemented and Zn-deficient diets, respectively, and after four weeks the animals received an injection of testosterone enanthate (20 mg I.M.). All the animals were maintained on the diets for two more weeks. Rats on Zn-deficient diets became hypophagic after 2-3 days on the diet. To ensure equicaloric consumption of food, pair-feeding procedures were instituted. Food allowed to the control animals (group 1) and groups 3 and 5 on Zn-supplemented diet was equal to the amount consumed by the Zn-deficient groups (groups 2, 4, 6). Animals were housed in stainless steel cages and all materials with which the rats came in contact were stainless steel, glass or plastic. Rats were killed by a blow on the head. Livers and a section of jejunum from all groups and testes from rats in groups 1 and 2 were immediately removed and placed into iced 0.25 M sucrose solution.

Preparation of Subcellular Fractions

Livers were homogenized in 0.33 M sucrose, 0.01 M Tris (pH 7.4) in a Potter-Elvehjem homogenizer (1 g liver/10 mL). The microsomal 105,000 G supernatant fractions were isolated from the liver homogenates as described by Vereyken et al. (13). The microsomal pellets were resuspended in 0.125 M KCl and 0.1 M Tris (pH 7.4). Microsomal membranes from small intestine were harvested by the procedure described by Ray (14). The mucosa was desquamated, scraped (15), and homogenized gently in a medium containing 250 mM sucrose, 0.2 mM EDTA and 2 mM piperazine-N, N^1-bis(2-ethanesulfonic acid) buffer (pH 7.0) using a loose pestle. The homogenate was centrifuged at 8,000 G for 5 min. The supernatant was then centrifuged at 100,000 G for 2 hr and the pellet was resuspended in the homogenizing medium. Testes were excised, decapsulated and pooled (3-4). They were homogenized with 6 vol of 0.15 M KCl, 5 mM $MgCl_2$, 1.5 μM glutathione, 62 mM phosphate buffer (pH 7) and 0.25 M sucrose in 0.1 mM EDTA. The homogenate was centrifuged at 10,000 G for 20 min, and the supernatant recentrifuged at 10,000 G for 1 hr. The pellet was suspended in the homogenizing solution. Protein contents of the various subcellular fractions were estimated by the method of Lowry et al. (16). Homogenization and centrifugation procedures were done at 0 C.

Determination of Lipids in Microsomes

Lipids were extracted as described earlier (17). The amount of phospholipid was determined by measuring the inorganic phosphate after hydrolysis by sulfuric acid as described by Bartlett (18). Cholesterol content of microsomes was measured as described by Abell et al. (19). Diglyceride was isolated by thin layer chromatography (TLC) on Silica Gel G using petroleum ether (bp 40-60 C)/diethyl ether/acetic acid (80:20:2, v/v/v) as developing solvent and recovered from the silica gel with diethyl/ether as already described (14).

Measurements of Enzyme Activities

sn-Glycerol 3-phosphate acyl-CoA acyltransferase activity was determined with 50 μM palmitoyl-CoA and 400 μM [^{14}C]*sn*-glycerol 3-phosphate (20). Diacylglycerol acyltransferase was assayed using endogenous diglycerides as substrates. Microsomes (0.5 mg protein) were incubated at 37 C in a medium described by Groener et al. (9) using 0.1 mM [1-^{14}C]-palmitoyl CoA. After the extraction of lipids (21), triglycerides were isolated by TLC and assayed for radioactivity. Choline phosphotransferase and ethanolamine phosphotransferase were also assayed in the microsomal fractions, using endogenous diglycerides as substrates, as described by Groener et al. (9). We used 0.2 mM CDP-[methyl-^{14}C]choline or 0.2 mM CDP-[2-^{14}C]ethanolamine. Formation of radioactive phosphatidylcholine or phosphatidylethanolamine was monitored by a filter disk method (22) and the filter disks transferred into 5 mL of liquid scintillation medium (23). Phosphatidate phosphatase was measured in the 105,000 G supernatant as described by Mangiapane et al. (24), using as substrate microsomal-bound [2-^{14}C]phosphatidate (25). Stearoyl-CoA desaturase activity was assayed by using 15 nmol of [1-^{14}C]stearoyl-CoA, 100 nmol of NADH, 30 μmol of potassium phosphate (pH 7.2), 50 μg of microsomal protein, and water to a final vol of 0.5 mL (12). The reaction was carried out for 20 min at 37 C and terminated by the addition of 2 mL alcoholic KOH.

Radioactive Compounds

[^{14}C]*sn*-Glycerol-3 phosphate (120 Ci/mol), [1-^{14}C]palmitoyl CoA (56 Ci/mol), CDP-[methyl-^{14}C]-choline (40 Ci/mol) and CDP[2-^{14}C], ethanolamine (28 Ci/mol) were purchased from Amersham.

RESULTS

Influence of Zinc Deficiency and Castration on Phospholipid, Cholesterol and Diglyceride in Liver Microsomes

Since some of the difference in various enzyme activities of hepatic microsomes in zinc-depleted rats could be due to an altered lipid environment, the phospholipid and cholesterol contents were estimated (Table I). Castrated rats fed the Zn-deficient diet had ca. 65% of the normal levels of hepatic microsomal phospholipids. A slight decrease in phospholipid was also observed in castrated rats on Zn-supplemented diet and in normal rats on Zn-deficient diet. Testosterone treatment restored the phospholipid to normal values (Table I). Since Zn deficiency decreases gain in body weight and liver size, phospholipid content in total liver would be higher in Zn-deficient rats as has been reported (6,26). Cholesterol remained almost unchanged in all the groups. Since castrated rats on Zn-deficient diet had the lowest phospholipid level, the percent molar ratio of cholesterol to phospholipid in this group was also the highest. Diglyceride content in liver microsomes varied with the zinc nutritional state. It increased in all groups on Zn-deficient diets but decreased in the Zn-sufficient state (Table I). However, this increased availability of diglyceride as a substrate was not seen in the total amount of diglyceride in liver, mostly because Zn-deficient rats had a decreased liver weight.

Influence of Zn Deficiency on Phospholipid and Cholesterol in Small Intestine and Testes Microsomes

Intestine and testes microsomes showed more variation in the content of phospholipid than did the hepatic microsomes (Tables II and III). Zn deficiency, as well as castration, decreased the phospholipid content but cholesterol levels remained unchanged. Consequently, Zn deficiency in the normal (group 2) and castrated rats treated with testosterone (group 6) showed a large increase in cholesterol-to-phospholipid ratio from 0.11 to 0.19 and 0.11 to 0.17, respectively (Table II). Because of the decreased phospholipid content, this ratio was also increased significantly in testes of rats on Zn-deficient diets (Table III).

Influence of Zn Deficiency on the Activity of Enzymes Involved in the Formation of Triglyceride, Phosphatidylcholine and Phosphatidylethanolamine

Specific activity (sp act nmol/min/mg protein) of glycerol phosphate acyltransferase which catalyzed the first step in glycerolipid synthesis from fatty acids remained unchanged except in castrated rats on the Zn-deficient diet where the activity was slightly increased (Fig. 1A). Since liver weight was decreased in Zn-deficient rats, total transferase activity per liver decreased slightly. Phosphatidic phosphatase which converts phosphatidic acid to diglyceride is present in larger concentrations in the 105,000 G supernatant than in the microsomal residue fraction. Figure 2 shows the changes in

TABLE I

Phospholipid, Cholesterol and Diacylglycerol Contents in Liver Microsomes of Rats Fed Different Zn Diets[a]

Groups of rats[b]	Phospholipid (μg/mg protein)	Cholesterol (μg/mg protein)	Cholesterol / phospholipid (molar ratio)	Diglyceride (nmol/mg protein)	Total liver diglyceride (nmol)
1. Normal + Zn (7)	242 ± 11.6	12.2 ± 0.2	0.12	12.5 ± 2.6	3812 ± 793
2. Normal − Zn (7)	210 ± 10.8	12.8 ± 0.3	0.15	17.4 ± 2.2	2871 ± 363
3. Castrated + Zn (5)	218 ± 8.6	12.5 ± 0.2	0.14	12.8 ± 3.3	3712 ± 957
4. Castrated − Zn (7)	158 ± 14.2	13.0 ± 0.2	0.20	18.6 ± 3.0	2641 ± 426
5. Castrated + testosterone + Zn (8)	258 ± 13.0	12.0 ± 0.1	0.11	6.8 ± 1.2	2312 ± 408
6. Castrated + testosterone − Zn (8)	239 ± 10.0	12.0 ± 0.2	0.12	10.4 ± 2.0	3224 ± 620

[a]Methods of estimating are described in the text and the values are given as mean ± SD.

[b]Numbers in parentheses represent the number of rats in each group.

TABLE II

Phospholipid and Cholesterol Contents in Small Intestine Microsomes of Rats Fed Different Zn Diets

Groups[a]	Phospholipid (μg/mg protein)	Cholesterol (μg/mg protein)	Cholesterol / phospholipid (molar ratio)
1. Normal rats + Zn (7)	196 ± 9.0	9.1 ± 0.3	0.11
2. Normal rats − Zn (7)	120 ± 5.6	9.3 ± 0.2	0.19
3. Castrated rats + Zn (5)	139 ± 12.2	9.4 ± 0.1	0.16
4. Castrated rats − Zn (7)	134 ± 8.6	9.9 ± 0.2	0.18
5. Castrated rats + testosterone + Zn (8)	205 ± 11.0	9.0 ± 0.3	0.11
6. Castrated rats + testosterone − Zn (8)	178 ± 18.0	8.8 ± 0.1	0.17

[a]The number of rats in each group is given in parentheses.

TABLE III

Phospholipid and Cholesterol Contents in Testes Microsomes of Rats Fed Different Zn Diets[a]

	Normal rats + Zn	Normal rats − Zn
Phospholipid (μg/mg protein)	322 ± 14	212 ± 8
Cholesterol (μg/mg protein)	14.5 ± 0.2	14.9 ± 0.3
Cholesterol / phospholipid (molar ratio)	0.11	0.17

[a]The number of rats in each group was 7, and the results are given as mean ± SD.

the phosphatase measured as specific (Fig. 2A) and total (Fig. 2B) activities in the supernatant fraction after feeding Zn-deficient diets to the rats. Zn depletion in both normal and castrated animals resulted in a significant increase in both specific and total activity of the enzyme. The effect of Zn deficiency and castration on the specific and total activities of diacylglycerol acyltransferase, choline phosphotransferase, and ethanolamine phosphotransferase, which catalyze the conversion of diglyceride into triglyceride, phosphatidylcholine and phosphatidylethanolamine, respectively, are shown in Figure 1. These enzymes are localized in the microsomal fraction of liver and were measured using endogenous diglycerides as substrates. Zn depletion increased the sp act of diacylglycerol acyltransferase and choline phosphotransferase, but the increase was more significant in castrated rats than in normal rats (Fig. 1A). However, total activities per liver decreased in Zn-deficient states for diacylgycerol acyltransferase (Fig. 1B). Ethanolamine phosphotransferase activity remained almost unchanged (Figs. 1A and 1B).

Influence of Zn Deficiency and Castration on Stearoyl-CoA Desaturase

Since stearoyl-CoA desaturase was shown to be more effective than fatty acid synthetase as a control enzyme for hepatic lipogenesis (12), we examined the activity of the desaturase in different Zn and testosterone states (Fig. 3). Zn deficiency increased the enzyme by 213% in normal rats, by 217% in castrated rats and by 124% in castrated rats treated with testosterone.

DISCUSSION

The first objective of this study was to understand whether Zn deficiency is related to the lipid composition of microsomal membranes of liver, testes and small intestine. Zn deficiency has been shown to markedly increase triglycerides and, to some extent, phospholipid and cholesterol in serum (4). Zn depletion did modify the lipid composition of liver microsomes, and to a greater extent, testes and small intestine microsomes. These and other changes, i.e., enhanced lipid peroxidation (26), suggest

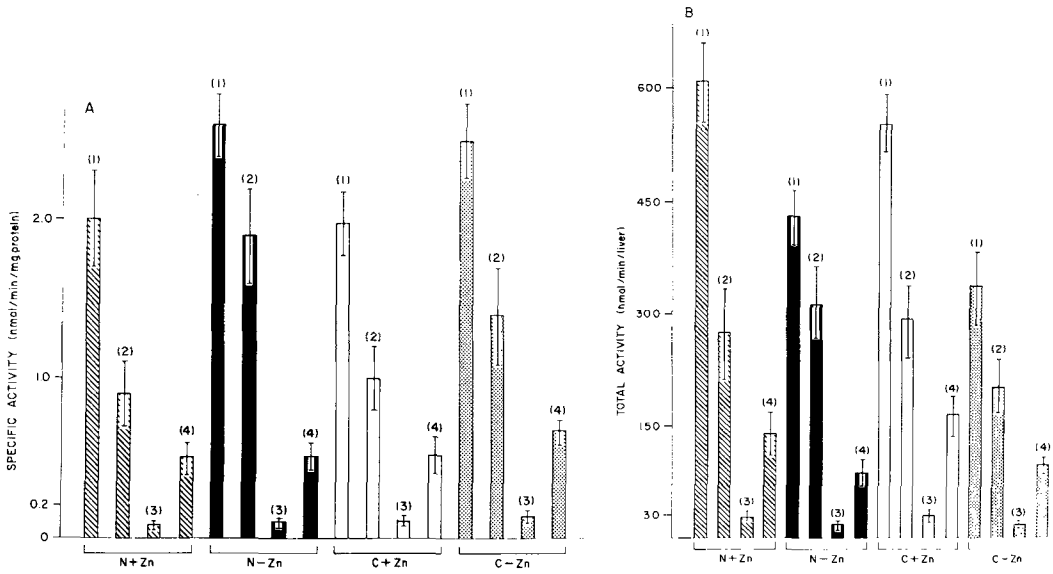

FIG. 1. The activities of 1) diacylgycerol acyltransferase, 2) choline phosphotransferase, 3) ethanolamine phosphotransferase, and 4) glycerol 3-phosphate acyltransferase in liver of ▨ normal and □ castrated rats on Zn-sufficient diet and in ■ normal and ⊡ castrated rats on Zn-deficient diet. The data are reported as mean ± standard deviation (vertical bars). A) specific activity in nmol/min/mg protein, and B) total activity in nmol/min/liver. The number of rats in each group was four.

that Zn deficiency may modify hepatic metabolism in a way that could lead to harmful effects to membranes. Increased amounts of Zn in the diet has been proposed as having a stabilizing effect on different membranes (27). Recent investigations have shown that the essential step in Zn absorption involves low molecular weight chelating agents in the small intestine (8). Thus, changes in the microsomal composition of small intestine observed here may have added importance. From the point of view of this discussion, the important difference is the increase of the percent molar ratio of cholesterol to phospholipid. The increased cholesterol phospholipid ratio could significantly modify thermotropic properties of microsomal membranes of Zn-deficient rats. On the basis of studies of cholesterol:phospholipid mixed vesicles, it has been concluded that increasing cholesterol content, in addition to its condensing effect, will also induce changes in structural, hydrodynamic (28) and thermotropic proper-

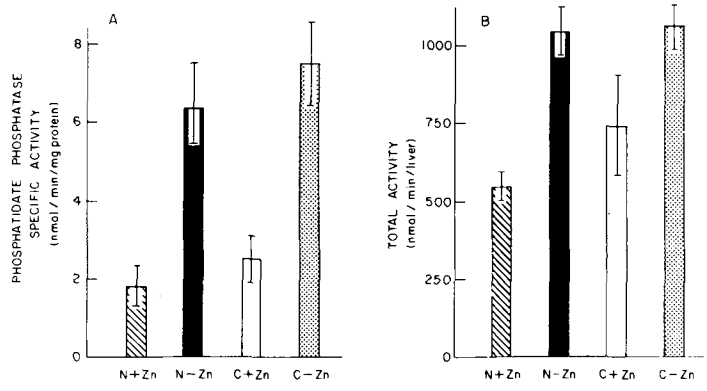

FIG. 2. A) Specific activity, and B) total activity of phosphatidate phosphatase in liver of ▨ normal and □ castrated rats on Zn-sufficient diet and in normal ■ and ⊡ castrated rats on Zn-deficient diet. Data represent mean ± SD.

ties of the vesicles (29). Rate of permeation (30) and enzyme activities (31) are found to change with altered cholesterol:phospholipid ratio. Therefore, the increased molar ratio of cholesterol:phospholipid in Zn deficiency may have a profound effect on every membrane-associated process.

The second objective of this investigation was to study the relationship between the influence of Zn deficiency and/or castration on hepatic glycerolipid metabolism in vivo, and correlate with the changes in the activity of relevant enzymes. Among the enzymes studied, phosphatidate phosphatase appears to be important since its activity responds to various environmental changes (32). There is evidence that this enzyme is rate-limiting for the synthesis of triglycerides in vitro (33). As shown in Figure 2, total activity of the phosphatase was increased almost two-fold by Zn deficiency which may explain the increased serum triglyceride. Furthermore, the accumulation of diglyceride in the liver of Zn-deficient rats, as observed here (Table I) may also increase the availability of substrates for triglyceride synthesis. It could therefore be proposed that the overall control of triglyceride synthesis in Zn deficiency state is exerted both by the activity of phosphatidate-phosphatase as well as by the substrated diglyceride. Changes in the activity of choline phosphotransferase (Fig. 1), but not ethanolamine phosphotransferase, in response to Zn status correlated well with the reported changes in the synthesis of phosphatidylcholine and phosphatidylethanolamine (10). Our previous results (4) have shown an enrichment of diglycerides with saturated fatty acids in Zn deficiency. Therefore, it is possible that this class of diglyceride is used as a substrate for the synthesis of phosphatidylcholine but less for phosphatidylethanolamine because choline phosphotransferase has more affinity for this substrate than ethanolamine phosphotransferase. Since stearoyl CoA desaturase was also increased in Zn-deficient states and since this enzyme regulates the amount and type of mono- and polyunsaturated fatty acids available for triglyceride synthesis, one could expect an increase in the amount of oleic acid present in the triglyceride fractions in Zn deficiency. Experiments in progress corroborate this point (unpublished observations).

From these studies, it could be concluded that diglyceride levels and activities of stearoyl CoA desaturase, phosphatidate phosphatase, and choline phosphotransferase are altered by Zn in the diet. The data will help explain hypertriglyceridemia and high levels of serum phospholipids seen in Zn deficiency (4). However, the mechanism by which Zn deficiency and/or testosterone deficiency alters these lipids and enzyme activities is unclear. Both Zn and testosterone are known to be associated with many metabolic changes which might affect complex lipid and lipoprotein synthetic rates.

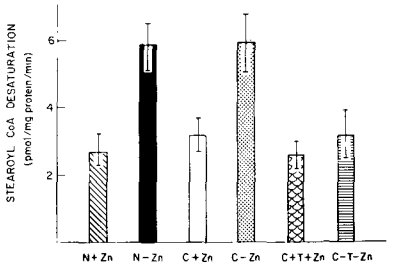

FIG. 3. Stearoyl CoA desaturase in pmol/mg protein/min in ▨ normal, □ castrated, and ⊠ castrated injected with testosterone rats on Zn-sufficient diet and in liver of ■ normal, ⊡ castrated, and ▤ castrated-injected with testosterone rats on Zn-deficient diet. Data represent mean ± SD.

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METHODS

Application of Methoxy-Bromomercuri-Adduct Fractionation to the Analysis of Fatty Acids of Partially Hydrogenated Marine Oils

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ABSTRACT

The fatty acids of a refined and of a partially hydrogenated menhaden oil, iodine value (IV) 84.5, were separated into different classes (e.g., monoene, diene, including pentaene and hexaene) by thin layer chromatography (TLC) of their methoxy-bromomercuri-adducts (MBM). In the solvent system hexane:dioxane, the separation of fatty acids occurred according to the degree of unsaturation. No influence was exerted by either the geometry or the position of the ethylenic bonds. The effect of the various chain lengths (C_{14}-C_{22}) was to broaden the bands, but no overlap occurred among the chain lengths. A wide range of C_{20} unsaturated fatty acids were prepared by the hydrazine reduction of 20:5-Δ5,8,11,14,17. These were separated into groups as MBM adducts and identified by comparison of their experimental and calculated equivalent chain lengths (ECL) in gas liquid chromatography (GLC) on SILAR-5CP and SILAR-7CP columns. This confirmed that GLC did not totally separate all groups of isomers of different degrees of unsaturation. The quantitative analysis of both refined and partially hydrogenated (IV-84.5) menhaden oils by GLC was effected by the recovery of the fatty acid methyl esters from the MBM adduct TLC bands with the addition of methyl heptadecanoate (17:0) as an internal standard, followed by analysis of the different fractions on open-tubular columns coated with SILAR-5CP. For methylene- and nonmethylene-interrupted unsaturated acids, 100% recovery from the MBM adducts was achieved, but in the case of the conjugated dienes the maximal recovery was 70%.

INTRODUCTION

Marine oils for human consumption are most widely used as partially hydrogenated oils in margarines and shortening (1). These oils can be divided roughly into two classes, low iodine value (IV) oils such as herring, or high IV oils such as menhaden. The basic difference in fatty acid composition is the presence or near absence of eicosenoic and docosenoic acids in addition to the ubiquitous 16:1 and 18:1 acids (2). Herring oil is rich (3) in the longer chain monoenoic fatty acids, 20:1 and 22:1, whereas in menhaden oil (4), these are replaced in part by highly unsaturated fatty acids (e.g., 20:5-Δ5,8,11,14,17, 22:6-Δ4,7,10,13,16,19). A low iodine value oil, upon partial hydrogenation, gives a mixture of saturated and *cis* and *trans* monoenoic fatty acids, but contains relatively minor quantities of polyenoic acids, including nonmethylene-interrupted dienoic and trienoic fatty acids (5,6). Conversely, menhaden oil would be expected to include higher proportions of residual polyenoic acids of all types. We have applied a method, the fractionation of methoxy-bromomercuri-adducts (MBM) described earlier for the study of vegetable oils (7,8), and also for marine lipids (9,10), to execute a comprehensive study of partially hydrogenated menhaden oil (PMHO) containing high proportions of *trans* unsaturated fatty acids in numerous positional dienoic, trienoic and tetraenoic isomers, but only minor quantities of conjugated acids.

MATERIALS AND METHODS

A refined menhaden oil, IV 159, was partially hydrogenated according to Canadian commercial practice with nickel catalyst (0.2%) at 175-215 C, and ca. 70 kPa hydrogen pressure. Samples were collected at intervals. Each sample was saponified (AOCS method Ca-6b-53), the unsaponifiables removed, and the fatty acids converted to the methyl esters by refluxing in a solution of 7% BF_3 in MeOH for 15 min.

Gas Liquid Chromatography (GLC)

The gas liquid chromatographic analyses were effected on stainless steel wall-coated open-tubular columns, 47 m in length, 0.25 mm id, coated with SILAR-5CP and/or SILAR-7CP and operated in a Perkin Elmer 910 series apparatus with flame ionization detector. A Linear Inst. Co. Model 252A 1 mV recorder with stepping-pen integrator provided quantitative data.

Separation of Fatty Acid Classes Using Bromomercuri-Adducts

The method described by White (11) was modified slightly as follows: 20 to 50 mg of a mixture of fatty acid methyl esters were weighed into a screw-cap (10 mL) centrifuge tube, an excess (100%) of mercuric acetate solution in methanol (0.25 M) was added, and the capped tube was heated at 100 C for 2 hr. These conditions were necessary to obtain complete reaction of the *trans* and highly unsaturated fatty acids. The reaction mixture was dissolved in 10 mL of chloroform in a 50-mL screw-cap tube, a 10% excess of sodium bromide in MeOH (0.5 M) was added, and the tube contents mixed with a vortex mixer for 2 min. Following the addition of water (30 mL), the adducts were extracted 3 times into chloroform (20 mL) which was washed several times with water. The chloroform solution was dried over sodium sulfate, concentrated under nitrogen, and the adducts in solution were streaked on a thin layer chromatography (TLC) plate ($\sim$10 mg/plate, Prekotes; Adsorbosil-5, Applied Science Laboratories). Development was in hexane/dioxane (60:40, v/v). The bands were visualized by spraying a solution of diphenylcarbazone (0.2%) in ethanol. The individual bands were removed into 50-mL centrifuge tubes and 5 mL of a mixture of concentrated HCl/MeOH (1:2) was added to destroy the MBM. A known amount (0.01% solution) of methyl heptadecanoate (17:0) was added to each tube prior to addition of water and recovery of methyl esters into hexane for the GLC analysis.

Hydrazine Reduction of 20:5-Δ5,8,11,14,17

In a typical hydrazine reduction (12), 60 mg of 20:5-Δ5,8,11,14,17 fatty acid (Nu-Chek-Prep, 99% pure) were dissolved in 80 mL of 96% ethanol in a two-necked, round-bottom flask fitted with a condenser and a magnetic stirrer. The flask was immersed in a water bath at 40-45 C and 3 mL of 95% hydrazine solution was added. Oxygen was passed over the stirred solution for 1 hr. Following the addition of H_2O, the fatty acids were extracted with diethyl ether, then converted to methyl esters with BF_3-MeOH.

RESULTS AND DISCUSSION

Comparisons of Mercuric Adducts of Different Types

The detailed monoenoic acid isomer compositions of partially hydrogenated oils originally of low IV, e.g., herring oil (5), have been studied by a combination of silver-nitrate TLC, GLC on open-tubular columns and oxidative fission. However, it was not possible to meet our objective of a complete breakdown of PHMO with the techniques just mentioned due to the numerous geometrical and positional isomers of dienoic, trienoic and tetraenoic acids formed during the progressive hydrogenation of the highly unsaturated fatty acids (13). Comparable quantitative results could not be obtained by GLC of total methyl esters, or fractions, on open-tubular columns coated with the very useful SILAR-5CP (14), or on Apiezon-L (6,15) and SILAR-7CP (14) (both of which usually give good resolution between *cis* and *trans* fatty acids), due to the extensive overlaps among the polyunsaturated isomers. The fractionation of methyl esters by silver nitrate-TLC ($AgNO_3$-TLC) depends on four factors: the number, position and geometry of the ethylenic bonds and the chain length. When this method was applied to the total esters of PHMO, there was overlapping among the diene, triene and tetraene bands and no clean separation of the fatty acid classes was possible.

Two types of mercuri-adduct formation (7,11) have been proposed for the separation of unsaturated fatty acids. The TLC fractionation of methoxy-acetoxymercuri-adducts (MAM), as described by Mangold (7), applied with double development, permitted separation of unsaturated fatty acids containing up to three ethylenic bonds. However, no separation of the highly unsaturated fatty acids (20:4, 20:5 and 22:6), typical of marine oils, was possible. A modification of this technique, developed by Pohl et al. (10), extended the fractionation to the highly unsaturated acids, but separations in this system were also influenced by the chain length, resulting in an overlap among classes of different chain lengths. Improved fractionation of unsaturated fatty acids of marine origin containing up to six ethylenic bonds was achieved by the conversion of the methoxy-acetoxymercuri-adducts to the MBM as described by White (11) and later by Minnikin and Smith (16).

We applied the method described by White

TABLE I

Reference Isomeric Fatty Acids Produced by the Hydrazine Reduction of 20:5-Δ5,8,11,14,17

Fatty acids	Peak no.[a]	Fatty acids	Peak no.[a]
Monoenes (Fig. 1B)		Trienes (Fig. 1D)	
Δ5	1	Δ5,8,11	11
Δ8	2	Δ5,8,14	13
Δ11	3	Δ5,11,14	14
Δ14	4	Δ5,8,17	16
Δ17	6	Δ5,11,17	16
		Δ8,11,14	16
Dienes (Fig. 1C)		Δ5,14,17	17
Δ5,8	5	Δ8,11,17	18
Δ5,11	5	Δ8,14,17	19
Δ5,14	6	Δ11,14,17	20
Δ8,11	7		
Δ8,14	8	Tetraenes (Fig. 1E)	
Δ5,17	9	Δ5,8,11,14	19
Δ11,14	10	Δ5,8,11,17	21
Δ8,17	11	Δ5,8,14,17	22
Δ11,17	12	Δ5,11,14,17	22
Δ14,17	15	Δ8,11,14,17	23

[a]In Fig. 1 (analysis on SILAR-7CP at 160 C).

(11) to a mixture of fatty acid methyl ester standards of roughly equal proportions: 18:0, *cis* and *trans* 18:1-Δ9, 18:2-Δ9,12, 18:3-Δ9,12,15, 20:4-Δ5,8,11,14 and 20:5-Δ5,8,11,-14,17. The TLC of the MBM adducts of this mixture gave six bands with the following R_f values: saturate, 0.83; monoenes, 0.72; diene, 0.62; triene, 0.50; tetraene, 0.40; and pentaene, 0.14. However, the more mobile bands were impure due to inclusion of incompletely formed adducts of more unsaturated acids. Relative to the saturate content, only 95% of *cis* 18:1, 75% of *trans* 18:1, 90% of 18:2, 70% of 18:3, 42% 20:4 and 30% 20:5 had formed the MBM adducts on all bonds.

Two parameters of the reaction, time and temperature, were altered until there was almost complete reaction for the *cis* and *trans* ethylenic bonds, as well as for the highly unsaturated fatty acids with four and five ethylenic bonds. More than 98% of 18:1 (*cis* and *trans*), 18:2, 18:3, 20:4 and 95% of 20:5 were converted to the MBM adducts under the reaction conditions of 100 C for 2 hr. As determined previously (10,11), neither the geometry of the ethylenic bond nor the chain length were critical for this MBM-adduct separation. The noninfluence of the geometry of the ethylenic bond for the MBM-adduct fractionation was further proven with C_{20} dienoic isomers. A mixture (c=*cis*, t=*trans*) of methoxy-bromercuri-adducts of *c,c*20:2-Δ11,-14, *c,t* + *t,c*Δ11,14 and *t,t*Δ11,14, synthesized by nitrous acid isomerization (17) of *c,c*20:2-Δ11,14 showed only one band of R_f 0.65 when submitted to TLC fractionation using dioxane/hexane as the eluting solvent.

TLC Fractionation of MBM-Adducts of Positional Fatty Acids of Different Classes

A complex mixture of fatty acids with differing degrees of unsaturation and both methylene- and nonmethylene-interrupted unsaturations was obtained by the hydrazine reduction of pure 20:5-Δ5,8,11,14,17. The hydrazine reaction products, totaling 31 components, are listed in Table I (21 peak, in Figure 1). The total reaction mixture (Fig. 1A) was then subjected to MBM-adduct formation. The TLC separation of the adducts gave 6 bands with the following R_f values: 20:0, 0.88; monoenes (Fig. 1B), 0.77; dienes (Fig. 1C), 0.68; trienes (Fig. 1D), 0.54; tetraenes (Fig. 1E), 0.28; and pentaene, 0.10. Each band was 95-98% pure. All of the fatty acids were identified by a comparison of the experimental and the calculated equivalent chain lengths (18) by the method described by Ackman et al. (19-21). These identifications confirmed that the position of the ethylenic bond does not influence the migration of the adducts in this particular separation of one chain length. It is also important to note the GLC overlap, even on open-tubular columns (Fig. 1), between isomeric fatty acids of different classes. In peak 6, the diene 20:2-Δ5,14 had the same retention time as 20:1-Δ17; peak 11 included both 20:2-Δ8,17 and 20:3-Δ5,8,11; and peak 19, both 20:3-Δ8,14,17 and 20:4-Δ5,8,11,16.

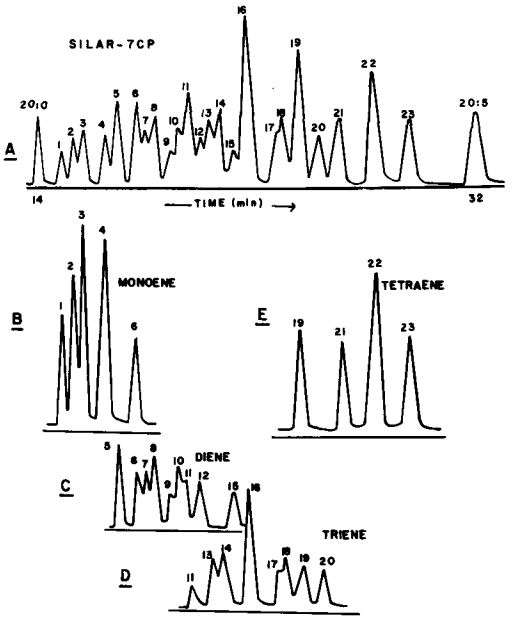

FIG. 1. Gas liquid chromatogram (SILAR-7CP) of the hydrazine reduction products of 20:5-Δ5,8,11,-14,17 and of individual bands resulting from MBM-adduct fractionation; (A) total reaction product, (B) monoene band, (C) diene band, (D) triene band, and (E) tetraene band.

MBM-Adducts of Conjugated Dienoic Acids

The C_{18} conjugated acids were prepared by alkali isomerization of 18:2-Δ9,12 using potassium-*t*-butoxide in *t*-butanol as described by Mounts and Dutton (22). These acids were identified by comparison of their ECL on SILAR-5CP and 7CP with the ECL values reported by Ackman et al. (23), and by Scholfield and Dutton (24) as a mixture of *c*9,*t*11-, *t*10,*c*12-, *t*9,*t*11- and *t*10,*t*12-18:2. A mixture of these conjugated dienes (32.8%), with 18:1-Δ9 (33.3%) and 18:2-Δ9,12 (33.9%) was submitted to MBM-adduct formation as described earlier. One part of the adducts was submitted to TLC fractionation whereas the other part was treated by HCl in order to regenerate the original methyl esters which were further analyzed by GLC. Only two TLC bands were obtained, one containing 18:1-Δ9 (R_f 0.70) and one containing the 18:2-Δ9,12 and the C_{18} conjugated dienes (R_f 0.60). However, the GLC analysis of the regenerated methyl esters showed that only 70% of the original conjugated acids was recovered after MBM-adduct formation if compared to 18:2-Δ9,12 or 18:1-Δ9. This loss was nonselective and seems to indicate the presence of a secondary reaction giving compounds which could not be detected by open-tubular GLC. The same phenomena was earlier observed by Planck et al. (25), when reacting mercuric acetate with methyl α-eleostearate in acetic acid. They concluded that the addition of mercuric acetate to the conjugated system was followed by decomposition giving a compound of lower mercury content than expected and that an ethylenic bond was oxidized to an oxygen-containing group which was not further identified. Despite this loss, the bromomercuri-adduct fractionation will be applicable without any restriction to oils containing a low proportion of conjugated acids such as marine oils partially hydrogenated according to Canadian practice (13), since conjugated acids are virtually absent. The quantitative analysis of the total of these conjugated acids could be readily executed by UV spectroscopy effected on the total methyl esters before MBM-adduct fractionation (26).

Fractionation of Fatty Acids of Refined and Partially Hydrogenated Menhaden Oils

Two different methods were used to determine the fatty acid composition of a refined menhaden oil and of the same oil after partial hydrogenation. In the case of the refined oil (Table II) the total fatty acid methyl esters were analyzed by open-tubular GLC and were also converted to the corresponding MBM-adducts. The TLC fractionation gave six bands with the R_f values comparable to those described earlier. An internal standard, 17:0 was added to each band, and each component of each class was related to the 17:0. Great care must be taken when collecting the saturate band because of its tendency to trail on the plate. This could result in contamination of the monoene band. However, with the unhydrogenated oil, the monoene band was very narrow and no contamination was observed. The quantitative results for the refined menhaden oil (Table II) obtained from direct GLC analysis of the starting material, and from reconstitution of the GLC analysis of each MBM-adduct TLC band are in very good agreement, indicating the quantitative recovery of all fatty acids from the MBM-adduct.

The fractionation of the esters of the partially hydrogenated oil (IV-84.5) was slightly more difficult. The MBM-adducts were prepared from the total methyl esters and then fractionated on a TLC plate. As observed earlier, the saturate band tended to spread and trail on the plate. However, in this case, the monoene band was broadened, probably due to both the higher monoene content (Table II) and the numerous

cis and *trans* positional isomers formed during partial hydrogenation. This effect resulted in some contamination of the monoene band by a small quantity of 14:0 and 16:0, but no overlap occurred between any of the other fatty acid classes (e.g., monoene, diene, triene). In view of this contamination, the internal standard approach was used to determine the fatty acid compositon of the partially hydrogenated oils. Each MBM-adduct band, except the saturate, was analyzed on a SILAR-5CP column after addition of a known quantity of 17:0 methyl ester. Thus, it was possible to determine for each chain length (C_{14}, C_{16}, C_{18}, C_{20}, C_{22}) the relative amount of each fatty acid class (monoene, diene, triene, tetraene, pentaene). The total fatty acid methyl esters were also run on the same column. The fatty acid composition was reconstituted using the results of the MBM-adduct band analysis (except for the saturates which were directly determined from the GLC analysis of the total fatty acid methyl esters). The results obtained are summarized by number of bonds in Table II.

All of the chain length totals were calculated

TABLE II

Comparison of the Fatty Acid Composition by the Direct GLC Analysis of the Refined Menhaden Oil and Following MBM-adduct Fractionation of the Refined and of the Partially Hydrogenated Menhaden Oils

Fatty acids	Refined		Partially hydrogenated, reconstituted from MBM-adduct fractionation[d]
	Direct GLC analysis	Reconstituted from MBM-adduct fractionation[d]	
14:0	10.8	9.8	10.5
16:0	23.2	21.6	24.1
18:0	4.2	4.0	5.2
20:0	0.4	0.3	0.7
22:0	trace	0.1	0.3
Others[a]	3.0	3.2	3.0
Σ Saturates	41.6	39.0	43.8
16:1	11.4	13.0	15.0
18:1	10.5	11.3	12.5
20:1	1.3	1.5	4.9
22:1	0.2	0.3	1.7
Others[b]	0.5	0.2	0.1
Σ Monoenes	23.9	26.3	34.2
C_{16} Dienes	1.5	1.6	0.9
C_{18} Dienes	1.8	2.3	2.4
C_{20} Dienes	0.6	0.3	6.6
C_{22} Dienes	–	–	3.3
Σ Dienes	3.9	4.2	13.2
C_{16} Trienes	2.2	2.1	–
C_{18} Trienes	1.7	1.7	0.2
C_{20} Trienes	0.3	0.2	3.8
C_{22} Trienes	–	–	4.3
Σ Trienes	4.2	4.0	8.3
C_{16} Tetraenes	1.0	1.2	–
C_{18} Tetraenes	2.1	2.4	–
C_{20} Tetraenes	2.3	2.1	0.1
C_{22} Tetraenes	0.2	0.1	0.3
Σ Tetraenes	5.6	5.8	0.4
20:5-Δ5,8,11,14,17	11.9	12.2	–
21:5-Δ6,9,12,15,18	0.5	0.4	–
C_{22} pentaenes + hexaenes	8.8	8.6	trace
Σ Conjugated acids[c]	0.7	0.7	0.5
IV (Wijs)	159.0	159.0	84.5
IV Calculated	148.0	149.0	79.0

[a]Includes 12:0, 19:0, iso 14:0, iso and anteiso 15:0, iso 16:0, iso and anteiso 17:0, iso 18:0.

[b]Includes 7Me-16:1, 17:1, 19:1 and 24:1.

[c]AOCS official method Cd-7-58.

[d]Average of 2 analyses.

TABLE III

Chain Length Totals for Fatty Acid Methyl Esters in Unhydrogenated and Partially Hydrogenated Menhaden Oils[a]

Chain lengths (wt %)	Refined menhaden oil	Refined menhaden oil reconstituted from MGM-adducts	Partially hydrogenated menhaden oil (IV-84.5) reconstituted from MBM-adducts
ΣC_{14}	10.8	9.8	10.5
	10.5	*10.5*	*10.5*
ΣC_{16}	39.3	39.5	40.0
	38.9	*38.9*	*40.7*
ΣC_{18}	20.3	21.7	20.3
	19.9	*19.9*	*19.7*
ΣC_{20}	16.8	16.6	16.1
	16.1	*16.1*	*15.2*
ΣC_{22}	9.2	9.1	9.9
	10.6	*10.6*	*9.4*

[a]Data in Roman type is summed from total analysis of Table II and that in italics is from analysis of fatty acid methyl esters completely hydrogenated on PtO_2 (SILAR-5CP, 180 C).

from the MBM-adduct/GLC analyses and were found to be in very good agreement (Table III) with the chain length totals of the original total ester samples completely hydrogenated in the laboratory (27).

Previously (14), semi-arbitrary decisions were necessary in dividing the total GLC envelope among monoene, diene, triene and tetraene areas in the GLC of partially hydrogenated marine oils on SILAR-5CP. The GLC analysis on SILAR-5CP of the total C_{20} acids (Fig. 2), and the total C_{22} acids (Fig. 3) of PHMO (IV-84.5) and of the peaks resulting from individual MBM-adduct bands showed that due to the extensive overlap it is definitely impossible to quantitate, using only GLC, the changes among fatty acids produced by partial hydrogenation. This overlap between the different fatty acid classes was especially noticeable in the dienoic and trienoic fatty acids where the diene band contained a late eluting peak probably containing acids with *cis* ethylenic bonds close to the methyl end of the molecule, e.g., Δ14,Δ17.

UV spectroscopy revealed the presence of small amounts (totals 0.5-0.7%) of conjugated acids in the various menhaden oil samples, including the partially hydrogenated oil with the low IV of 84.5. These were not identifiable

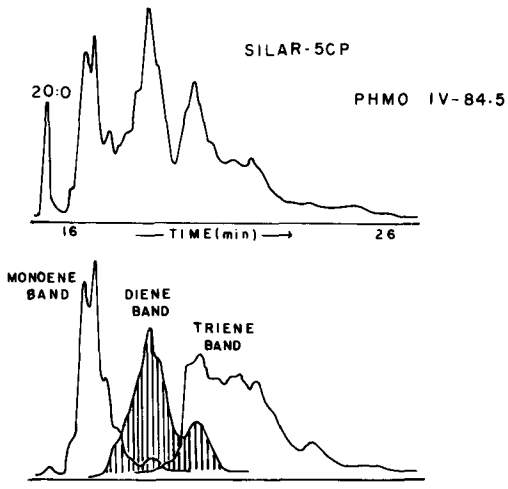

FIG. 2. Gas liquid chromatograms of methyl esters of total C_{20} fatty acids (*above*) from partially hydrogenated menhaden oil (IV-84.5), and of respective individual bands resulting from MBM-adduct fractionation (*below*), on SILAR-5CP. Note portion of diene band under triene band and effect on total envelope. Fraction (band) envelopes are approximately correct in proportion relative to total.

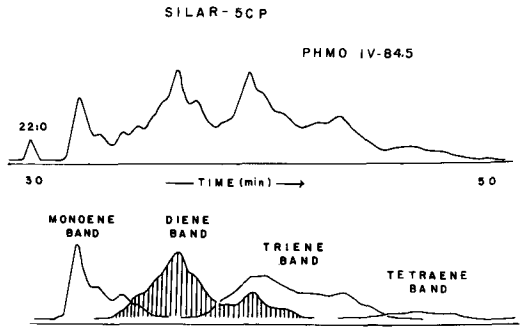

FIG. 3. Gas liquid chromatograms of methyl esters of total C_{22} fatty acids (*above*) from partially hydrogenated menhaden oil (IV-84.5), and of respective individual bands resulting from MBM adduct fractionation (*below*), on SILAR-5CP. Fraction (band) envelopes are approximately correct in proportion relative to total.

as discrete peaks.

With MBM-adduct fractionation, all fatty acids having the same degree of unsaturation are found in the same TLC band irrespective of the position or geometry of their unsaturation. Thus, the TLC fractionation of MBM-adducts should be mandatory in any study on structural elucidation and/or fatty acid composition of oils produced by the partial hydrogenation of marine oils. The basic technique is, of course, fully applicable to natural lipid mixtures including fatty acids of unusual structure such as the nonmethylene-interrupted fatty acids (28).

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The OV 275-Packed Stainless Steel Column in *Trans*-Fatty Acid Research: A Note of Caution

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ABSTRACT

Gas chromatographic analysis of fatty acid methyl esters in a 6.1 m stainless steel column packed with 15% OV 275 and operated at 220 C resulted in poor recovery of polyunsaturated esters relative to their saturated or monoenoic counterparts. Relative responses of the esters declined with increasing unsaturation, were concentration-dependent at low concentrations and changed as the column aged. Decomposition of polyunsaturated compounds in the hot stainless steel column appeared to be the major reason for their poor recovery and renders this particular column of doubtful value in analysis of complex ester mixtures.

Introduction of highly polar silicone phases has facilitated gas chromatographic analysis of geometric isomers of fatty acids. Ottenstein et al. (1) used a 6.1 m (20 ft) × 2 mm id (1/8 in. od) stainless steel column packed with 15% OV 275 on Chromosorb P and operated at 220-230 C for the fractionation of *cis*- and *trans*-octadecenoic acids and the analysis of margarine samples. This column appears to have gained acceptance among researchers in the hydrogenated fat field. Recent experience in my laboratory has indicated that caution is necessary when using this column for analysis of samples containing polyunsaturated acids, since these compounds appeared to be underestimated (area % basis) in comparison with their analysis on a packed polyester column. This prompted a re-examination of the OV 275 column.

MATERIALS AND METHODS

Five gas chromatographic columns were employed: a new 6.1 m × 2 mm id stainless steel column packed with 15% OV 275 on 100-120 mesh Chromosorb P, AW/DMCS (Supelco, Inc., Bellefonte, PA); an "old" 6.1 m × 2 mm id stainless steel-OV 275 column, used intermittently for 3 years; a 3.05 m × 2 mm id stainless steel-OV 275 column (half of the previous column); a 3 m × 2 mm id glass column packed with 15% OV-275 on 100-120 mesh Chromosorb P; a 2 m × 2 mm id glass column packed with 15% EGSS-X polyester on 100-120 mesh Gaschrom P. The first four columns were operated at 220 C, the last one at 180 C, unless otherwise stated. Columns were mounted in a Hewlett-Packard 5710A chromatograph using the same glass-lined injector-hydrogen flame ionization detector combination for each column. Helium was the carrier gas, unless otherwise stated, at flow rates of 10 mL min^{-1} for the 6.1 m columns, 14-18 mL min^{-1} for the shorter OV 275 columns and 30 mL min^{-1} for the polyester column. The carrier gas line was equipped with an oxygen trap. Hydrogen, air and make up carrier gas were supplied for optimal detector operation (Hewlett-Packard Application Note, GC 2-73). Injector and detector were maintained at 250 C. Peak areas were measured with a Spectra-physics SP4100 computing integrator. Electrometer settings were kept constant throughout the study at sensitivity 10, attenuation 8x (except where indicated in the test), yielding a full-scale deflection of ca. 6×10^{-10} amps on a 1-mV recorder.

Samples consisted of mixtures of high purity methyl esters (Nu-Chek-Prep, Elysian, MN) prepared by weight in my laboratory and containing approximately equal amounts of the component esters (5-7 components). Known amounts of esters were injected into the column in 2 μL of CS_2 (but were not stored in this solvent). The relative response of the ester (18:0 = 1.0) was calculated as follows: relative response = (area % ester/area % 18:0) ÷ (wt % ester/wt % 18:0).

RESULTS AND DISCUSSION

Methyl oleate, linoleate and linolenate standards yielded ca. 2, 2 and 5% of minor components, possibly isomers, eluting before the major peaks from OV 275 (Fig. 1); principal and minor peak areas were combined in calculating area % or relative responses. A single peak eluted from EGSS-X for each ester.

Simple area % composition and actual weight % composition of an ester mixture were similar for the EGSS-X column (Table I); maximal relative deviation was 2.6% for 18:1. In contrast, polyunsaturated esters were underestimated with a new OV 275 column and area % differed markedly from actual weight % composition.

Relative responses for several esters were obtained by analyzing standard mixtures on the silicone and polyester columns (Table II). For EGSS-X, relative responses were within ± 4% of unity for all esters studied. With OV 275, all unsaturated esters were detected less efficiently than methyl stearate, although the effect was small for C-18 monoenes. The deficit increased sharply, however, with increasing unsaturation. Less than 70% of methyl linolenate and ca. 40% of methyl arachidonate were detected upon elution from OV 275. Sample sizes for the EGSS-X column were ca. 1/3 those analyzed on OV 275 in order to maintain similar peak heights for the two columns (50-75% full scale for 2 μg 18:3). Relative response tended to decrease with increasing retention time on OV 275 (cf. saturated esters, Table II), probably because of less efficient detection of

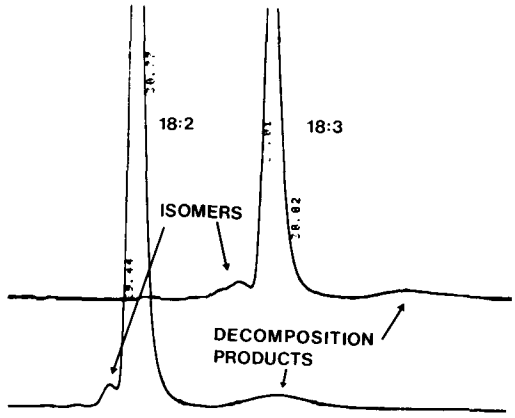

FIG. 1. Gas chromatograms of methyl linoleate and methyl linolenate standards. Isomeric materials and probable decomposition products are indicated.

TABLE I

Analysis of Fatty Acid Methyl Esters on OV 275[a] and EGSS-X Columns

Ester	Weight %	OV-275 (area %)	EGSS-X (area %)
16:0	21.34	24.42 ± 0.10[b]	21.20 ± 0.11
18:0	18.31	20.57 ± 0.05	18.27 ± 0.11
18:1	18.31	19.51 ± 0.06[c]	17.93 ± 0.09
18:2	19.87	18.49 ± 0.03[c]	20.12 ± 0.06
18:3	22.17	16.99 ± 0.06[c]	22.50 ± 0.08

[a]Recently purchased or "new" OV 275 column.

[b]Mean of 5 analyses ± SDM.

[c]Includes minor isomeric material.

TABLE II

Relative Responses of Various Esters on OV 275[a] and EGSS-X

	OV 275		EGSS-X	
Ester	Rel ret time[b]	Rel response[c]	Rel ret time	Rel response
16:0	0.69	1.02	0.57	1.00
18:0	1.00	1.00	1.00	1.00
18:1 *trans*	1.12	0.98	1.16	1.03
18:1 *cis*	1.20	0.95	1.18	0.98
18:2	1.51	0.83	1.52	1.02
18:3	1.97	0.68	2.05	1.02
20:0	1.48	0.95	1.77	0.98
20:1	1.73	0.92	2.09	0.97
20:2	2.16	0.68	2.67	0.96
20:3	2.60	0.62	3.18	1.02
20:4	2.99	0.41	3.65	0.96
22:0	2.21	0.89	3.13	1.01

[a]"New" OV 275 column.

[b]Retention times of 18:0 were ca. 21 and 7 min on OV 275 and EGSS-X, respectively.

[c]Based on 3-5 analyses of mixtures containing 1.6-2.4 μg of each ester for OV 275 or 0.6 - 0.8 μg for EGSS-X.

broader peaks by the integrator. However, polyunsaturated esters exhibited much lower responses than less unsaturated esters with similar retention times (cf. 18:2 vs 20:0 or 20:1; 18:3 vs 20:1; 20:2 vs 22:0).

Long retention times may have contributed to but were not the major cause of the low responses of polyunsaturates. Analysis of individual esters on OV 275 yielded a broad, flat peak following the major component (Fig. 1) and substantially wider than expected for its retention time. This is typical of a decomposition product formed during passage through the column. No similar evidence of decomposition was seen for 18:1, nor did analysis of 18:2 or 18:3 in the glass EGSS-X column yield decomposition products.

The effect of column material on relative response was determined by analyzing polyunsaturated esters in 3 m stainless steel or glass columns packed with OV 275 (a 6 m glass column was unavailable for comparison with the original 6 m stainless steel column). In spite of much shorter retention times, destruction of 18:2 and 18:3 occurred in the steel column, whereas the glass-OV 275 combination gave excellent relative responses (Table III). The combination of steel and high temperature appeared to cause ester decomposition, although metal-catalyzed interaction of ester and stationary phase cannot be ruled out. Substitution of nitrogen for helium carrier gas or of petroleum-ether for CS_2 as injection solvent was without effect with the stainless steel-OV 275 column, indicating no contribution of these components to ester decomposition. Reducing residence time in the steel column improved polyunsaturated ester recovery (cf. 3.05 and 6.1 m columns, Table III). Reducing retention time by operating the 6.1 m OV 275 column at 230 C also improved response slightly (by 5-10%).

Calibration of the OV 275 column was complicated by the concentration dependence

TABLE III

Relative Responses of 18:2 and 18:3 Analyzed in Metal or Glass Columns Containing OV 275

OV 275 Column	Relative response[a] 18:2	18:3
Stainless steel[b], 6.1 m	0.70	0.48
Stainless steel[b], 3.05 m	0.77	0.59
Glass, 3 m	0.98	0.96

[a]Mean of 3 analyses; ca. 2 μg of ester injected into 6.1 m column, 1 μg into 3 m columns. 18:0 = 1.00.
[b]"Old" OV 275 column.

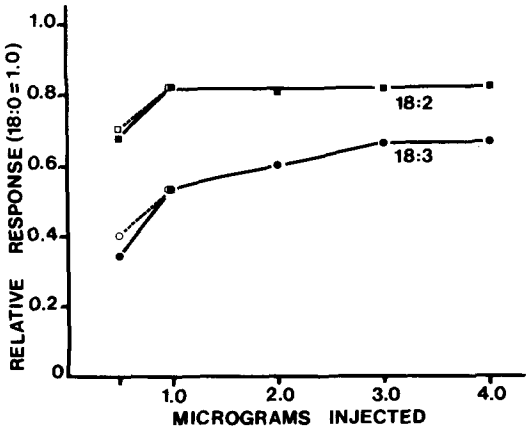

FIG. 2. Relative responses of methyl linoleate and methyl linolenate as functions of amount injected into OV 275 Column. "New" 6.1 m OV 275 column was employed. Open symbols and broken lines indicate the effects of decreasing electrometer attenuation in proportion to amount of ester injected.

of polyunsaturated ester relative responses, which declined when less than 1 μg of 18:2 or 3 μg of 18:3 was injected (Fig. 2). Electrometer attenuation was varied with sample size below 2 μg of ester to compensate for any decline in integrator sensitivity to smaller peaks. No effect of this manipulation was evident at the 1 μg level but some improvement occurred with 0.5 μg of ester (Fig. 2, broken line). Over the range of 2-0.5 μg, relative responses of 20:0 and 22:0 (with similar retention times to 18:2 and 18:3) declined by 4-5% (no modification of attenuation), indicating that loss of integrator sensitivity to small peaks was a minor factor in the loss of response to polyunsaturated esters. Destruction of proportionately more polyunsaturated ester probably accounted for most of the decline in response at low concentrations. Detection of minor isomer peaks undoubtedly contributed to the effect of attenuation observed with 18:2 and 18:3 in Figure 2. Decline in response to sample size was also seen with methyl arachidonate: 1 and 2 μg yielded relative responses of 0.41 and 0.28, respectively.

Comparison of data in Table II, obtained with a "new" OV 275 column, and that in Table III, obtained with a column used sparingly over a 3-year period, indicates an aging effect, with the older column yielding lower responses. Indeed, the "new" column yielded response factors after 7-8 week operation which were noticeably lower than those recorded after 3-4 weeks of use, indicating that the aging effect may be quite rapid.

Although the stainless steel-OV 275 column

separated *cis* and *trans* 18:1 efficiently and yielded excellent quantitative results for these two acids (cf. Table II), analysis of samples containing polyunsaturated esters was contraindicated. Not only were relative responses for these esters low but they were variable as a result of their concentration dependence and the column aging effect, making calibration difficult. Moreover, decomposition products may interfere with detection of esters eluting later. For example, corn oil (61% 18:2, 1.2% 18:3) yielded a low value for 18:2 (53%) on OV 275 but a variable and often high value for 18:3 (1.3-3%), in spite of the expected low response for this ester. The decomposition product of 18:2 (Fig. 1) was superimposed on the 18:3 peak, resulting in the anomalous value. The main problem appeared to be decomposition of esters on the hot stainless steel. No other metals were investigated and aluminum columns may not show the disadvantages recorded here for stainless steel. Long glass columns (6 m) were not used in this study but may provide a reasonable alternative to stainless steel. Heckers and his coworkers have investigated a variety of glass columns containing OV 275 and similar silicones (2,3).

ACKNOWLEDGMENTS

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COMMUNICATIONS

Inhibition of Hepatic Cholesterol Biosynthesis by Chloroquine

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ABSTRACT

Chloroquine is shown to be a potent inhibitor of cholesterol biosynthesis by isolated rat hepatocytes. Half-maximal inhibition of cholesterogenesis occurs at ca. 10 μM chloroquine. Chloroquine does not affect fatty acid synthesis by isolated hepatocytes. This suggests that chloroquine acts on the cholesterol biosynthetic pathway beyond the cytosolic acetyl-CoA branchpoint of cholesterol and fatty acid synthesis.

INTRODUCTION

Chloroquine is widely recommended as an antimalaria drug. The compound is also used as a tool in biochemical studies. In isolated hepatocytes, chloroquine inhibits proteolysis (1) and intracellular lipolysis (2). These effects are most likely explained by an elevation of the intralysosomal pH upon accumulation of chloroquine within the lysosomes (1). The enzymes concerned with both proteolysis and lipolysis are located within these organelles.

In this communication, we present evidence that in isolated hepatocytes, chloroquine inhibits specifically cholesterogenesis, a process which does not occur within the lysosomes.

METHODS AND MATERIALS

Hepatocytes were obtained from meal-fed male Wistar rats according to Seglen (3), with modifications described previously (4). Isolated hepatocytes (6-8 mg protein/mL) were suspended in Krebs-Henseleit bicarbonate buffer, supplemented with 3.5% bovine serum albumin (charcoal-treated and dialyzed) and 10 mM glucose. Incubations (final vol 3 mL) were done in 25-mL Erlenmeyer flasks under an atmosphere of 95% oxygen, 5% carbon dioxide in a gyratory water bath (90 cycles/min). To monitor rates of fatty acid and cholesterol synthesis, [^{3}H]H_2O (1 mCi/mL) was added to the incubation vessels.

Incubations were terminated by the addition of 0.4 M (final concentration) perchloric acid. Fatty acids were extracted according to Harris (5). The nonsaponifiable fraction of total lipids was subjected to thin layer chromatography after addition of carrier cholesterol. Chromatography was performed on Silica Gel G with petroleum ether (bp 40-60 C)/diethyl ether/acetic acid (80:20:2, v/v) as developing agent. The silica containing the cholesterol was scraped from the plate, mixed with scintillation fluid and counted for its radioactivity.

Fraction V bovine serum albumin, collagenase and chloroquine diphosphate were purchased from Sigma Chemical Co., St. Louis; [^{3}H]H_2O from the Radiochemical Centre, Amersham; Silica Gel G from Merck; other chemicals were from Baker.

RESULTS AND DISCUSSION

The synthesis of fatty acids and cholesterol by isolated liver cells was monitored by the incorporation of ^{3}H from [^{3}H]H_2O into these lipids. The [^{3}H]H_2O method is considered the most reliable method available to assess rates of fatty acid and cholesterol synthesis (6).

Figure 1 shows the incorporation of [^{3}H]-H_2O into fatty acids (panel A) and cholesterol (panel B) as a function of incubation time. As demonstrated before (4,5), fatty acid synthesis has a lag phase of ca. 15 min (Fig. 1A). Likewise, the rate of cholesterogenesis increases with incubation time (Fig. 1B). Chloroquine (50 μM) does not affect fatty acid biosynthesis (Fig. 1A), but drastically inhibits cholesterol synthesis by isolated hepatocytes (Fig. 1B).

Figure 2 illustrates that chloroquine is a very potent inhibitor of cholesterol synthesis. At a concentration of ca. 10 μM, chloroquine reduces cholesterogenesis to 50% of the control

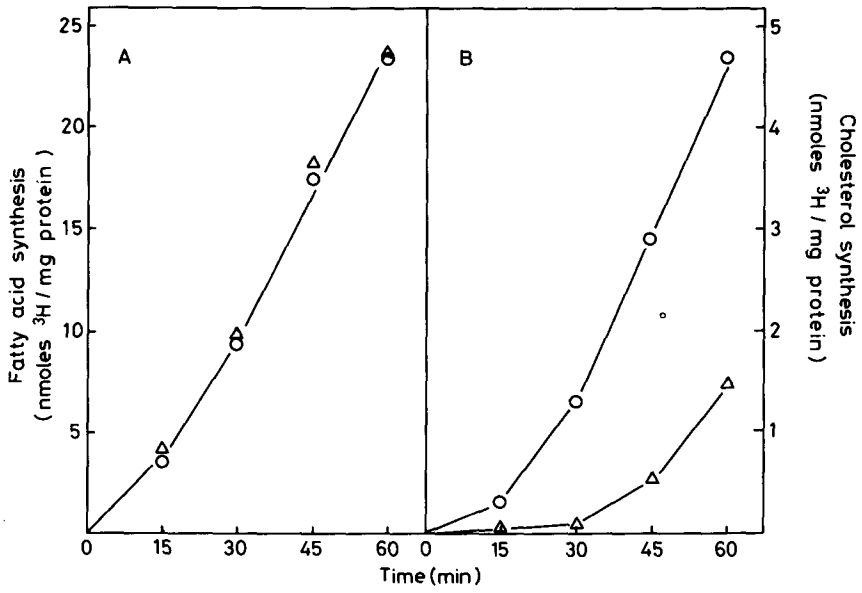

FIG. 1. Time course of $[^3H]H_2O$ incorporation into fatty acids (panel A) and cholesterol (panel B) by isolated rat hepatocytes. Control (○), chloroquine, 50 μM (△).

value. It should be stressed that in Figure 2, data on the incorporation of $[^3H]H_2O$ into cholesterol during the 60-min incubation period were used. These data do not represent true rates of cholesterogenesis since the incorporation of ^3H into cholesterol (both in the presence and absence of chloroquine) is not linear during the entire 60-min incubation period. In comparison, known inhibitors of hepatic cholesterol synthesis, such as (−) hydroxycitrate (7) and clofibrate (8), induce half-maximal inhibition at concentrations of ca. 1 and 2 mM, respectively. However, in contrast to chloroquine, these compounds also inhibit fatty acid synthesis.

In isolated hepatocytes, chloroquine inhibits gluconeogenesis, probably by impairment of lysosomal proteolysis. This impairment prevents replenishment of amino acids, which are required for shuttling reducing equivalents into the mitochondria during gluconeogenesis (1). It could be argued that chloroquine prolongs the lag in cholesterogenesis seen in the untreated cells (Fig. 1B). This lag phase may reflect the time required for the hepatocytes to accumulate the lipogenic substrates lactate and pyruvate (cf. ref. 5). However, addition of lactate and/or acetate significantly inhibits cholesterol synthesis by hepatocytes from meal-fed rats (9). Furthermore, chloroquine does not affect fatty acid synthesis, a process that is stimulated by the addition of lactate and/or acetate (5,9). Since cholesterol and fatty acid synthesis draw upon the same cytoplasmic acetyl-CoA pool, our observations indicate that chloroquine acts beyond this branchpoint on the cholesterol biosynthetic pathway per se. At present, it is

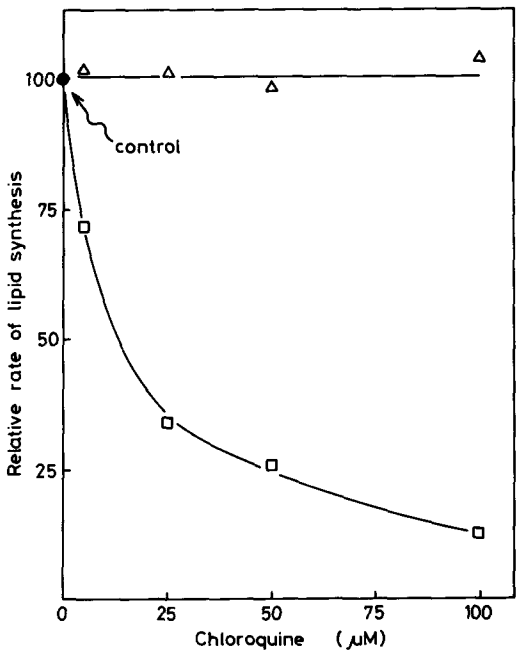

FIG. 2. Influence of chloroquine on fatty acid (△) and cholesterol (□) synthesis. Hepatocytes were incubated for 60 min.

unknown at which enzymatic site(s) in the conversion of acetyl-CoA into cholesterol chloroquine exerts its inhibitory action. Our results suggest that chloroquine may be a specific hypocholesterolemic agent.

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Obesity: Can Some Fat Cells Enlarge while Others Are Shrinking?

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ABSTRACT

CBA mice were made obese by injection with gold thioglucose (GTG). After receiving transplants of "lean" and "GTG-obese" fat under separate kidney capsules, the host mice were fed a restricted diet for three weeks. Over this period, the fat cells in the lean grafts enlarged whereas the fat cells in the GTG-obese grafts shrank. Concurrent fat loss and fat gain can therefore take place.

INTRODUCTION

With increasing age, anterior leg fat thickness has been reported to diminish in both men and women, whereas trochanteric fat thickness increases (1). A photographic study of fat distribution in 90 women, aged between 13 and 69 years (2), showed older, fatter women were more likely to have a central type of fat distribution. Thus, both of these cross-sectional studies indicate that increasing age and/or obesity can produce a possible redistribution of fat among body depots. Cross-sectional studies such as these do not answer the question, "Can fat be lost from some sites at the same time as it is gained by others, or must the process of fat mobilization or fat storage be dominant at all sites at the same time?"

We have previously described a technique for transplanting small pieces of adipose tissue under the kidney capsule of mice (3). Here, we use this technique to look for concurrent fat loss and fat gain during dietary restriction in mice which had previously been made obese by injection with gold-thioglucose (GTG). GTG induces obesity probably via its ability to cause lesions in the ventromedial part of the hypothalamus (4,5) which consequently leads to overeating.

EXPERIMENTAL PROCEDURES

All mice were CBA strain females aged 6-7 weeks. They received an intraperitoneal injection (0.1 mL) of GTG (80 mg/mL in experiment I and 70 mg/mL in experiment II). In both experiments, a few mice that were not injected with GTG were kept as control "lean" mice. After six weeks, when the injected mice were approximately double the weight of the control lean mice, "GTG-obese" mice received transplants of lean gonadal fat under their left kidney capsule and "GTG-obese" gonadal fat under their right kidney capsule. Samples of donor fats and host gonadal fat were taken at this stage for the estimation of mean fat cell size by the method previously described (3,6). Immediately after transplantation, dietary restriction was begun and instead of free access to stock diet (ca. 3.8 g/day), the mice in experiment I were allowed an average of 2.2 g/day and the mice in experiment II were allowed ca. 3.3 g/day. Three weeks later, when their weights had reached a level which was intermediate between the original weights of GTG-obese and lean mice, the host mice (three in experiment I and 13 in experiment II) were killed by cervical dislocation. The kidneys plus fat grafts were removed and fat cell size was determined not only in these fat grafts but also in samples of the "slimmed" host mice gonadal fat.

RESULTS

Donor Mice

Results are depicted in Table I and Figure 1.

At transplantation, the body weights of the GTG-obese fat donors were 39 g (experiment I) and 41 g (experiment II) and the body weights of the lean fat donors were 20 g (experiment I) and 18 g (experiment II). Fat donors were not subjected to dietary restriction and neither their body weights or fat cell weights changed significantly during the 3-week experimental period.

Host Mice

The mice in experiment II were subjected to less severe dietary restriction so that body weight and fat cell size decreased less than in Experiment I. However, the body weights of the host mice and their gonadal fat cell weights decreased significantly in both experiments ($p<0.001$, paired t-test).

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Behavior of Grafted Fat

During the transplantation period, the lean grafts increased in fat cell size whereas the GTG-obese grafts decreased in fat cell size. The difference in behavior of the two types of grafts was significant. Fat cell size of the slimmed hosts and fat cell sizes in either of the grafts were not significantly different. Thus, dietary restriction resulted in equalization of graft fat cell sizes to a size typical of their hosts.

The smaller cells in the lean grafts were able to increase in weight even though the body weights of the host mice and the weights of the larger grafted cells were decreasing.

DISCUSSION

During dietary restriction of host mice, fat mobilization must have taken place with the overall effect of reducing body weight. Thus, it was not surprising that host gonadal fat cell size decreased during this period. However, it was less easy to predict the changes in fat cell size in the grafts. Results from in vitro experiments (7) have indicated that fat mobilization is greater from larger cells than from smaller cells. Indeed, in some of our preliminary experiments in which the host's fat cells shrank to a size even smaller than those of the lean donor, we observed that the fat cells in the lean grafts shrank less than the fat cells in the GTG-obese grafts.

Studies of fat mobilization in vivo have also shown that the decrease in fat cell size during starvation in animals (8,9) or dietary restriction in man (10) is proportional to initial cell size. When fat storage is induced in rats by insulin injection (11,12) or by hypothalamic ventromedial lesions (13), the greatest increase in fat cell size is seen in the region with the smallest cells. Thus, there is a general tendency during fat mobilization or fat storage for "fat cell size equalization" to take place and the only reported exceptions to this rule occur when fat changes are induced by hormones such as corticosteroids (14) or estrogens (9) which appear to exert specific regional effects.

The transplantation model is unique because it gives an opportunity to study changes in fat cell size at different fat sites in the same animal. Fat loss was greatest from the largest cells (in the GTG-obese grafts) and fat gain was greatest in the smallest cells (in the lean grafts). Our results have therefore not only confirmed the tendency to fat cell size equalization but are the first to demonstrate that it can be brought about by fat loss from some cells at the same

TABLE I

Expt.	Host body weights (g)		Host fat		Fat cell weights (μg)[a]				Changes in fat cell weights (μg)		
					"Lean" fat		"GTG obese" fat				
	At transplant	When killed	At transplant	After diet restriction	At transplant	When removed from host	At transplant	When removed from host	"Lean" fat	"GTG obese" fat	p[b]
I	40.5 ± 2.1	25.5 ± 0.7	0.52 ± 0.14	0.18 ± 0.03	0.09	0.15 ± 0.01	0.42	0.15 ± 0.03	0.06 ± 0.01	-0.27 ± 0.03	<0.01
II	36.4 ± 2.0	25.6 ± 1.7	0.60 ± 0.09	0.44 ± 0.05	0.14	0.42 ± 0.09	0.80	0.40 ± 0.09	0.28 ± 0.09	-0.40 ± 0.09	<0.001

[a]Means ± standard deviations.
[b]Change in "lean" fat cell weight vs change in "GTG-obese" fat cell weight (paired t-test).

time as fat gain in other cells.

Insulin is the main hormone controlling fat synthesis and the catecholamines are the main hormones controlling fat mobilization from rodent fat cells (15). It is possible that the small cells of the lean grafts were more sensitive to the fat storage effects of insulin and that the large cells in the GTG-obese grafts were more sensitive to the fat mobilizing effects of catecholamines. Indeed, in vitro experiments have shown the fat cells of different sizes respond to hormones such as insulin and epinephrine to differing extents (15).

Finally, the demonstration of concurrent fat loss and fat gain in this experimental model indicates that this is a theoretically possible mechanism for the redistribution of human fat in situ.

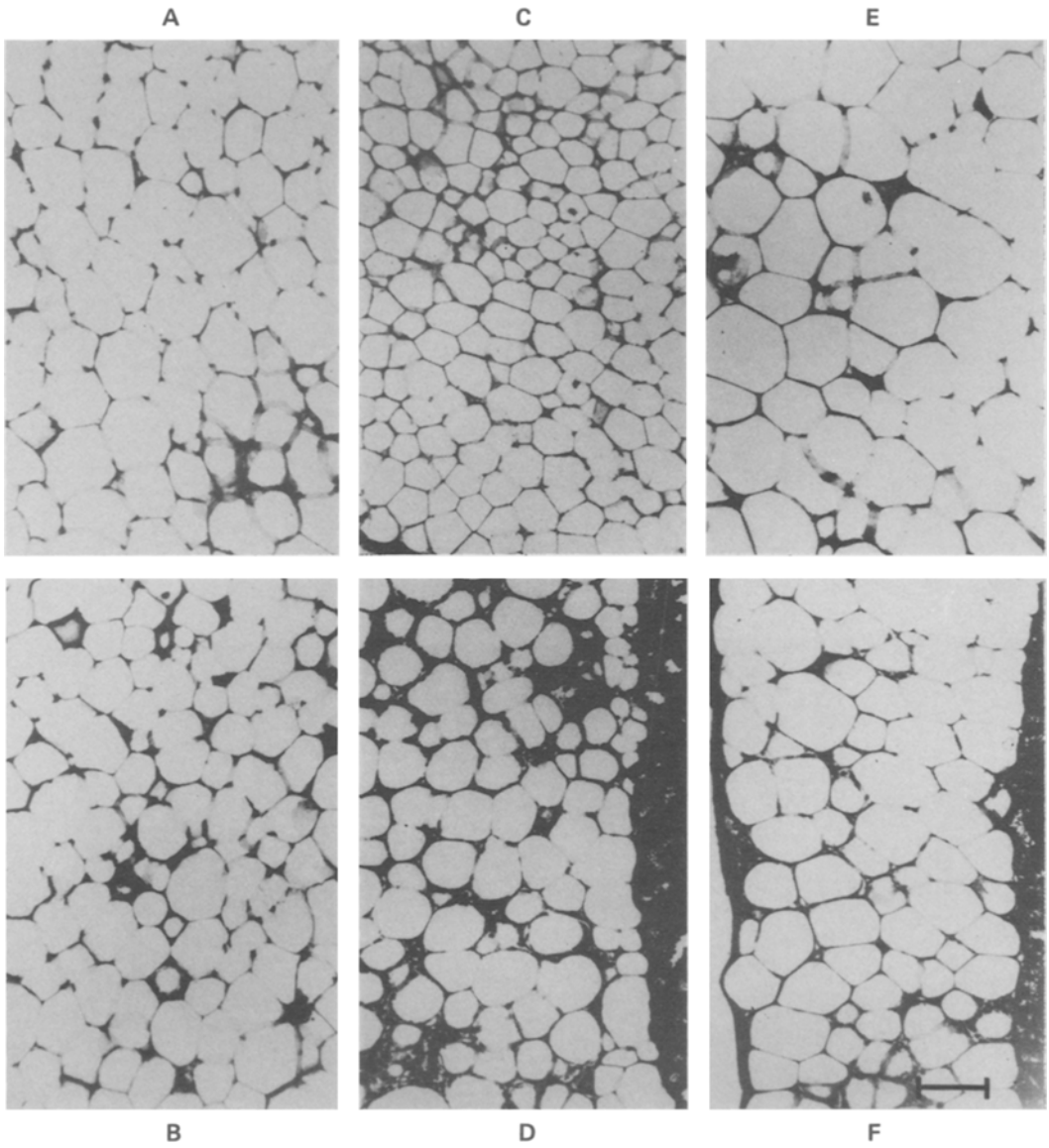

FIG. 1. Photomicrographs of adipose tissue samples from a typical mouse (experiment II) before and after dietary restriction: (A) host gonadal fat at transplantation; (B) host gonadal fat after dietary restriction; (C) "lean" donor fat at transplantation; (D) "lean" fat graft after dietary restriction of "GTG-obese" host; (E) "GTG-obese" donor fat at transplantation; (F) "GTG-obese" fat graft after dietary restriction of "GTG-obese" host. Scale marker = 100 μm.

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Positional Specificity of *trans* Fatty Acids in Fetal Lecithin

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ABSTRACT

Differences in the positional incorporation of 9-*trans*[1-^{14}C] octadecenoic (elaidic) and 9-*trans*,12-*trans*[1-^{14}C] octadecadienoic (linoelaidic) acids in fetal lecithin of rats were demonstrated. On the 20th day of gestation, a ^{14}C-labeled albumin complex of elaidic or linoelaidic acid was injected into the jugular vein of pregnant rats. For comparative purposes, 9-*cis*[1-^{14}C] octadecenoic (oleic) or 9-*cis*,12-*cis*[1-^{14}C] octadecadienoic (linoleic acid) was injected into the maternal circulation of rats. Animals were killed 6 hr later. Distribution of label in total lipids and phospholipids (PL) of fetal tissue was measured by TLC. Irrespective of the label, the highest percentage of total radioactivity was associated with PL-59 to 67%. Within PL, the major portion of radioactivity was found in choline phosphoglycerides (CPG)-53 to 67%, and in ethanolamine phosphoglycerides (EPG)-18 to 33%. While linoelaidic acid was predominantly esterified in the 2-position of CPG, elaidic acid was nearly equally distributed between positions 1 and 2 of lecithin. Distribution of radioactivity within fatty acid methyl esters (FAME) of CPG measured by radio-GLC suggested that oleic and possibly linoleic acids may be converted to nervonic and arachidonic acid, respectively, in the rat by the 20th day of gestation. Following injection of elaidate, radioactivity of FAME was distributed between palmitate and elaidic acid indicating that rat fetal tissue may metabolize elaidic acid via β-oxidation. In contrast, following injection of linoelaidate, radioactivity of FAME was primarily associated with *tt*-18:2, suggesting little biotransformation to other fatty acids by fetal tissues.

INTRODUCTION

Phospholipids (PL) usually exhibit asymmetric positional distribution of their constituent fatty acids (1-4) as well as tissue specificity of molecular composition (1). Selectivity of acyltransferases which control incorporation of dietary fatty acids into PL is believed to be of major importance for the maintenance of normal membrane properties and biological function (1,5,6). Generally, saturated fatty acids are preferentially esterified at position 1, and unsaturated fatty acids are primarily esterified at position 2 of glycerophospholipids. Likewise, *trans* fatty acids exhibit positional specificity in esterification reactions; however, they differ from their corresponding *cis* isomers regarding incorporation into triglycerides (TG) and PL. Several investigations of the intramolecular distribution of fatty acids have demonstrated that a higher percentage of *trans* isomers are primarily esterified at the 1- and 3-position of TG, and the 1-position of PL (3,7-9).

Incorporation of *trans* fatty acids into membrane lipids may alter membrane function (5,6,10). The lack of information regarding the esterification of *trans* fatty acids in fetal PL during the most critical and vulnerable period of intrauterine growth led us to undertake this investigation. Our results demonstrate differences in the positional incorporation of *trans* and *cis* octadecenoic and octadecadienoic acids in fetal lecithin (CPG) of rats. While linoelaidic acid (*tt*-18:2) is predominantly esterified in the 2-position, elaidic acid (*t*-18:1) is nearly equally distributed between positions 1 and 2 of fetal lecithin.

MATERIALS AND METHODS

9-*trans*[1-^{14}C] octadecenoic (elaidic) acid, 55 mCi/mM was obtained from Applied Sciences (Inglewood, CA) and 9-*trans*,12-*trans*[1-^{14}C]-octadecadienoic (linoelaidic) acid, 58 mCi/mM was obtained from Rosechem (Los Angeles, CA). 9-*cis*[1-^{14}C] octadecenoic (oleic) acid, 57 mCi/mM was obtained from New England Nuclear (Boston, MA). Radiopurity of *trans* and *cis* fatty acid isomers was greater than 98% as determined by thin layer chromatography (TLC) on silver nitrate impregnated Silica Gel-60 plates using 5% acetone in toluene as the developing solvent and by radioGLC (11).

On the 20th day of gestation, 150 μCi each of ^{14}C-labeled oleic, elaidic, linoleic, or linoelaidic acids in the form of a fatty acid albumin complex (12) were injected into the jugular veins of 2 rats. Six hr later, animals were anesthetized and maternal blood was collected by open heart puncture. Each rat had 12 fetuses; fetuses were removed and placed on ice. Fetal brain and body were separated,

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weighed and frozen at -70 C until extraction for lipid analysis. Analysis was carried out on pooled (groups of 4) samples.

Total lipids of plasma and fetal body were extracted and purified by the Folch et al. method (13). Distribution of radioactivity in various lipid components was determined by TLC methods using pentane/ether/acetic acid (80:20:1, v/v) for neutral lipids and chloroform/methanol/acetic acid/water (100:60:16:8, v/v) as the solvent system for PL (14). Areas indicated by brief exposure to iodine vapors were scraped directly into scintillation vials containing Aquasol (New England Nuclear, Boston, MA) and counted using a Beckman Liquid Scintillation Counter Model 800.

Isolation and Purification of Lecithin

Total lipids were fractionated by column chromatography according to the methods described by Rouser et al. (15). Purity of isolated fetal lecithin was confirmed by TLC. Lecithin was subjected to hydrolysis by venom *Crotalus adamanteus* (Sigma, St. Louis, MO) as proposed by Robertson and Lands (16). Erroneous results will be obtained if hydrolysis is not complete because different fatty acids may be hydrolyzed at varying rates. Therefore, the reaction was monitored every 1/2 hr by withdrawing an aliquot from the incubation mixture and by checking for the completeness of hydrolysis on a small 1.5 cm x 10 cm TLC plate using chloroform/methanol/formic acid/water (70:28:7.5:2.5, v/v) as the developing solvent (17). When no more of the starting material could be detected, the reaction was stopped by the addition of 40% methanol in chloroform. If the enzymatic hydrolysis would have split FA from the 1-position in addition to the 2-position, then the FA composition of the 2-position would have shown a very much higher percentage of .18:0. An aliquot of the mixture of free fatty acids liberated from the 2-position and lysolecithin were separated on Silica Gel-60 TLC plates using the solvent system just described. Areas indicated by brief exposure to iodine fumes were scraped into scintillation vials and counted as described previously. The remaining portion was plated on several TLC plates. Visualized bands were collected to obtain sufficient amounts of lysolecithin and fatty acids. Lysophosphatidylcholine was subjected to methanolysis overnight with 1% sulfuric acid in methanol (14). Analysis of fatty acid methyl esters (FAME) obtained from lysolecithin represented fatty acids at the 1-position whereas methyl esters obtained following hydrolysis with snake venom represented fatty acids at the 2-position. Completeness of the hydrolysis at the 2-position was also checked by fatty acid analysis (Table 1). Distribution of radioactivity of FAME was analyzed with a Packard Radio-Gas Chromatograph Model 804 in combination with a Packard Gas Proportional Counter Model 894 fitted with a 6 ft x 1/8 in. column packed with Silar 10C (Applied Science Laboratories, State College, PA) at 185 C (18).

RESULTS

Completeness of hydrolysis of lecithin by phospholipase A_2 was reassured from the close correlation noted between fatty acids in the 1- and 2-positions combined together (Table 1) and the fatty acids of unhydrolyzed lecithin following gas liquid chromatographic (GLC) analysis.

Distribution of radioactivity in the components of maternal plasma at 1- and 6-hr intervals is given in Table 2. Except in the case when labeled oleic acid was administered, the general trend seems to be a reduction in the

TABLE 1

Positional Distribution of Fatty Acids in Fetal Lecithin[a]

Fatty acid	1-Position	2-Position	Reconstituted (pos. 1 + pos. 2) / 2	Total PC
14:0	2.0	1.1	1.3	1.7
16:0	61.4	20.2	40.8	35.0
16:1	2.7	6.8	4.7	6.2
18:0	24.4	1.0	12.7	11.3
18:1	7.6	33.3	21.8	23.7
18:2	–	11.0	5.7	7.2
20:4	–	13.9	8.8	8.1
22:6	–	5.3	2.6	2.3

[a]Average of duplicate determinations of 3 pooled fetal body lecithin.

TABLE 2

Distribution of Radioactivity in Maternal Plasma[a,b]

Tracer injected	*c*-18:1		*t*-18:1		*c,c*-18:2		*t,t*-18:2	
	1 hr	6 hr	1 hr	6 hr	1 hr	6 hr	1 hr	6 hr
CE	14.9	4.0	1.3	4.0	2.6	12.2	1.6	1.9
TG	37.7	79.8	62.3	51.1	74.3	54.3	84.5	8.5
FFA	30.8	4.0	8.7	4.0	8.5	2.5	6.2	3.0
Chol	9.4	4.0	23.4	5.6	5.3	2.9	2.9	10.1
PL	7.3	8.4	4.3	35.4	9.2	28.1	4.8	76.5

[a]Values reported as the average of duplicate analysis

[b]1-hr values obtained from an earlier study (19).

TABLE 3

Percent Distribution of Radioactivity in Maternal Plasma FAME[a,b]

Tracer injected	*c*-18:1		*t*-18:1		*c,c*-18:2		*t,t*-18:2	
	1 hr	6 hr	1 hr	6 hr	1 hr	6 hr	1 hr	6 hr
16:0	–	–	16.7	15.9	5.9	–	–	27.8
c-18:1	100	100	–	–	–	–	–	–
t-18:1	–	–	83.3	84.1	–	–	–	–
c,c-18:2	–	–	–	–	94.1	80.4	–	–
t,t-18:2	–	–	–	–	–	–	100	72.2
c-20:4	–	–	–	–	–	19.6	–	–

[a]Values reported as the average of duplicate analysis.

[b]1-hr values obtained from an earlier study (19).

proportion of radioactivity in the TG and an increase in the proportion of radioactivity in the PL. The decrease in trend is also noticed in the cholesterol, free as well as esterified, fraction.

Table 3 gives the percentage distribution of radioactivity in maternal plasma total lipid fatty acids. There appears to be little difference in the pattern of distribution of label 1 and 6 hr after injection of *cis* and *trans* monoenes. Even 6 hr following injection, oleic acid remained the only radioactive component of maternal plasma fatty acids, whereas elaidic acid was partly oxidized and provided acetyl CoA for synthesis of palmitate. In contrast, differences were observed in the pattern of distribution of label 1 and 6 hr after injection of *cis* and *trans* dienes. One hr following injection of linoleic acid, no radioactivity was associated with 20:4; however, at the end of 6 hr, ca. 20% of radioactivity was found in arachidonic acid. In comparison with the all-*cis* 18:2, all-*trans* 18:2 was not converted to tetraenoic fatty acid, a finding which agrees with our earlier observation (19).

Distribution of radioactivity in nonpolar and polar lipids of fetal body lipids 6 hr following injection of *t*-18:1, *c*-18:1, *tt*-18:2 or *cc*-18:2 is presented in Table 4. Irrespective of the injected tracer, the major portion of radioactivity in total lipids was found in PL (59-67%) and to a lesser extent in TG (18-28%). The distribution of radioactivity in various components of fetal total lipids indicated that free fatty acids contained only a relatively small percentage (4-7%) of the total radioactivity, demonstrating nearly complete incoporation of injected isotopes into fetal lipids. Distribution of radioactivity within the major PL (Table IV) indicated that ^{14}C-labeled fatty acids were primarily found in choline phosphoglycerides (53-67%) and in ethanolamine phosphoglycerides (18-33%).

Table 5 summarizes the positional distribution of fatty acids in isolated fetal lecithin. Values are reported as the average of duplicate determinations of 3 pooled fetal samples. Radioactive fatty acids containing more than 2 double bonds were not found at position 1.

Positional dsitribution of radioactivity in isolated products of the phospholipase-A_2 hydrolysis are summarized in Table 5. Following injection of elaidic acid (*t*-18:1), a nearly equal distribution of radioactivity was observed between positions 1 and 2. After oleic acid injection, twice as much radioactivity was

present at the 2-position compared to the 1-position. In contrast, when ^{14}C-labeled linoelaidic or linoleic acid were administered, more than 80% of the radioactivity was located in the 2-position.

Since total radioactivity at any position may be associated with the injected tracer or with metabolic products, radio-GLC was used to identify the labeled fatty acids (Table 5). Six hr following injection, results indicated that tracers had undergone metabolic transformation. After injection of elaidic acid (*t*-18:1), radioactivity was distributed between palmitic (16:0) and elaidic (*t*-18:1) acids. At position 1, radioactivity was contributed by both 16:0 (13%) and *trans* 18:1 (37%). Likewise, at position 2, radioactivity was associated with 16:0 (17%) and *trans* 18:1 (32%). Thus, these findings suggest that elaidic acid was metabolized and palmitate was synthesized from acetyl CoA derived from β-oxidation of the tracer fatty acid.

Following injection of oleic acid, a major portion of the radioactivity at position 2 was contributed by *c*-18:1. Furthermore, nearly twice as much total radioactivity was found at position 2 compared to position 1. These findings, therefore, suggest a higher preferential esterification of oleic acid at the 2-position of rat fetal lecithin. In contrast, this specificity of esterification was not displayed for elaidic acid, as reflected by the nearly equal distribution of total radioactivity and distribution of label between 1- and 2-positions of fetal lecithin.

After injection of oleic acid, about 9% of the radioactivity at position 2 was found in nervonic (24:1ω9) acid. Earlier studies have shown that the oleic acid is a precursor for nervonic acid (20). Since no radioactivity was found in the nervonic acid of the circulating mternal plasma, it would appear that by the 20th day of gestation, rat fetal tissues may have the enzymes required for elongation of *c*-18:1 to *c*-24:1.

Following injection of linoelaidic acid, only 17% of the total radioactivity was found at position 1 whereas 83% of the radioactivity at position 2 consisted of *tt*-18:2 (Table 5). Results suggest preferential esterification of linoelaidic acid at position 2 and a limited metabolic transformation of the injected tracer.

When linoleic acid (*cc*-18:2) was given to pregnant rats, nearly 100% of the radioactivity was found at position 2 (Table 5). RadioGLC indicated that ca. 67% of the label at position 2 was associated with the injected tracer (*cc*-18:2) and ca. 11% of the label was present in arachidonic acid (*cis*-20:4).

TABLE 4

Distribution of Radioactivity in Nonpolar and Polar Lipids of Fetal Body Lipids[a,b]

Tracer injected	*c*-18:1		*t*-18:1		*c,c*-18:2		*t,t*-18:2	
	1 hr	6 hr	1 hr	6 hr	1 hr	6 hr	1 hr	6 hr
% Distribution in total lipids								
Sterol esters	8.2 ± 2.1	2.0 ± 0.3	1.7 ± 0.2	2.8 ± 2.7	8.1 ± 4.1	1.3 ± 0.1	2.7 ± 0.3	1.8 ± 0.6
Acyl glycerides	19.7 ± 0.9	28.4 ± 0.8	18.0 ± 1.6	23.6 ± 1.4	13.7 ± 3.8	18.1 ± 1.8	26.6 ± 2.4	23.4 ± 0.8
Free fatty acids	14.4 ± 1.6	4.5 ± 0.4	7.1 ± 2.8	3.8 ± 0.9	15.4 ± 2.6	4.5 ± 0.7	6.5 ± 0.5	7.0 ± 0.7
Free sterols	10.2 ± 1.5	5.8 ± 0.6	23.5 ± 1.0	10.1 ± 0.4	13.2 ± 2.3	9.1 ± 0.3	6.4 ± 0.6	4.1 ± 0.1
Polar lipids	47.5 ± 5.3	59.3 ± 2.0	49.8 ± 1.2	59.7 ± 2.3	49.5 ± 11.9	67.0 ± 0.3	57.8 ± 2.4	63.9 ± 0.4
% Distribution in major PL								
Choline phosphoglycerides		67.5 ± 1.4		53.0 ± 0.2		66.9 ± 1.8		53.6 ± 1.9
Ethanolamine phosphoglycerides		21.9 ± 2.1		25.8 ± 1.2		18.9 ± 1.2		32.5 ± 2.0
Serine phosphoglycerides		9.9 ± 0.7		15.7 ± 0.5		11.8 ± 0.5		12.0 ± 0.4

[a]Values reported as the average of duplicate analysis of 3 pooled fetal body samples.

[b]1-hr values from an earlier study (19).

TABLE 5

Positional Specificity of Fatty Acids in Fetal Body Lecithin (PC)[a]

Tracer injected	Position 1		Position 2	
	% Rad. in FA	% Rad. in PC	% Rad. in FA	% Rad. in PC
t-18:1	16:0-13.3 *t*-18:1-37.0	50.2	16:0-17.4 *t*-18:1-32.4	49.8
c-18:1	16:0-10.6 *c*-18:1-20.3	30.9	16:0- 9.8 *c*-18:1-50.2 *c*-24:1- 9.3	69.2
t,t-18:2	16:0-14.1 *t,t*-18:2- 3.2	17.3	16:0- 9.9 *t,t*-18:2-72.8	82.7
c,c-18:2	16:0- 2.7 *c,c*-18:2- 1.2	3.9	16:0-15.4 *c*-18:1- 3.6 *c,c*-18:2-66.8 *c*-20:4-10.5	96.1

[a]Average of duplicate analysis of 3 pooled samples 6 hr after injection of labeled fatty acids.

DISCUSSION

Uptake and esterification of *trans* fatty acids into fetal lipids was recently demonstrated following injection of ^{14}C-labeled elaidic and linoelaidic acids into the maternal circulation of rats (21). Regardless of the fatty acid injected, *trans* fatty acids were primarily incorporated into PL of the rapidly growing cell populations of the fetus. In this investigation, ca. 50% of the radioactivity in total lipid was associated with the PL fraction. Following injection of ^{14}C-labeled fatty acids, the greatest proportion of radioactivity within PL was found in choline phosphoglycerides of the fetus. This study also demonstrated preferential incorporation of linoelaidic acid in the 2-position of fetal lecithin whereas elaidic acid was esterified at positions 1 and 2 to a nearly equal extent. The positional specificity of *tt*-18:2 agrees with our earlier observation in the developing brain (22); however, in the developing brain (15-day-old rats) elaidic acid was mainly esterified at the 1-position of brain lecithin. Patterns of distribution of radioactivity within fatty acids of CPG in this investigation suggest that by the 20th day of gestation, fetal rat tissues have the enzyme systems for elongation of oleic to nervonic acids. Since the pattern of distribution of radioactivity in the maternal plasma total fatty acids after injection of oleic acid was different from that found in the fetal lecithin, it is assumed that fatty acid transformation and esterification occurs in the fetus, and does not merely reflect transport of maternal products to fetus. This may not have been the case with linoleic acid, since the circulating radioactive plasma fatty acids also contained both 18:2 and 20:4.

Asymmetric distribution of *trans* fatty acids in glycerophospholipids has been reported by several investigators. *Trans* isomers of octadecenoic and octadecadienoic acid have been found to predominantly occupy the 1-position of rat liver CPG (7-9). However, it has been recognized that distribution of fatty acids between positions 1 and 2 are also influenced by competition with other fatty acids. Privett et al. (23) found that *tt*-18:2 was esterified primarily in the 1-position of CPG except in those molecular species in which *tt* 18:2 was a constitutent along with saturated fatty acids. Under such conditions, linoelaidic acid was predominantly located at the 2-position. However, these CPG species represented only 3.5% of the incorporated *tt* 18:2.

Molecular composition of PL is thought to reflect selectivity of acyltransferases. Studies by Lands (24) and Okuyama et al. (25) reported acyl transfer to the 1-position of PL was sensitive to configurational differences among *cis* and *trans* octadecenoic isomers, whereas transfer to the 2-position did not display this selectivity. Lands has suggested that melting point may not determine enzyme specificity, but instead, the acylating enzyme for the 2-position favors 18-carbon chains that have π bonds located at positions 5, 9 and 12. *Trans* octadecenoic acid has been reported to be primarily esterified in the 1-position of rat liver lecithin (7-9). However, in this investigation, *t*-18:1 was found to be fairly equally distributed between positions 1 and 2 of fetal lecithin. Studies indicate that oleic acid displays a less

pronounced positional distribution in CPG than saturated and polyunsaturated fatty acids (26). Therefore, the lack of a positional specificity of elaidic acid for position 1 of fetal lecithin may reflect a lag in the development of selectivity of acyltransferases for configurational differences among octadecenoic isomers.

Acyltransferases have been reported to discriminate between *cis* and *trans* isomers of octadecadienoate as well (27). While acyltransferases from rat liver microsomal fractions incorporated linoleic acid predominantly in the 2-position of TG andCPG, *trans,trans* octadecadienoate was esterified at the 1-position and *cis,trans* isomers gave intermediate results (27). However, in this investigation, both *cc*-18:2 and *tt*-18:2 acids were predominantly incorporated into the 2-position of fetal CPG. Thus, differences in this study and those in the liver may be attributed to tissue differences.

Following injection of elaidic acid, radioactivity was associated with both palmitate (16:0) and *t*-18:1. Results suggest that fetal tissues can metabolize elaidic acid and contribute acetyl CoA for de novo synthesis of palmitic acid. Likewise, previous studies in our laboratory (21) have demonstrated placental transfer of elaidic acid with subsequent oxidation of *t*-18:1 and synthesis of palmitate in the rat. Thus, elaidic acid may provide a source of energy for the fetus. On the other hand, the minimal biotransformation of ^{14}C-linoelaidic acid suggests a difference in the fetal metabolism of *trans* octadecenoic and octadecadienoic acids. Linoelaidic acid may not undergo β-oxidation to any significant extent in the fetus and, therefore, may tend to accumulate in growing tissues.

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Major Hydrocarbons of the Post-Pharyngeal Glands of Mated Queens of the Red Imported Fire Ant *Solenopsis invicta*

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ABSTRACT

Thin layer and column chromatographic analyses showed that hydrocarbons were the major lipoidal components of post-pharyngeal glands of mated queens of the red imported fire ant, *Solenopsis invicta*. Gas liquid chromatographic analyses on an OV-17 column showed four major hydrocarbons which have been identified and confirmed by synthesis and comparative mass spectral analyses as 13-methylheptacosane, 13,15-dimethylheptacosane, 3-methylheptacosane, and 3,9-dimethylheptacosane. When microgram quantitites of the natural alkanes on filter paper were placed in colonies of ants, the ants clustered on the paper about the sample and proceeded to masticate the paper in the area containing the alkanes.

INTRODUCTION

The post-pharyngeal glands (PPG) that occupy a large portion of the head overlaying the brain with openings in the pharnyx (1-3) are an important pair of structures in ants. In the red imported fire ant, *Solenopsis invicta*, the PPG of the queen are more highly developed than the PPG of other castes (B.M. Glancey, unpublished observations) and they become disproportionately large in queens and are filled with fluid prior to the mating flight (B.M. Glancey, unpublished observations). Although no definite roles or functions have been established for the PPG, it has been recently suggested that they function in much the same way as a gastric caecum (4). We have examined the lipids of PPG of queens 1 to 8 weeks after the mating flight and report in this paper on the identification and organic synthesis of the major lipoidal components of the PPG.

EXPERIMENTAL PROCEDURES

Instrumentation

Melting points were observed on a Kofler block, and IR spectra were obtained with a Perkin-Elmer Model 221 prism-grating spectrophotometer. Gas liquid chromatographic (GLC) analyses were made on a Barber-Colman Model 10 chromatograph; the GLC systems were 0.75% SE-30 and 1.0% OV-17 coated on Gas-Chrom P. The mass spectra of the various intermediates and hydrocarbons were obtained by using an LKB Model 9000 (Electron Impact) gas chromatograph-mass spectrometer (GC-MS), (LKB Produkter AB, Stockholm, Sweden) equipped with a Varian Spectro System 100 MS data system; the samples were introduced directly into the ionization chamber of the mass spectrometer through the GLC system and the ionization energy was 70 eV. The hydrocarbons were also analyzed by an analytical GC-MS LKB Model 2091, Electron Impact/Chemical Ionization GC-MS-EI, using an SE-30 glass capillary column 0.2 mm id x 25 m. The spectrometer was equipped with an LKB-2130 GC-MS Data system. In addition, chemical ionization mass spectra of the hydrocarbons were obtained with a Finnigan 4000 mass spectrometer fitted with 0.25 mm id x 30 m, SP-2100 glass capillary column. Isobutane chemical ionization was used for production of ions. Data were collected and analyzed via an Incos Data system.

Extractions and Isolation of Hydrocarbons from Post-Pharyngeal Glands

The PPG were removed from queens 1 to 8 weeks after the mating flight (ages mixed within this range) and stored and homogenized in benzene (initially, PPG were extracted with chloroform/methanol (2:1); because the major lipid was hydrocarbons, benzene was used for subsequent extractions). The solution was filtered through a fritted disc funnel and the benzene was evaporated under a stream of nitrogen. The residue was chromatographed over 2.0 g of Unisil and the column was eluted with 15 mL of hexane followed by 15-mL fraction of hexane containing 2, 5, 10, 25, and 50% diethyl ether, then 100% diethyl ether.

The fractions were analyzed on two separate thin layer chromatography (TLC) plates; one plate was developed in the solvent system of benzene/hexane (1:1) and the other in benzene/ethyl acetate (9:1). The hexane fraction which contained the hydrocarbons was analyzed by argentation TLC.

Bioassay of PPG Hydrocarbons

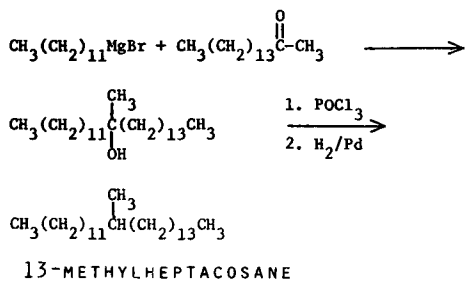
Either a 10- or 20-μg sample in hexane (1 μg/μL) of chromatographically pure hydrocarbons from the PPG was spotted on the center of a 5 × 5 cm piece of filter paper. A blank was similarly treated with the appropriate volume of hexane. After the hexane had evaporated, the papers with and without hydrocarbons were placed in laboratory colonies of about 10,000 ants. The ants were observed for 2 hr.

Materials and Chemical Synthesis of Intermediates Used in Schemes I-IV

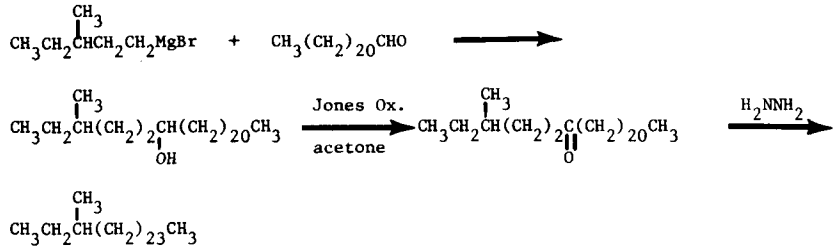
Silicic acid (Unisil 100 to 200 mesh) was obtained from Clarkson Chemical Co. (Williamsport, PA). Neutral alumina (Woelm) was obtained from ICN Nutritional Biochemicals (Cleveland, OH), and the required amount of water was added to make activity Grade II alumina. Docosanol was purchased from Lachat Chemicals, Inc. (Mequon, WI), Dodecyl bromide was purchased from Aldrich Chemical Co. (Milwaukee, WI) and 3-methyl-1-pentanol from Albany International Chemicals Division (Columbus, OH). The reaction of 3-methyl-1-pentanol with triphenylphosphine dibromide (5) gave 1-bromo-3-methylpentane, bp 47-49 C, 20 mm (used in Scheme III). The reaction of dodecyl magnesium bromide with acetaldehyde gave in nearly quantitative yield 2-tetradecanol, (mp 33-34 C) which, when allowed to react with triphenylphosphine dibromide (5), gave 2-bromotetradecane, bp 152-155 C, 9 mm. This material was used as starting material in Scheme II. The other starting material in Scheme II, 2-methyltetradecanal, was prepared via the reaction of dodecyl bromide and the sodium salt of diethyl methylmalonate (6); this was followed by decarboxylation and treatment of the resulting monocarboxylic acid with diazomethane to give the methyl 2-methyltetradecanoate. Reduction of the ester with lithium aluminum hydride in ether gave the alcohol which, upon oxidation with anhydrous chromium trioxide-pyridine in methylene chloride (7), gave 2-methyltetradecanal. A similar oxidation of docosanol yielded docosanal (starting materials, Scheme III). The required starting material (4-methyldocosanol) in Scheme IV was also prepared by the condensation of octadecyl bromide and the sodium salt of diethylmalonate (6), followed by decarboxylation to the monocarboxylic 2-methyleicosanoic acid, mp 62-66 C. Treatment of the acid with diazomethane followed by reduction with lithium aluminum hydride gave 2-methyleicosanol, mp 48-50.5 C, which was converted by oxidation with anhydrous chromium trioxide-pyridine in methylene chloride (7) to 2-methyleicosanal. The reaction of this aldehyde with malonic acid in pyridine containing 0.4% piperidine gave the 4-methyl-2-docosenoic acid, which upon treatment with diazomethane and subsequent catalytic reduction with 10% palladium on charcoal in cyclohexane followed by a reduction with lithium aluminum hydride, gave 4-methyldocosanol, mp 48-49 C.

Preparation of 13-Methylheptacosane (Scheme I)

To a solution of dodecyl magnesium bromide, prepared by refluxing 0.4 g of magnesium and 4.15 g of dodecyl bromide in 75 mL of anhydrous diethyl ether until all magnesium had been consumed (4 hr), was added 4.0 g of 2-hexadecanone in 75 mL of ether and the mixture was refluxed for 8 hr. The solution was cooled and 10 mL of water was added by drops, followed by 15 mL of a solution of 6 N sulfuric acid. The ethereal phase was separated, washed with water, dried over anhydrous sodium sulfate and reduced to dryness under vacuum to give 6.28 g of crude 13-methyl-13-heptacosanol. The material was chromatographed over 120 g of hexane-washed neutral alumina and the following 100-mL fractions were collected: 1, hexane, 2, hexane/benzene (9:1), 3, hexane/benzene (1:1), 4, benzene, and fraction 5 and 6 of benzene/ether (3:1). On the basis of TLC analyses, fractions 4-6 were combined to give 4.3 g of material. Recrystallization of a sample from acetone-methanol yielded needles, mp 50-51 C. To 4.2 g of the 13-methyl-13-heptacosanol in 75 mL of dry pyridine and 25 mL of benzene was added 3.0 mL of phosphorus oxychloride and the solution was refluxed for 1 hr and then 40 mL of



SCHEME I. Synthesis of 13-methylheptacosane.



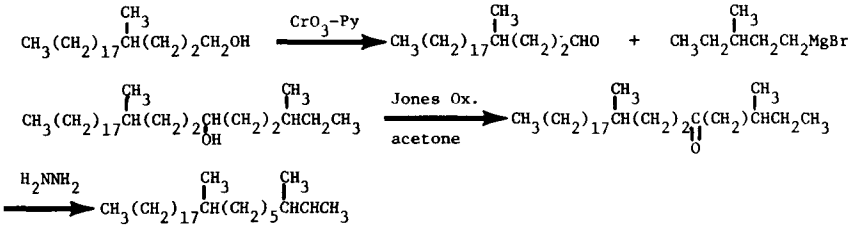
3-METHYLHEPTACOSANE

SCHEME III. Synthesis of 3-methylheptacosane.

added 6.0 g of freshly prepared docosanal over a 30-min period. The mixture was refluxed and, when worked up as in the preparation of 13-methyl-13-heptacosanol, yielded 7.65 g of crude alcohol. Purification of the material by column chromatography over 153 g of activity Grade II neutral alumina gave 5.63 g of 3-methyl-6-heptacosanol, mp 62-63 C. The oxidation of 3-methyl-6-heptacosanol (5.0 g) in 200 mL of acetone with an 8 N solution of chromic acid in dilute sulfuric acid (Jones reagent [8]) gave 4.6 g of 3-methyl-6-heptacosanone, mp 54-55 C. To a 3-neck flask equipped with a reflux condenser and a thermometer was added 150 mL of triethylene glycol and 0.5 g of sodium and the mixture was heated below 90 C until the sodium had been consumed (9). The mixture was cooled, 4.5 g of 3-methyl-6-heptacosanone in 50 mL of anhydrous diethyl ether and 4.0 mL of hydrazine was added; the mixture was gently heated and the ether was removed by distillation. The temperature of the solution was gradually raised to 140 C, maintained between 140-145 C overnight, then gradually raised to 212 C and kept at that temperature for 3 hr. After cooling, the solution was poured into a cold solution of dilute hydrochloric acid; the precipitate was collected and dried to give 4.3 g of 3-methylheptacosane, mp 45-46 C. A sample recrystallized from acetonitrile/ether melted at 46-47 C.

Preparation of 3,9-Dimethylheptacosane (Scheme IV)

To a magnetically stirred solution of 8.3 g of pyridine in 200 mL of methylene chloride was added 5.25 g of chromium trioxide and the deep burgundy solution was stirred for 15 min at room temperature (7). At the end of this period, a solution of 3.0 g of 4-methyldocosanol in 75 mL of methylene chloride was added and the mixture was stirred for an additional 15 min. The solution was decanted from the tarry black deposit which was washed with 200 mL of ether. The combined organic phases were washed with solutions of 5% sodium hydroxide, 5% hydrochloric acid, 5% sodium bicarbonate and then with water, and dried over sodium sulfate. After solvent removal under vacuum, 2.6 g of the 4-methyldocosanal was obtained. The aldehyde (2.6 g) in 100 mL ether was added to a refluxing solution of 1.45 g of 3-methylpentyl magnesium bromide in 50 mL of ether and the mixture was refluxed for 8 hr. The work-up of the Grignard in the usual manner gave the crude 3,9-dimethyl-6-heptacosanol, which, when chromatographed over 75 g of activity Grade II neutral alumina and eluted with the solvent systems used in the chromatography of 13-methyl-13-heptacosanol (Scheme I), yielded 2.2 g of chromatographically pure 3,9-dimethyl-6-heptacosanol, mp 37-39 C. The oxidation of 3,9-dimethyl-6-heptacosanol (2.1 g) in 100 mL



3,9-DIMETHYLHEPTACOSANE

SCHEME IV. Synthesis of 3,9-dimethylheptacosane.

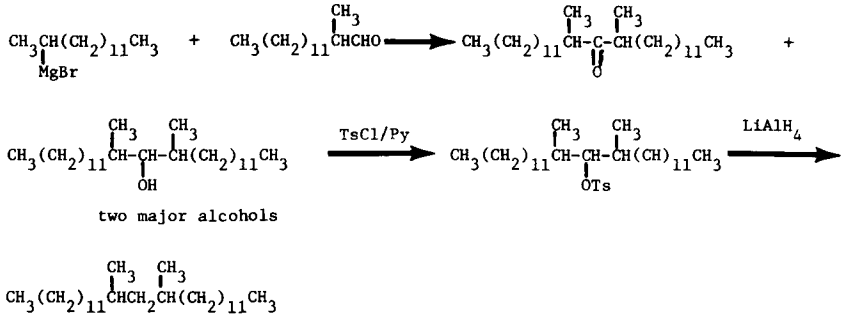
solvent was distilled off. The reaction mixture was cooled, diluted with water, extracted with hexane, and the hexane solution was washed with water, dilute sulfuric acid solution, water and dried over sodium sulfate. Removal of the hexane under vacuum gave 3.65 g of a mixture of 13-methylheptacosenes which was filtered in hexane through a column of 90 g of alumina. The residue (3.4 g) in 120 mL of cyclohexane, and 340 mg of 10% palladium on charcoal was treated with hydrogen for 3 hr at room temperature and atmospheric pressure. The solution was filtered and the solvent was removed under vacuum; the residue in hexane was chromatographed over 40 g of Unisil impregnated with 20% siliver nitrate. The first two 100-mL fraction yielded 3.3 g of 13-methylheptacosane, mp 29-30 C. Argentation TLC indicated that it did not contain any unsaturated hydrocarbons.

Preparation of 13,15-Dimethylheptacosane (Scheme II)

To a solution of 2-tetradecyl magnesium bromide, prepared by refluxing 0.72 g of magnesium and 8.18 g of 2-bromotetradecane in 100 mL of tetrahydrofuran (THF) until most of the magnesium was consumed (6 hr), was added 5.4 g of 2-methyltetradecanal in 100 mL of THF over a period of 1 hr and the mixture was refluxed for 8 hr. The Grignard reaction mixture worked up as in the preparation of 13-methyl-13-heptacosanol yielded 11.53 g of residue of which the bulk consisted of an undesired more polar compound. The residue was chromatographed over 200 g of hexane-washed neutral alumina (activity Grade II) and the column was eluted with 200-mL fractions (100-mL fractions collected) of increasing concentrations of 10, 15, 25, 50, 75, 80% benzene in hexane and finally benzene. The first fraction of 10% benzene in hexane yielded 500 mg of hydrocarbon; the second collected fraction of 10% benzene through 25% benzene in hexane gave 500 mg of the 13,15-dimethyl-14-heptacosanone (which when reduced with lithium aluminum hydride gave nearly a 1:1 mixture of the two diastereoisomers of 13,15-dimethyl-14-heptacosanol). The fractions eluted with 80% benzene in hexane yielded 624 mg of a 13,15-dimethyl-14-heptacosanol with a mp 33-34 C and an R_f of 0.50 in the solvent system of benzene/ethyl acetate (96:4). Further elution of the column with benzene gave 366 mg of a second 13,15-dimethyl-14-heptacosanol with an R_f of 0.36 in the solvent system just described. This compound did not crystallize at room temperature. The tosylates of both alcohols were prepared. Lithium aluminium hydride reduction of the tosylate in ether of the alcohol with an R_f of 0.5 gave 423 mg of 13,15-dimethylheptacosane with an equivalent carbon length on an OV-17 column of 27.5 which was identical to that of the hydrocarbon of the second peak of hydrocarbons of PPG of the imported red fire ant. A similar reduction of the tosylate of the more polar alcohol (R_f 0.36) gave 300 mg of the 13,15-dimethylheptacosane that eluted earlier on the OV-17 than the 13,15-dimethylheptacosane obtained via the alcohol with an R_f of 0.50. Both samples were filtered through 15 g of Unisil impregnated with 20% silver nitrate. The desired 13,15-dimethylheptacosane melted at 25-27 C; the other was a liquid at room temperature. The two hydrocarbons, possibly diastereoisomers, gave identical mass spectral data which supported this conclusion.

Preparation of 3-Methylheptacosane (Scheme III)

To a solution of 3-methylpentyl magnesium bromide, prepared by refluxing 4.0 g of 1-bromo-3-methylpentane and 590 mg of magnesium in 120 mL of anhydrous ether for 5 hr until the magnesium had been consumed, was



SCHEME II. Synthesis of 13,15-dimethylheptacosane.

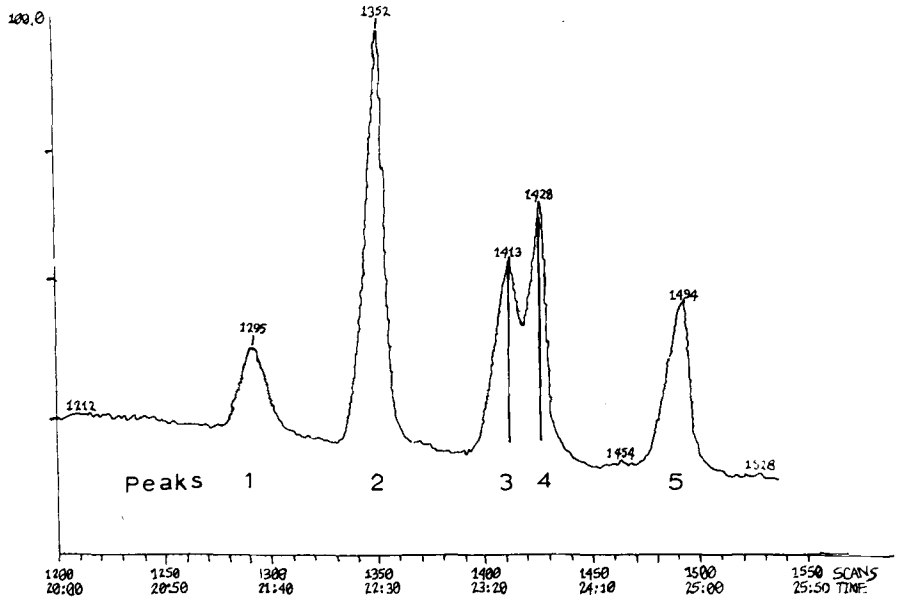


FIG. 1. GC-MS total ion monitor trace of the hydrocarbons from PPG of *S. invicta* obtained with a Finnigan 4000 mass spectrometer.

of acetone with Jones reagent (8) gave 2.0 g of 3,9-dimethyl-6-heptacosanone, mp 30-31 C. A modified Wolf-Kishner reduction of 3,9-dimethyl-6-heptacosanone (9) as in preparation of 3-methylheptacosane (Scheme III) gave 1.69 g of crude product, which, when filtered in hexane through 30 g of Unisil, gave in the second 50 mL fraction 1.57 g of 3,9-dimethylheptacosane, refractive index at 15 C 1.4519.

RESULTS

Surprisingly, TLC and column chromatographic analyses showed that the major lipoidal components of PPG from queens 1 to 8 weeks after the mating flight were hydrocarbons. In a typical experiment, 15 PPG yielded 1,000 μg of lipids, which, when chromatographed over 2 g of Unisil and the residue from each fraction weighed, showed that the fraction eluted with hexane yielded 601 μg of saturated hydrocarbons as indicated by argentation TLC analyses. The remaining 40% of lipids consisted of triglycerides, waxes, sterol esters, alcohols and sterols. GLC analyses of the hydrocarbons on SE-30 showed a minor peak with an equivalent chain length (ECL) of 27 and three major peaks with ECL of 27.5, 27.7 and 28, respectively. The GLC analyses of the hydrocarbons on an OV-17 column, however, yielded a minor peak with an ECL of 27 and four major peaks (peaks 2, 3, 4, and 5) with ECL of 27.3, 27.5, 27.7 and 28, respectively. Thus, the second major peak on the SE-30 column separated into two peaks, the third and fourth peaks on the OV-17 system. Peaks 1 through 5 on the OV-17 column represented ca. 6, 35, 17, 22 and 15%, respectively, of the total hydrocarbons. Minor peaks eluting both before and after accounted for the remaining 5%.

The hydrocarbons, when analyzed by GC-MS on a Finnigan spectrometer equipped with an SP-2100 glass capillary column, also separated into four major peaks similar to those on the OV-17 column. A typical total ion monitor trace is shown in Figure 1. The chemical ionization (CI) mass spectra of these hydrocarbons are shown in Figures 2 and 3. The mass spectra of hydrocarbon peaks 2-5 (Fig. 1) indicated that all were methyl branched alkanes. The CI-mass spectrum of peak 1, with a base peak at $(M-1)^+$ at m/z 379 and the ECL of 27, identified peak 1 as heptacosane. The EI spectrum (Fig. 4) also supports this structure. The $(M-1)^+$ peak at m/z 393 of the compound with an ECL of 27.3 (peak 2, Fig. 1) and fragment ion pairs at m/z 224/225 and 196/197 which arise from cleavage on each side of a methyl branch, established the structure for this component as 13-methylheptacosane (Fig. 2, spectrum 2).

The second major branched alkane (peak 3, Fig. 1) with an ECL value of 27.5 on OV-17 was identified as 13,15-dimethylheptacosane (Fig. 2, spectrum 3). The $(M-1)^+$ peak at m/z 407 established the molecular weight as 408.

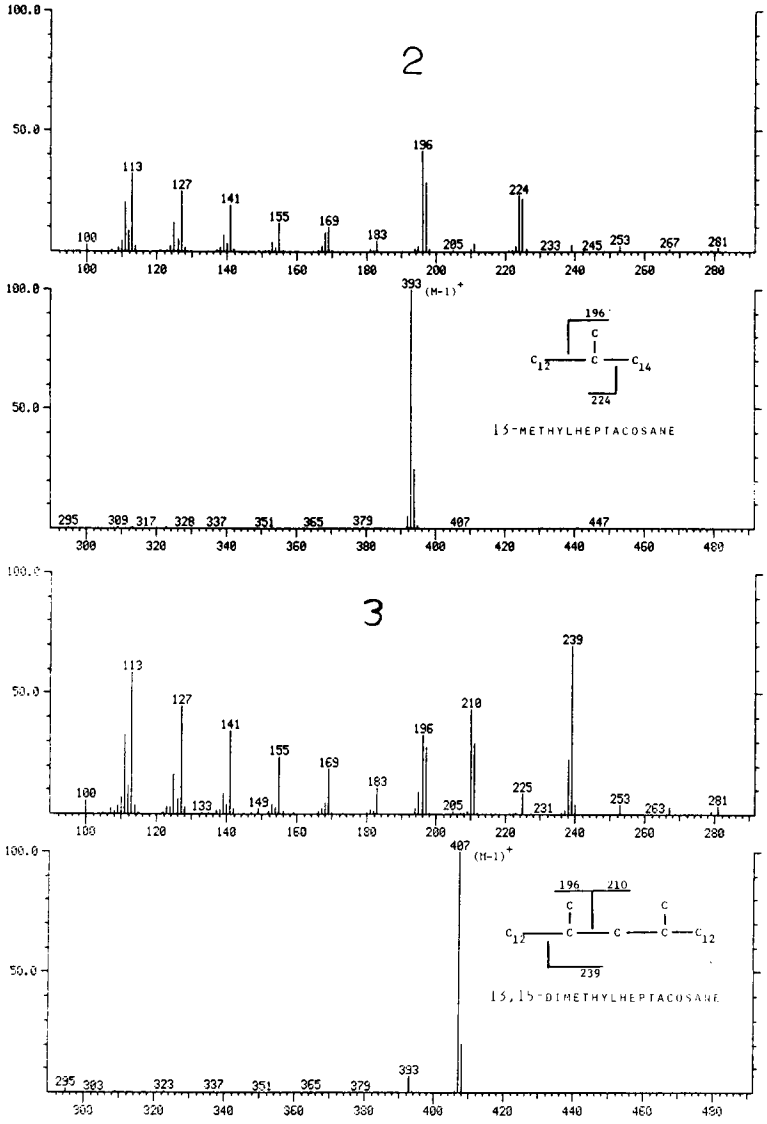


FIG. 2. CI mass spectra of GLC hydrocarbon peaks 2 and 3, respectively, from PPG of *S. invicta*, (2) 13-methylheptacosane and (3) 13,15-dimethylheptacosane.

Cleavage internal to either methyl branch yielded a secondary carbonium ion that gave the ion pairs at m/z 196/197 whereas cleavage external to the methyl branch gave the 17-carbon secondary carbonium ion at m/z 239. The ion pairs at m/z 210/211 resulted from cleavage internal to the methyl branch which formed a 15-carbon primary carbonium ion. The small peak in the spectrum at m/z 393 $(M-1)^+$ most likely results from a small amount of contamination of the alkane of peak 4.

The GC-CI-MS analyses of peak 4 (Fig. 1) with an ECL of 27.7 showed $(M-1)^+$ ion peak at m/z 393 and a strong fragment ion at m/z 365 which indicated cleavage at the methyl branch (Fig. 3, spectrum 4). Thus, this compound was identified as 3-methylheptacosane.

The CI-mass spectrum of peak 5 (Fig. 1) showed a $(M-1)^+$ at m/z 407 which indicated a carbon number of 29. Thus, the ECL of 28 and the mass spectral data of this hydrocarbon suggested a methyl branch at 2, 3 or 4 positions and a second methyl group farther down the chain. The ion pairs at m/z 155/154 and 281/280 suggested that the major hydrocarbon of peak 5 was 3,9-dimethylheptacosane (Fig. 3,

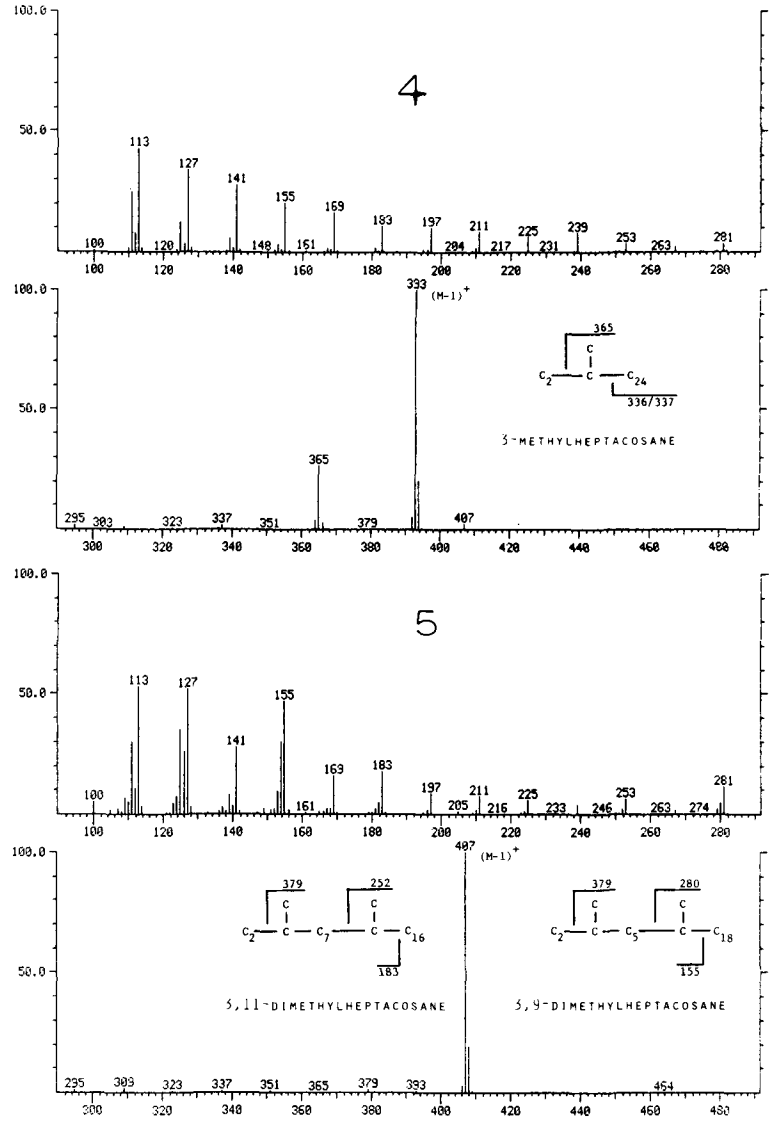


FIG. 3. CI mass spectra of GLC hydrocarbon peaks 4 and 5, respectively, from PPG of *S. invicta*, (4) 3-methylheptacosane and (5) 3,9-dimethylheptacosane and 3,11-dimethylheptacosane.

spectrum 5). Unlike the CI-mass spectra of the other hydrocarbons, the mass spectrum of this peak was not readily interpretive; that is, fragments indicating branch points were not very obvious. The EI-mass spectrum (Fig. 8), however, confirms that the major hydrocarbon of this peak is indeed 3,9-dimethylheptacosane. The fragment ion pairs at m/z 183/182 and 253/252 indicate another component in peak 5 with the structure of 3,11-dimethylheptacosane.

The EI-mass spectral analyses of the hydrocarbons of peak 1-5 and of respective synthetic compounds (Fig. 4-8) confirm the structures and identities of the major hydrocarbons of the PPG of the red imported fire ant. The ECL of the synthetic and naturally occurring respective hydrocarbons were identical.

Schemes I-IV show the syntheses of the red imported fire ant hydrocarbons and all were obtained in reasonable yields except for the 13,15-dimethylheptacosane. The structures of intermediates and final products were confirmed by IR and mass spectroscopy of the

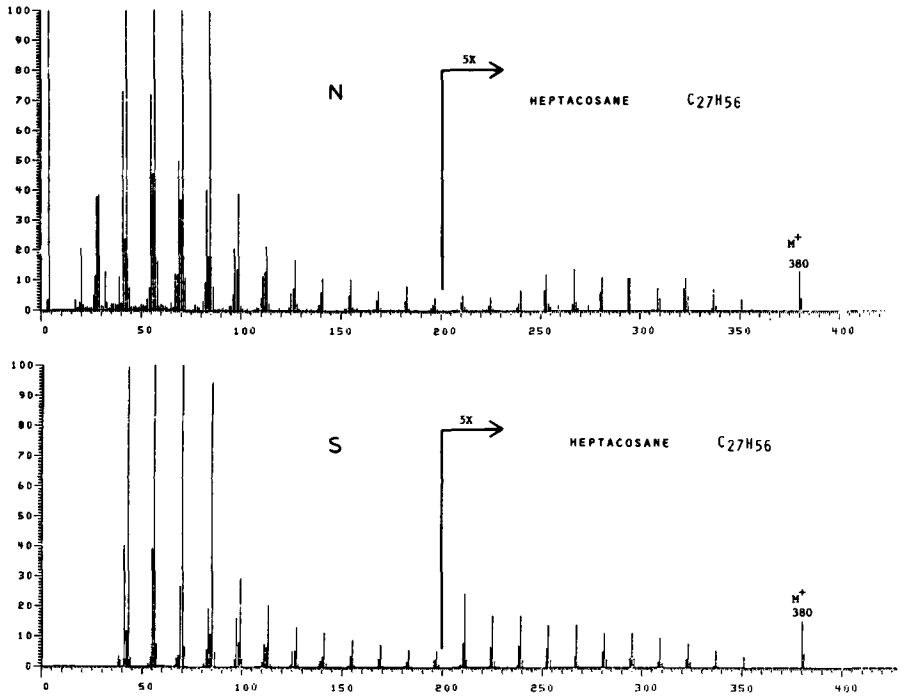


FIG. 4. EI mass spectra of GLC peak 1. (N) Heptacosane from PPG of *S. invicta* and (S) synthetic heptacosane.

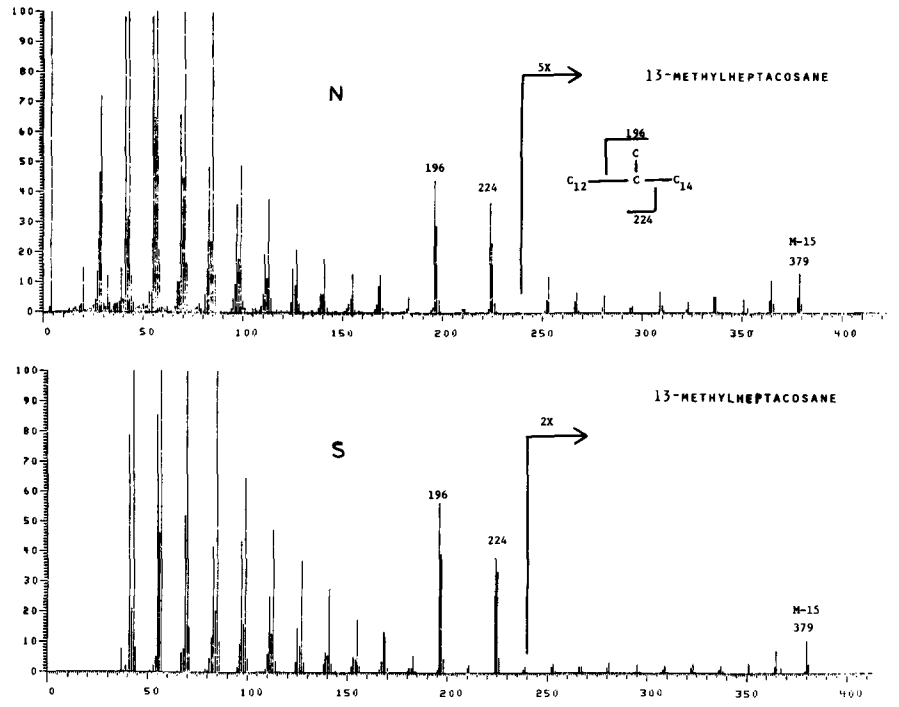


FIG. 5. EI mass spectra of GLC peak 2. (N) 13-Methylheptacosane from PPG of *S. invicta* and (S) synthetic 13-methylheptacosane.

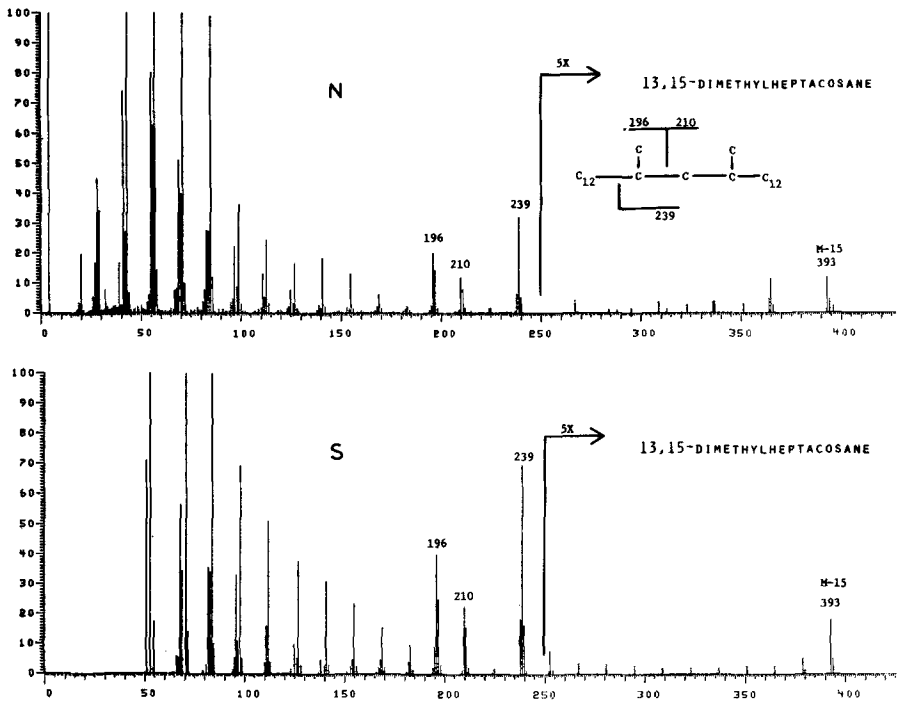


FIG. 6. EI mass spectra of GLC peak 3. (N) 13,15-Dimethylheptacosane from PPG of *S. invicta* and (S) synthetic 13,15-dimethylheptacosane.

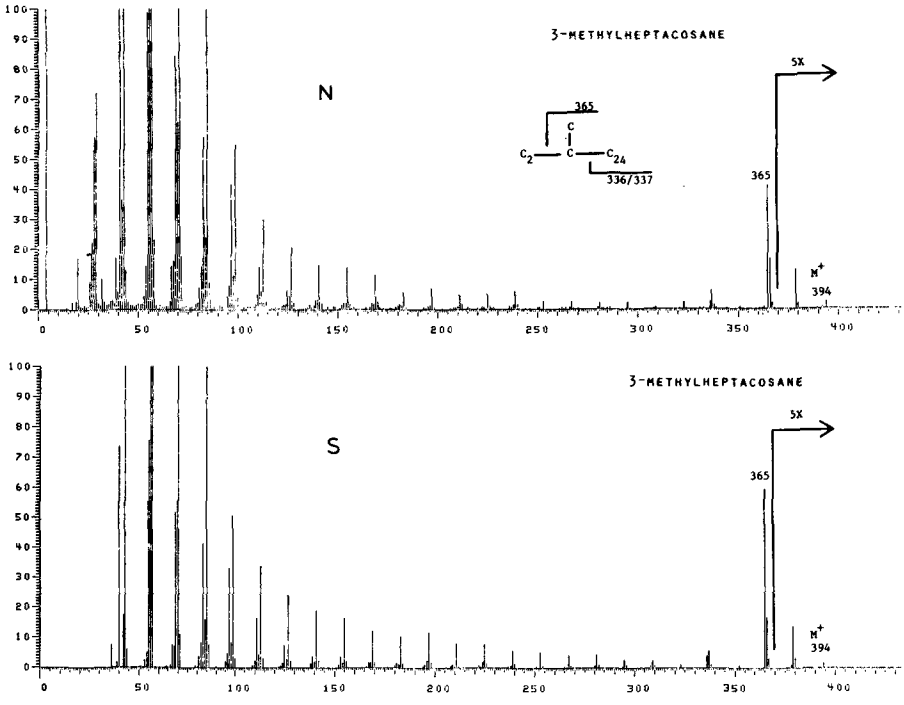


FIG. 7. EI mass spectra of GLC peak 4. (N) 3-Methylheptacosane from PPG of *S. invicta* and (S) synthetic 3-methylheptacosane.

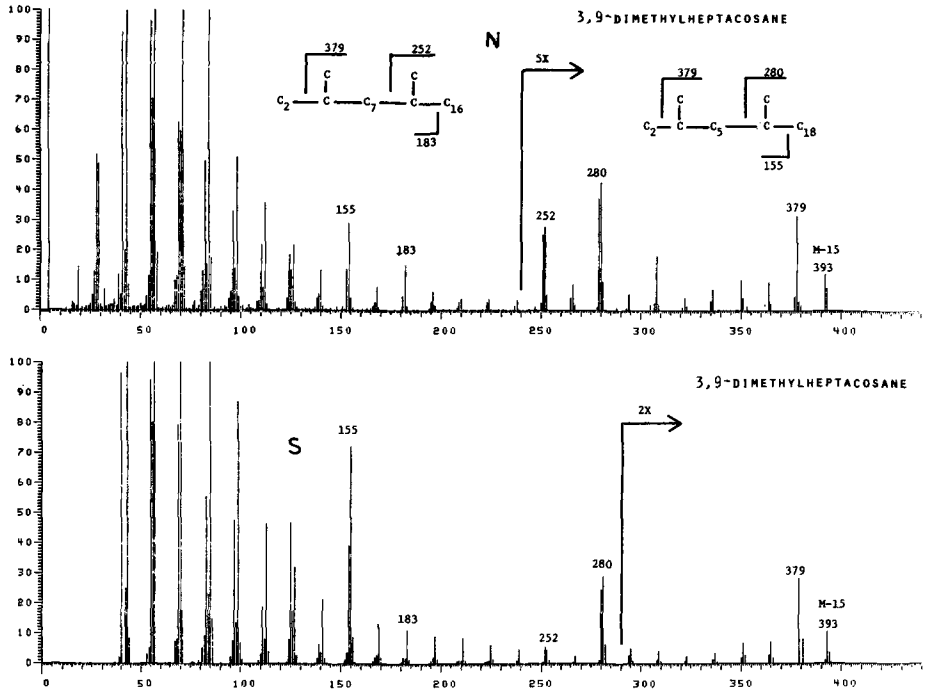


FIG. 8. EI mass spectra of GLC peak 5. (N) 3,9-Dimethylheptacosane and 3,11-dimethylheptacosane from PPG of *S. invicta* and (S) synthetic 3,9-dimethylheptacosane.

EI-MS of the synthetic hydrocarbons are presented in Figures 4-8. The purity of the final products as determined by GLC and argentation TLC was >98% except for 13,15-dimethylheptacosane which was of 90% purity. It contained 10% of the earlier-eluting hydrocarbon.

DISCUSSION

As previously noted, GC-CI-MS is the method of choice for determining the molecular weight of every component in complex alkane mixtures such as those found in insect cuticle (10) and it also provides branch point information (11,12). Thus, the GC-CI-MS (Figs. 2 and 3) readily show the molecular weights and branch points of the components of the peaks 1-5 (Fig. 1), and thus enhance the identification of these hydrocarbons. For example, the molecular weight of 394 and point branch which resulted in the fragment at m/z 365 (Fig. 3, spectrum 4) identified the component of peak 4 as 3-methylheptacosane. In this spectrum, the $(M-1)^+$ peak at m/z 407 indicated the presence of a small amount of an alkane with a molecular weight of 408 that most likely is derived from the component of peak 5. Although EI-MS often provides the best information for determining the location of a methyl branch in long chain alkanes, it does not always show the molecular ion. The use of both CI and EI mass spectra greatly facilitated the structural analyses of the hydrocarbons of the PPG.

The results of this study indicate that hydrocarbons represent about 60% of the lipoidal components of the post-pharyngeal glands of queens 1 to 8 weeks after the mating flight. Although repetitive GC-MS scans of peaks 1-5 suggest the presence of several minor hydrocarbons other than those identified, the major hydrocarbons of peaks 1-5 that account for nearly 90% of the hydrocarbons of the PPG of mated queens are 13-methylheptacosane, 13,15-dimethylheptacosane, 3-methylheptacosane and 3,9-dimethylheptacosane. Proof of their structures has been confirmed by synthesis and comparative mass spectral analyses. The results of capillary GC-MS analyses of peaks 1-5 (Fig. 1) suggest that a longer SP-2100 capillary column perhaps would show a physical separation of these minor hydrocarbons, or at least would allow for even better GC-MS analyses and interpretations.

The relative percentages of the hydrocarbons of PPG of queens 2, 3, 4, 5 and 8 weeks after mating remain fairly constant with only a

noticeable change occurring in the relative amounts of 13,15-dimethylheptacosane; it decreased with time. 13-Methylheptacosane remained the predominant hydrocarbon of PPG of queens 2-8 weeks after mating. Interestingly, heptacosane, the smaller peak that precedes the major hydrocarbons of the PPG of mated queens, is a major hydrocarbon in PPG of virgin queens.

We have conducted GC-MS analyses only on the hydrocarbons of the PPG of virgin and mated queens. We have found similar GLC patterns, however, in the hydrocarbon fractions of PPG of repletes, of crops of major workers, and of Dufour's glands of queens 8 weeks after mating, and these results suggest that these hydrocarbons, although minor lipoidal components of crops and Dufour's glands, are present in other organs of the fire ants. In a reanalysis of the cuticular methylalkanes of *S. invicta*, they have been identified as the major hydrocarbons (13).

No interrelationship has been determined or established for the presence of these alkanes in the PPG of mated queens and certain organs or other ant castes. Interestingly, when either 10- or 20-μg samples of the hydrocarbons in hexane were spotted on filter paper, the hexane evaporated and the paper placed in colonies of fire ants, the ants clustered about the sample and proceeded to masticate the paper in the area that contained the alkanes.

Since the PPG in the virgin queens are much larger than the PPG of other castes (B.M. Glancey, unpublished observations) and since the major hydrocarbons of the PPG are also the major cuticular hydrocarbons of *S. invicta* (13), it is possible that the secretion or contents of these glands may have unique functions in relation to colony organization, caste determination, species and/or case recognition, food exchange or queen brood tending. Work is underway to develop assay systems to determine what biological and physiological function, if any, the major hydrocarbons from the PPG of queens have in maintaining social organization, caste determination and interaction in colonies of red imported fire ants.

ACKNOWLEDGMENTS

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Hyperlipidemia in Rats Fed Retinoic Acid[1]

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ABSTRACT

This report describes a series of experiments that attempt to characterize the lipidemia accompanying retinoic acid administration. After feeding young adult male Sprague-Dawley rats, 1.2 Retinol Equivalents (R.E.) retinyl acetate plus supplemental retinoic acid (100 μg/g dry diet) for three days and fasting for 6-8 hr, triglyceride, cholesterol, and phospholipid content of various serum lipoprotein fractions were determined. When compared to unsupplemented controls, both the serum very low density lipoprotein (VLDL) and the high density lipoprotein (HDL) fractions of the retinoic acid-fed rats were found to harbor an elevated triglyceride content. While VLDL cholesterol and phospholipid content were also elevated, total serum cholesterol and phospholipids were not statistically altered. The detergent Triton WR-1339 was used to depress serum triglyceride clearance in order to assess the effects of retinoic acid feeding on serum triglyceride levels. Triglyceride accumulation started earlier after Triton treatment and was greater when rats were fed 100 μg/g retinoic acid for three days prior to testing. Red and white gastrocnemius muscle, cardiac ventricular muscle, and perirenal adipose tissue were removed from rats following retinoic acid feeding. Analysis of these tissues for lipoprotein lipase (EC 3.1.1.3) activity showed a decrease in adipose tissue, a large depression in both areas of gastrocnemius muscle and no change in cardiac muscle as a result of retinoic acid feeding.

INTRODUCTION

Several reports have appeared documenting hypertriglyceridemia in rats fed or injected with supplemental vitamin A or retinoic acid (1-3). Previous work by Erdman et al. (4,5) has pointed to alterations in hepatic lipid synthesis in vitro when either retinyl acetate, retinol, or retinoic acid was added to a liver homogenate system, resulting in increased incorporation of lipid precursors into triglycerides. Setty and Misra (6) fed supplemental vitamin A for two days and observed a decreased uptake of intraportally injected palmitate-1-^{14}C by heart, kidney, and adipose, implicating an impairment in the uptake of plasma lipids by extrahepatic tissues.

The experiments reported here were performed to study the mechanism whereby all-*trans* retinoic acid feeding results in hyperlipidemia. Young adult male rats were fed 100 μg retinoic acid/g dry diet for 3 days; a regimen which has been shown to consistently induce hypertriglyceridemia (7). A lipoprotein profile of the sera was done to determine the triglyceride, cholesterol and phospholipid content of lipoprotein fractions. Several experiments assessed the effect of retinoic acid on serum triglyceride accumulation when Triton WR 1339 was used to depress extrahepatic triglyceride breakdown. In another experiment, lipoprotein lipase (EC 3.1.1.3) activity was measured in several key tissue types, as an indicator of extrahepatic capacity for triglyceride uptake.

[1]Portions of this work have appeared earlier in an abstract, Gerber, L.E., and Erdman J.W., Jr. (1980) Fed. Proc. 39, 437.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats (Harlan Industries, Inc., Cumberland, IN) were placed into individual stainless steel cages in a controlled temperature environment, with a light-dark cycle of 0600 to 1800 hr light and 1800 to 0600 hr dark. Rats were fed daily 6% corn oil, 22% casein, 22% sucrose, 44% starch, 2% agar, 4% mineral mix, and 1% vitamin mix as gel diet ad libitum at the start of the dark cycle. All ingredients were supplied by Teklad of Madison, WI. Preparation of the diet as well as the composition of the vitamin and mineral mix has been described previously (3,8). All diets contained vitamin A as retinyl acetate (Sigma Chemical Company, St. Louis, MO) at a level suggested by the NRC, 1.2 Retinol Equivalents (R.E.)/g diet (9). (One R.E. is equal to the biological activity associated with 1 μg of retinol.) Crystalline all-*trans* retinoic acid, (gift of Hoffmann-LaRoche, Inc., Nutley, NJ) was dissolved in a small amount of 95% ethanol and added to the diets as needed to achieve a concentration of 100 μg/g dry weight of diet. An equivalent amount of the carrier ethanol was added to control diets.

Rats were acclimated to the control gel diet for several days prior to the experimental feeding period. During the 3-day experimental period, rats were fed 1.2 R.E./g dry diet with or without 100 μg/g dry diet all-*trans* retinoic acid. In all experiments, rats were fasted 4 to 8 hr prior to serum or tissue collection. Serum was obtained via cardiac puncture under light ether anesthesia. Tissue samples for enzyme analysis were removed postmortem, rinsed with

TABLE 1

Body Weights of Rats and Average Consumption of Retinyl Acetate and Retinoic Acid during Experiments 1, 2A, 2B, and 3[a]

Experiment number	Basal (retinyl acetate) diet			Retinoic acid-supplemented diet			
	n[b]	Final body wt (g)	Retinyl acetate intake (R.E./d)	n[b]	Final body wt (g)	Retinyl acetate intake (R.E./d)	Retinoic acid intake (μg/d)
1	12	333.7 ± 28.5	24.2 ± 1.3	12	327.8 ± 18.1	23.0 ± 1.1	1920 ± 90
2A	15	311.9 ± 17.7	26.0 ± 3.1	15	306.8 ± 23.6	24.2 ± 4.0	2020 ± 330
2B	32	273.3 ± 14.2	23.0 ± 4.2	28	272.4 ± 14.9	22.0 ± 4.3	1830 ± 360
3	10	398.8 ± 17.1	29.4 ± 3.2	10	410.7 ± 17.7	29.6 ± 3.2	2470 ± 270

[a]Mean ± SD.
[b]Number of rats consuming the diet.

saline, blotted dry, and immediately frozen in either liquid nitrogen or an acetone/Dry Ice mixture, whereas serum and liver samples were stored at -20 C until analysis. Lipoprotein separation (experiment 1) was done on fresh serum. The average consumption of retinyl acetate and retinoic acid in all experiments can be found in Table 1.

Experimental Design

Experiment 1. Twenty-four male rats, ca. 300-500 g, were randomly assigned to one of two groups, and fed either control or retinoic acid-supplemented diets for 3 days. Following a 6-8 hr fast, serum was taken and lipoprotein analysis was done on 4 pooled serum samples per group. The sera from 3 rats were pooled to provide an amount sufficient for lipoprotein separation and analysis.

Experiment 2. Two experiments were conducted to assess the effect of retinoic acid on Triton WR 1339-induced hyperlipidemia. In the first experiment (experiment 2A), 30 male Sprague-Dawley rats, between 300-350 g, were fed either the basal or the retinoic-acid-supplemented diet for 3 days. Rats were injected ip with 800 mg/kg body weight of Triton WR 1339 (Sigma Chemical Co., St. Louis, MO) in 0.9% saline. Each rat was sampled for serum twice, such that the rats sampled at the time of Triton injection were sampled again 4 hr later, one group at 2 and 5 hr post-Triton, and another at 3 and 6 hr. A second experiment (experiment 2B) using sixty 250- to 300-g male Sprague-Dawley rats was conducted similarly, but modified so that the rats sampled at the time of Triton injection were sampled again 1.5 hr later. At this time, another group was sampled, and then monitored again in 1.5 hr. This pattern was repeated up to 7.5 hr post-Triton.

Experiment 3. Twenty 400-g male Sprague-Dawley rats were assigned to groups and fed one of the two previously described diets for 3 days. Serum and tissues (perirenal adipose, cardiac ventricular muscle, and red and white gastrocnemius muscle) were removed after the rats were fasted 4 to 6-1/2 hr.

ANALYTICAL PROCEDURES

Lipids were analyzed by methods previously cited (3), with the exception of lipoprotein cholesterol and triglyceride analysis, which made use of Agent Clinical Chemistry Reagent (Abbott Laboratories, Pasadena, CA) (10) and Beckman Enzymatic Triglyceride Reagent (Carlsbad, CA).

Very low density lipoprotein (VLDL), high density lipoprotein (HDL), and HDL plus low density lipoproteins (LDL) fractions were prepared via a combination of ultracentrifugation and heparin-Mn^{+2} precipitation as outlined by the Lipid Research Clinic Program (11). LDL determination was indirect and represents the difference between HDL + LDL values minus HDL values. Extraction of lipoprotein lipase followed the procedures of Paik and Yearick (12), and analysis was done according to Nilsson-Ehle and Schotz (13), employing tri-[9,10-^{3}H] oleoylglycerol (Amersham Co., Arlington Heights, IL) and unlabeled trioleoylglycerol (Sigma Chemical Company, St. Louis, MO). Assays were carried out on duplicate aliquots of enzyme extract for the determination of total lipolytic activity, while an additional aliquot from each enzyme extract was assayed for lipolytic activity in the presence of 1 M NaCl. This concentration of NaCl has been previously shown to specifically inhibit lipoprotein lipase and not other lipases, so it provides a measure of nonlipoprotein lipase lipolytic activity (14). Correction for nonlipoprotein lipase lipolytic activity was made by subtracting nonspecific activity from the total lipase activity. Reactions were terminated by the addition of 3.25 mL of methanol/chloroform/*n*-heptane (1.41:1.25:1.00, v/v/v) (15), followed by addition of 1.05 mL of 0.5 M NaOH (16). The fatty acid was extracted into the methanol/water phase of this system after vortexing, and an aliquot was counted in 10 mL of Aquasol (New England Nuclear, Boston, MA) in a Beckman LS 9000 liquid scintillation system spectrometer. Enzyme activity was calculated as mU of lipoprotein lipase, defined as the release of 1 nmol of oleic acid/min at 37 C.

All data were subjected to Student's t-test or Least Significant Difference (LSD) test for statistical evaluation (17).

RESULTS

Experiment 1

This experiment was performed to quantify the lipid content in the various lipoprotein fractions in the serum of rats fed diets with or without 100 μg retinoic acid/g of diet for 3 days (Table 2). Analysis of VLDL showed an elevation in triglyceride, cholesterol and phospholipid in retinoic-acid-fed rats. Although LDL composition was unaltered by the treatment, an elevation of HDL triglyceride was evident. Total serum analysis revealed the presence of hypertriglyceridemia in retinoic-acid-fed animals, but no change in either total cholesterol or phospholipids.

Experiment 2

The time-course of retinoic acid-Triton WR 1339-induced hypertriglyceridemia (Table 3) was monitored in the two experiments, A and B, as described in the methods section. When rats were fed a control diet and injected with Triton WR 1339, significant accumulation of serum triglyceride was observed 6 hr later.

TABLE 2

Lipid Distribution among the Lipoprotein Fractions of Serum from Rats Fed Diets with or without Supplemental Retinoic Acid for 3 Days (experiment 1)[a]

Lipoprotein fraction	Lipid fraction (mg/dL)	Basal diet n=4[b]	Retinoic acid diet[c] n=4
VLDL	Triglyceride	57.2 ± 17.1^A	218.7 ± 61.7^B
	Cholesterol[d]	3.9 ± 2.3^A	19.5 ± 5.2^B
	Phospholipid	31.4 ± 7.2^A	46.9 ± 13.2^B
LDL	Triglyceride	6.5 ± 1.8	8.0 ± 8.0
	Cholesterol	33.7 ± 7.4	24.6 ± 13.1
	Phospholipid	112.9 ± 32.6	94.2 ± 33.8
HDL	Triglyceride	23.3 ± 6.3^A	40.8 ± 10.0^B
	Cholesterol	58.5 ± 6.6	63.2 ± 11.4
	Phospholipid	126.7 ± 19.0	95.4 ± 32.7
Total serum	Triglyceride	85.5 ± 17.6^A	292.7 ± 94.0^B
	Cholesterol	97.7 ± 19.8	114.2 ± 11.3
	Phospholipid	305.8 ± 63.6	300.3 ± 24.4

[a]Values represent mean ± SD. Treatment means across a row with different superscripts differ significantly ($p \leq 0.05$), as determined by Student's t-test.

[b]Samples are the pooled sera of 3 different rats from the same group.

[c]100 μg retinoic acid/g diet.

[d]Unesterified plus esterified cholesterol.

TABLE 3

Serum Triglycerides of Rats Fed Diets with or without Supplemental Retinoic Acid after Triton WR-1339 Administration (experiment 2A and 2B)[a]

Experiment 2A[b]			Experiment 2B[d]		
Hours post-triton	Triglycerides (mg/dL)		Hours post-triton	Triglycerides (mg/dL)	
	Basal diet	Retinoic acid diet[c]		Basal diet	Retinoic acid diet[c]
0	79.3 ± 19.1A	402.5 ± 166.4C	0	96.3 ± 21.6E (6)	219.9 ± 74.6G (6)
2	84.5 ± 47.2A	629.6 ± 366.0C,D	1.5	53.5 ± 40.2E (6)	212.7 ± 81.0G (6)
3	89.3 ± 46.3A	489.7 ± 168.1C	3	110.4 ± 40.4E (4)	495.6 ± 215.6G (3)
4	101.2 ± 4.6A	810.0 ± 436.1C,D	4.5	196.8 ± 187.6E (4)	1313.5 ± 334.4H (3)
5	174.3 ± 74.4A	1209.5 ± 640.7D			
6	364.4 ± 209.9B	1350.2 ± 1142.3D	6	927.9 ± 584.4F (6).	1558.5 ± 704.2H (5)
			7.5	1220.6 ± 635.3F (6)	2015.9 ± 550.0H (5)

[a]Values represent mean ± SD. Comparisons have been made between the serum triglyceride levels measured at different times post-Triton among rats fed the same diet. Treatment means in a column with different superscripts, therefore, differ significantly ($p \leq 0.05$), as determined by LSD test.

[b]$n=4$ for all groups.

[c]100 μg retinoic acid/g diet.

[d]n is indicated in parentheses.

Those rats injected with Triton and also fed retinoic acid had significant elevations ($p \leq 0.05$) of triglyceride by 4.5 and 5 hr post-Triton, at least 1 hr earlier than the rats not receiving retinoic acid.

Changes in serum triglycerides during a sampling period were calculated from the data accumulated in experiment B. The sampling procedure in this experiment allows accumulation to be calculated for a given rat during a 1.5-hr interval, whereas experiment A could only yield accumulation for a given rat over a 3- or 4-hr interval. Retinoic acid feeding results in significantly more accumulation of serum triglycerides in Triton-injected rats between 4.5 to 6 hr ($p \leq 0.025$) and 6 to 7.5 hr ($p \leq 0.01$).

Experiment 3

The effects of retinoic acid feeding on lipoprotein lipase activity in various tissues was evaluated (Table 4). Serum triglyceride analysis showed the retinoic-acid-fed rats to be hypertriglyceridemic and the controls normolipidemic. A significant depression of perirenal adipose tissue lipoprotein lipase activity occurred in retinoic-acid-fed rats. Lipoprotein lipase activity of red and white gastrocnemius muscle but not cardiac ventricular muscle was greatly depressed. The lipoprotein lipase activity from both red or white gastrocnemius muscle of retinoic-acid-fed rats correlated negatively ($p \leq 0.05$) with serum triglyceride levels.

DISCUSSION

The current work has employed techniques to characterize the retinoic-acid-induced hyperlipidemia, previously reported by our lab (3,7) as well as others (1,2). The current studies substantiate our previous work (7) which suggested VLDL-linked elevation of serum triglycerides. Not only VLDL, but HDL triglycerides as well, were elevated due to the feeding of retinoic acid. The increase in HDL triglyceride suggests that at least some of the hyperlipidemia comes from an increased hepatic production of triglyceride, as HDL and VLDL are both secreted by the liver, and therefore may be available outlets for excess hepatic triglyceride.

The results obtained when retinoic-acid-fed rats were subjected to Triton-WR 1339 treatment indicate an effect on triglyceride clearance and perhaps entry rate, as well. Studies to determine the effect of Triton WR-1339 on lipid metabolism have shown that this detergent inhibits lipoprotein lipase activity (18,19). The earlier induction of hypertriglyceridemia by

TABLE 4

Serum Triglycerides and Lipoprotein Lipase Activity Present in Tissues of Rats Fed Diets with or without Supplemental Retinoic Acid for 3 Days (experiment 3)[a]

	Basal-fed diet (n=10)	Retinoic acid[b] diet (n=10)	Correlation of LPL activity with serum triglyceride levels[c]
Serum triglycerides (mg/dL)	88.0 ± 32.2^{A}	382.6 ± 196.0^{B}	–
Lipoprotein lipase activity[d] (mU/g of tissue)			
Perirenal adipose	89.3 ± 18.9^{A}	75.7 ± 14.0^{B}	-0.11
Cardiac ventricular muscle	36.7 ± 10.2	36.5 ± 13.9	-0.18
Red gastrocnemius muscle	7.2 ± 3.8^{A}	1.7 ± 1.3^{B}	-0.60*
White gastrocnemius muscle	1.4 ± 0.7^{A}	0.5 ± 0.2^{B}	-0.52*

[a]Values represent mean ± SD. Treatment means across a row with different superscripts differ significantly ($p \leq 0.05$) as determined by Student's t-test.

[b]100 μg retinoic acid/g diet.

[c]Values denoted with an asterisk (*) indicate a negative correlation with serum triglyceride levels at a significance of $p \leq 0.05$.

[d]1 MU = 1 nM of oleic acid released/min at 37 C when incubated for 30 min (perirenal adipose, cardiac muscle, and red gastrocnemius muscle) or 60 min (white gastrocnemius muscle). Values represent total lipase activity corrected for nonspecific lipase activity present in each sample (see methods section of text).

Triton when retinoic acid has been fed may be due to an additional inhibition of lipoprotein lipase activity.

The triglyceride entry rate into serum for the rat has been estimated to be about 11 mg/hr/100 g B.W. (20,21). An entry rate of that magnitude could account for all of the triglyceride accumulating in the serum of the nonretinoic-acid-fed rats, even at the interval of greatest accumulation (6 to 7.5 hr). This would suggest that by 6 to 7.5 hr, the Triton was acting to block virtually all triglyceride breakdown. An entry rate of 11 mg/hr/100 g B.W., however, can account for only about 1/2 the triglycerides accumulating in retinoic-acid-fed rats at any of the intervals after 3 hr post-Triton. An increased in vitro synthesis of triglycerides by liver homogenates from lipid precursors in the presence of retinoic acid has been demonstrated previously (4,5). Other investigators have found a doubled incorporation rate of fatty acids into triglycerides in liver slices from rats fed large doses of retinyl acetate compared to slices from control rats (22). Increased hepatic synthesis of triglycerides could result in increased release into the serum contributing to the triglyceride accumulation.

When tissues were assayed for lipoprotein lipase, a small depression of activity in perirenal adipose tissue was found. This decrease, although only 15%, could account for a large component of the serum triglyceride elevation; as adipose tissue accounts for 1/4 to 1/3 of serum triglyceride uptake in the rat (23). Cardiac ventricular muscle enzyme activity was not depressed, perhaps due to efficient homeostatic regulation. Alternatively, since cardiac lipoprotein lipase is compartmentalized into available and unavailable enzyme (24), retinoic acid may alter compartmentation of LPL and thus affect in vivo activity without altering the activity determined in homogenized tissue.

Of major interest was the finding that the lipoprotein lipase activities of both red and white segments of gastrocnemius muscle were depressed. While activity in the red segment of retinoic-acid-fed rats was lowered to levels approximating those found in white muscle, the white muscle lipoprotein lipase activity was reduced to even lower levels. As there is very little information available on the regulation of lipoprotein lipase activity of skeletal muscle, it is difficult to compare our data with those of others. Since it is estimated that between 1/3 and 1/2 of serum triglycerides are removed by skeletal muscle (23), a decreased activity in muscle, as represented by these muscle segments, might account for a substantial part of the total elevation in serum triglycerides in retinoic-acid-induced hypertriglyceridemia.

It should be mentioned that an acute induction of hypertriglyceridemia might itself cause an apparent decrease in tissue lipoprotein lipase levels due to usage and depletion of existing enzyme without time for replacement. The fast turnover of several hours (25) for lipoprotein lipase, however, should allow for adaptation to the need for increased triglyceride breakdown

over the 3-day feeding period if retinoic acid is not directly affecting enzyme synthesis or activity.

The results of these and previous experiments suggest that the hypertriglyceridemia associated with retinoic acid administration stems from excess synthesis of hepatic triglycerides which are released in the VLDL and HDL fractions. Hepatic accumulation of excess triglycerides (fatty liver) does not occur following the feeding at similar levels to the present study of all-*trans* retinoic acid to rats for up to 28 days (3). Concurrently, depressed lipoprotein lipase activity in muscle and adipose prevents the excess serum triglyceride from being catabolized and taken up by these tissues. In rats, hypertriglyceridemia is maintained at least up to 28 days during continuous feeding of retinoic acid (3), and will return to normal 1-3 days after cessation of retinoic acid feeding (26).

Although the role of hypertriglyceridemia in the pathogenesis of atherosclerosis remains to be shown, individuals ingesting large amounts of retinoids should probably be monitored for hyperlipidemia. Treatment of several forms of acne as well as chemoprevention of epithelial cancer in humans has employed oral administration of 13-*cis* retinoic acid (27-29). Levels of 100 to 800 μg/100 g body weight have been given daily in these studies for up to 8 months, and in the case of cancer chemoprevention, administration may occur for the lifetime of the individual.

Katz et al. (29) have recently reported elevated serum triglyceride levels in their patients being treated for disorders of skin keratinization with oral 13-*cis*-retinoic acid. Six out of 9 previously normolipemic individuals developed hypertriglyceridemia whereas one individual with Type IV hyperlipidemia accumulated triglyceride levels greater than 1,000 mg/100 mL of serum.

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Myelin Subfractions Isolated from Mouse Brain: Analysis of the Lipid Composition at Three Developmental Stages

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ABSTRACT

Lipids were examined in whole myelin and 8 myelin subfractions isolated from mouse brain at 18-24, 44-48 and 80-90 days of age. Relative to protein, total lipid was lowest in whole myelin isolated from the oldest animals as well as from subfractions isolated at greater sucrose densities, thus partially accounting for the observed myelin subfraction distribution pattern which shifted during development from an average peak density banding between 0.55 and 0.65 M sucrose to one banding between 0.60 and 0.70 M sucrose. Whole myelin and each myelin subfraction isolated at one age contained nearly the same ratio of sterol and phospholipid to galactolipid; these ratios decreased uniformly during development suggesting enrichment with galactolipid in all myelin subfractions. Sulfatide, as percentage of total galactolipid, was relatively constant during development and appeared to be slightly enriched in the denser myelin subfractions. The findings suggest that regardless of the origin(s) of the subfractions, an age-related mechanism exists in the central nervous system which modifies myelin lipid composition relatively uniformly.

INTRODUCTION

In the past decade, there has been considerable interest in myelin subfractions isolated from brain tissue. Much of the interest centers on the origin and physiological significance of the myelin subfractions and the recognition that studies of myelin subfractions may provide an alternative approach to elucidating myelin development. In particular, it is believed that the myelin subfractions may represent different stages in the formation of compact mature myelin (1-4).

One measure of the attention devoted to myelin subfractions is the relatively large number of different isolation procedures that have been developed (1,2,5-13). Despite the various approaches that have been employed, the origin of the wide variety of subfractions and their possible role in myelin development remain obscure; however, there is evidence that the subfractions are not mere artifacts of the isolation procedures used (11).

A major difficulty has been the very nature of myelin. As it is isolated from the central nervous system (CNS), it apparently consists of a continuum of membranous particles distributed in a gaussian fashion (12,13); consequently, a seemingly endless variety and number of myelin subfractions can theoretically be isolated. Because of obvious practical considerations, only a limited number of myelin subfractions are actually isolated for physical and chemical studies. Most of the isolation procedures that have been developed result in 2-4 myelin subfractions which are characterized. With this relatively small number of subfractions, it is likely that subtle differences in myelin distribution patterns and significant trends among myelin subfractions might not be apparent. In addition, some important minor fractions will go unnoticed.

With this problem in mind, we used a procedure that resulted in 8 myelin subfractions isolated on discontinuous density gradients ranging from 0.32 M to 0.85 M sucrose (11). In our previous study employing myelin isolated from mouse brain, we were able to show that the subfractionation patterns obtained were quite reproducible but obviously related to the type of myelin applied to the discontinuous gradient. Among the results obtained in that study (11) was the observation that myelin subfractionation patterns were dependent on the age of the animal from which the myelin was isolated. We have now extended our studies to examine the lipid content and major lipid classes in these myelin subfractions. Our results are reported herein.

MATERIALS AND METHODS

Myelin Isolation and Subfractionation

The myelin used in these studies was obtained from brains of C57Bl/6J mice which were raised in our animal colony from stock originally purchased from Jackson Laboratory (Bar Harbor, ME). Details pertaining to the isolation and subfractionation of myelin have

been published (11). Briefly, purified myelin was isolated essentially as described by Norton and Poduslo (14) and subfractionated by layering the purified myelin, dispersed in 0.32 M sucrose, over discontinuous gradients containing 5 mL each of 0.85 M, 0.75 M, 0.70 M, 0.65 M, 0.60 M, 0.55 M and 0.45 M sucrose. After centrifugation at 75,000 G for 30 min, 8 fractions were obtained, a pellet (P) and 7 bands (A-G) floating at each sucrose interface; band A was the least dense subfraction and it floated at the 0.32-0.45 M sucrose interface. Each of the myelin subfractions was further purified by sucrose washout and osmotic shocking procedures detailed in our earlier paper (11).

Myelin and myelin subfractions were isolated from brain tissue obtained from mice at 3 different ages, 18-24, 44-48 and 80-90 days. Purified myelin was isolated from at least 25 mice such that resulting subfractions contained a minimum of 500 μg of protein. The protein content was estimated by the method of Lowry et al. (15) as modified by Hess and Lewin (16) using bovine serum albumin, fraction V (Sigma, St. Louis, MO) as the standard.

Lipid Extraction and Analyses

Solvents were reagent grade or better. Acetone was distilled and chloroform was washed with water, dried over $CaCl_2$ and distilled before use.

Lipid extraction was according to Folch et al. (17). Myelin containing 1-1.5 mg protein/mL water was stirred at 4 C with 19 vol of chloroform/methanol (2:1, v/v) and the extract was filtered through medium porosity filter paper. The insoluble residue and filter paper were rinsed twice with 2.0-mL portions of chloroform/methanol/water (12:6:0.9, v/v/v) and the rinses were pooled with the original filtrate. After the addition of aqueous 0.58% NaCl to adjust the solvent ratio for phase separation (17), the mixture was shaken and allowed to stand at 4 C overnight. The upper phase was removed and the interface was "rinsed" twice with 0.1 vol of Folch theoretical upper phase. The "rinses" were added to the original upper phase which was then washed once in 0.2 vol and twice with 0.1 vol of Folch theoretical lower phase. The resulting lower phases were pooled with the original lower phase and used as the source of lower phase lipids.

After the addition of butylated hydroxytoluene (5 μg/mg expected lipid), the lower phase lipids were concentrated *in vacuo* at less than 35 C using repeated additions of chloroform/methanol (2:1, v/v) to ensure removal of water. They were dissolved in a known volume of chloroform/methanol (2:1, v/v) and aliquots were used for analysis of phospholipid (18) and sterol (19). The remaining lower phase lipid was concentrated, redissolved in chloroform and submitted to silicic acid chromatography (20) prior to analysis of glycolipid. For 40 mg of expected lipid, 4 g of Biosil A (Calbiochem, LaJolla, CA) were used and chloroform (30 mL), acetone (100 mL) and methanol (30 mL) were employed to elute 3 fractions. Based on thin layer chromatography, material corresponding to galactosyl diglyeride, cerebroside and sulfatide was found only in the acetone fraction. The acetone fraction was concentrated in vacuo at less than 35 C, dissolved in chloroform/methanol (2:1, v/v) and aliquots were assayed for lipid galactose (16) and sulfatide (21).

RESULTS

Data obtained for the purified whole myelin preparations are summarized in Table 1. Based on the content of protein and lipid, the yield of myelin (mg/g brain) was calculated to be 9.76, 18.9 and 23.9 at 18-24, 44-48 and 80-90 days of age, respectively. Relative to protein, the amount of lipid was lowest for myelin obtained from the oldest animals. As can be seen in Table 1, the major factor contributing to the lower percentage of lipid at the oldest age was the lower amount of phospholipid relative to protein. Per mole of total lipid, phospholipid decreased from a value of 41% at 18-24 days of age to a value of 35% at 80-90 days of age. On the other hand, total galactolipid increased slightly (19-24%) whereas total sterol remained nearly constant (41-42%). These results are also confirmed by the decrease in the mole ratio of phospholipid and sterol to galactolipid between the 3 ages (Table 1).

The lower percentage of myelin lipid observed at the oldest age is consistent with the observed distribution of myelin in the various subfractions during development. These data displayed in Figure 1 as mg of myelin lipid/g brain wet weight show that the myelin subfraction distribution pattern shifts during development from a peak density banding between 0.55 and 0.65 M sucrose (fraction C) to one banding between 0.60 and 0.70 M sucrose (fractions D and E). This shift was also verified when total protein/g brain for each myelin subfraction was examined (11).

Other data (also contained in Fig. 1) verify that the subfractions isolated at the lower sucrose densities contain more lipid relative to protein (expressed as % lipid). Although there

TABLE 1

Protein and Lipid in Purified Whole Myelin Isolated from Mouse Brain at Several Ages[a]

Myelin component	18-24 Days	44-48 Days	80-90 Days
Protein (mg/g brain)	2.5, 3.1, 3.4	5.9, 5.3, 5.8	8.2, 8.4
Sterol (μmol/mg protein)	1.2, 1.7, 1.3	1.5, 1.4, 1.6	1.2, 1.3
Phospholipid (μmol/mg protein)	1.2, 1.7, 1.3	1.4, 1.3, 1.6	1.1, 0.99
Galactolipid (μmol/mg protein)	0.56, 0.72, lost	0.76, 0.81, 0.66	0.62, 0.76
Sulfatide (μmol/mg protein)	0.12, 0.15, 0.12	0.11, 0.15, 0.14	0.13, 0.13
Total lipid (%)[b]	66, 72, lost	70, 69, 72	66, 64
Mole lipid ratios[c]	2.2:2.2:1	1.9:2.0:1	1.5:1.8:1

[a]Myelin isolated from C57Bl/6J mice as described (11); individual values shown are for separate preparations.

[b]Based on the weights of protein, sterol, phospholipid and galactolipid using average molecular masses of 387, 775 and 846 for cholesterol, phospholipid and galactolipid, respectively, for calculating weight percents.

[c]Mole ratios are moles of phospholipid and sterol relative to galactolipid and are means of 2 or 3 separate preparations.

are some obvious discrepancies, in general, the calculated percentage of lipid appears to be unique to each myelin subfraction regardless of the age examined. In Figure 2, it can be seen that regardless of age, all lipid classes were present in relatively higher concentrations in the lighter myelin subfractions.

As also can be seen in Figure 2, the major lipid classes in any particular subfraction tended to reflect the purified myelin from which it was derived. For example, the lowest phospholipid values obtained for virtually all of the subfractions were for those isolated from the oldest age group and the lowest galactolipid values obtained for virtually all of the subfractions were for those isolated from myelin obtained from 18- to 24-day-old mice. This is more evident when the lipid data are expressed as mole ratios (data not shown). For example, the mole ratios for the major lipid classes in subfractions isolated from mice at one age were all similar to the lipid ratios in whole myelin isolated at the same age (Table 1). The only exceptions were higher ratios at 18-24 days for fraction G (2.4:2.4:1) and the pellet (2.8:3.1:1). As has been noted for whole myelin (Table 1), the ratios for the subfractions decreased uniformly during development suggesting enrichment with galactolipid at the older ages. Statistical analysis (22) was employed to analyze the lipid ratios obtained for the myelin subfractions at the different ages. Two-way analysis of variance revealed no statistically significant differences in the mole ratio among myelin subfractions within any one age group; however, significant age differences were observed ($p \leq 0.01$) for both sterol and phospholipid to galactolipid.

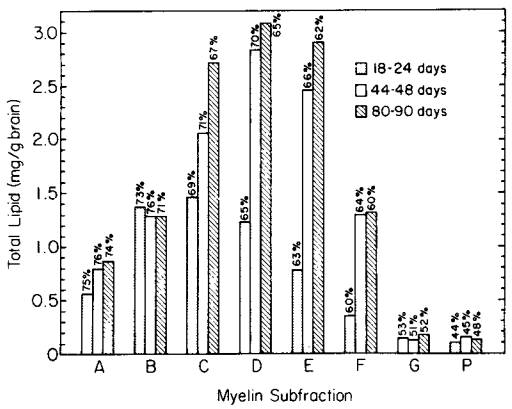

FIG. 1. Total lipid content of 8 myelin subfractions isolated from mouse brain at 3 ages. Total lipid is calculated from the sum of the weights of the 3 lipid classes calculated using molecular masses given in Table 1. Results are normalized/g wet wt of whole brain tissue and are means of 2 preparations at 18-24 days and 80-90 days and 3 preparations at 44-48 days. The percent values included in the figure are the percentage of lipid in each subfraction based on weights of protein, sterol, phospholipid and galactolipid.

Unlike total galactolipid, phospholipid or sterol, the sulfatide content of myelin relative to protein did not change with age (Table 1). This was also true for sulfatide content of myelin subfractions (Table 2). As mole % of total galactolipid, the highest values observed, 26, 22 and 23%, respectively, for 18-24, 44-48 and 80-90 days were for the densest myelin subfraction; thus, there is proportionately somewhat more sulfatide in the denser myelin fractions. These percentages are also higher

when compared with whole myelin in which sulfatide represents 20, 17 and 19%, respectively, for the same 3 ages. In addition, sulfatide represents a relatively constant portion of the total galactolipid in each subfraction and in whole myelin during the developmental period examined.

DISCUSSION

Compared with the work done on protein composition, relatively little attention has been devoted to the lipids in myelin subfractions. Differences in species, ages and brain regions examined as well as in the isolation procedures employed result in myelin subfractions that differ in number and relative amounts as well as in purity and composition making it difficult to strictly compare the available reports from different laboratories. There is one generalization that can be made. Relative to protein, total lipid, and/or cholesterol, phospholipid and galactolipid are reduced in the denser myelin subfractions (9,12,23-26). The ratios of the major lipid classes are generally reported to differ among the subfractions. Agrawal et al. (27) and Burton and Agrawal (23) have isolated a dense fraction called MPM which has no detectable cerebrosides. Further, the other two myelin subfractions isolated in these studies differed sufficiently suggesting enrichment of phospholipid and cholesterol relative to cerebrosides in the heavy myelin fraction. Other laboratories report a similar trend (9,12,23). Nevertheless, except for the dense subfractions, relative amounts of the major lipid classes reported by different laboratories for the myelin subfractions do not differ appreciably (9,12,23,24).

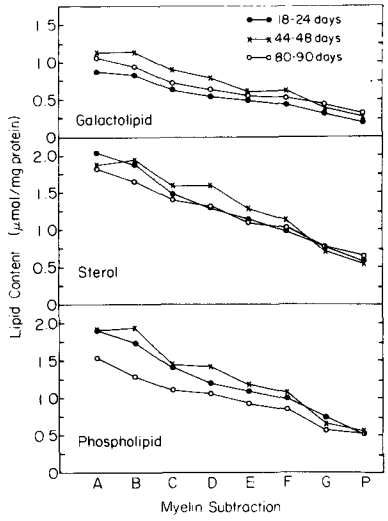

FIG. 2. Phospholipid, galactolipid and sterol content of 8 myelin subfractions isolated from mouse brain at 3 ages. The results for individual lipids in each subfraction are per mg myelin subfraction protein and are means of 2 preparations at 18-24 days, and 80-90 days, and 3 preparations at 44-48 days.

In this study, we have examined and compared the lipid composition of myelin and 8 myelin subfractions isolated from whole brains of mice at 3 age periods. As expected, compared to material banding at less dense sucrose layers, the denser fractions contained less total lipid as well as smaller amounts of the individual lipid classes relative to protein. Moreover, the percentage of lipid in any specific subfraction appeared to be unique for that fraction, regardless of the age of the animal from which the myelin was derived. At the same time,

TABLE 2

Sulfatide Content of Myelin Subfractions Isolated from Mouse Brain at Several Ages[a]

	Sulfatide					
Age	18-24 days		44-48 days		80-90 days	
Subfraction	μmol / mg protein	(%)	μmol / mg protein	(%)	μmol / mg protein	(%)
A	0.14,0.21	(20)	0.16,0.16,0.19	(15)	0.19,0.16	(17)
B	0.13,0.20	(19)	0.24,0.19,0.18	(18)	0.15,0.20	(19)
C	0.14,0.15	(22)	0.15,0.16,0.14	(17)	0.14,0.13	(20)
D	0.13,0.09	(20)	0.14,0.14,0.13	(18)	0.14,0.14	(22)
E	0.12,0.09	(20)	0.14,0.10,0.11	(19)	0.12,0.12	(21)
F	0.10,0.09	(20)	0.11,0.12,0.09	(18)	0.11,0.12	(21)
G	0.09,0.07	(25)	0.10,0.10,0.04	(21)	0.08,0.10	(21)
P	0.05,0.06	(26)	0.06,0.03,0.08	(22)	0.07,0.07	(23)

[a]Values are individual results for 2 preparations at 18-24 and 80-90 days and 3 preparations at 44-48 days. Percent values are mol % of total galactolipid.

during development, there was a shift in the subfraction pattern toward the greater sucrose density layers resulting in a greater average subfraction density at the older ages. The observed shift in the subfraction pattern is consistent with the decreased percentage of total lipid found in whole myelin at the oldest age period examined. Nevertheless, the change in average subfraction density does not appear to be entirely accounted for by a change in the percentage of lipid in myelin (relative to protein) since between 18-24 and 44-48 days a significant increase in average subfraction density was observed which was not paralleled by a decrease in the percentage lipid content of whole myelin at these ages.

The important unique findings from this study were, first of all, that the lipid composition of each of the myelin subfractions changed during development, and second, that within any one age group all of the myelin subfractions exhibited about the same ratio of sterol or phospholipid to total galactolipid. These ratios changed uniformly for each subfraction during the developmental period examined, suggesting enrichment of all myelin membrane fractions with galactolipid. Unpublished observations indicate that each subfraction also exhibited age-related changes in the myelin protein profiles, becoming more enriched in basic proteins and proteolipid proteins at older ages. (These developmental changes were clearly present; on the other hand, the levels of non-myelin markers were similar for whole myelin and the subfractions at all 3 ages [11].) It is emphasized that these results were not expected in that they appear to be incompatible with the hypothesis that conversion of heavy myelin subfractions to lighter myelin subfractions is a necessary requisite for compaction of myelin. Our results are consistent with the simultaneous addition of lipids to all of the myelin subfractions as reported by Benjamins et al. (3,4) for certain myelin components. If product-precursor relationships exist among the subfractions, new precursor must be formed in a manner such that the resulting lipid composition is different from that of previously existing precursor fractions. We believe that the observed age-related alterations in the chemical composition of the subfractions convincingly demonstrate that all 8 subfractions, isolated using our procedures, are modified during development, becoming progressively enriched in components characteristic of compact myelin.

The physiological significance and origin of the dense myelin subfractions have been of considerable interest. Compared to whole myelin, the low percentage of total lipid (Fig. 1), high levels of total ATPase (11) and the preponderance of high molecular weight proteins (unpublished observations) suggest that subfractions G and P are relatively impure. However, these fractions have high levels of 2′,3′-cyclicnucleotide-3′-phosphohydrolase (11). Furthermore, the lipid ratios are virtually identical to those of the other subfractions and whole myelin. If these fractions are simply mixtures of native myelin and other contaminating membrane fractions, the contaminating membrane fractions must contain extremely low levels of lipid relative to protein or they must contain lipid in mole ratios that are virtually identical to myelin. We conclude that the dense fractions are native to myelin or some myelin-related structure, such as fraction SN-4 (28). They may originate from regions of the membrane in closer proximity to the oligodendroglial cell, since some of the chemical features of these subfractions are similar to those observed for oligodendroglial membrane (29,30). However, the high relative amount of galactolipid in the heavy fractions preclude gross contamination with oligodendroglial plasma membrane per se (29).

In an earlier paper (11), we presented evidence that the myelin subfractions were not an artifact of the myelin preparation procedures and suggested that they represent physiologically significant membrane fractions. We also found that the subfraction pattern obtained for 18- to 24-day-old animals was similar to that obtained for the hind brain region of 44- to 48-day-old animals, an observation which allowed us to conclude that myelin isolated from young animals was probably mostly derived from the hind brain where myelination is known to commence earliest (30). Results of this study show that the myelin maturation phenomenon is expressed in each of the myelin fractions, regardless of their origin or physiological significance. If certain subfractions consist primarily of myelin membranes stemming from specific brain regions, it would appear that there is an age-related mechanism that modifies myelin lipid composition relatively uniformly in contrast to a brain-region-controlled phenomenon or to segregated genetic control suggested by other investigators (2). The myelin subfractions probably represent membranes originating from a common unit of control of their synthesis and compaction, most likely within the oligodendroglial cell.

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Acylglycerol Structure of Genetic Varieties of Peanut Oils of Varying Atherogenic Potential

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ABSTRACT

Detailed investigation was made of the triacylglycerol structure of three varieties of peanut oils of varying atherogenic activity. By means of chromatographic and stereospecific analyses, it was shown that all the oils had markedly nonrandom enantiomeric structures with the long chain saturated fatty acids (C_{20}-C_{24}) confined exclusively to the *sn*-3-position, whereas the palmitic and oleic acids were distributed about equally between the *sn*-1- and *sn*-3-positions, with the linoleic acid being found preferentially in the *sn*-2-position. On the basis of detailed studies of the molecular species of the separate *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diacylglycerol moieties, it was concluded that the fatty acids in the three positions of the glycerol molecule are combined with each other solely on the basis of their relative molar concentrations. As a result, it was possible to calculate the composition of the molecular species of the peanut oil triacylglycerols (including the content of the enantiomers and the reverse isomers) by means of the 1-random 2-random 3-random distribution. In general, the three peanut oils possessed triacylglycerol structures which where closely similar to that derived earlier for a commercial peanut oil of North American origin. Since their oil has exhibited a high degree of atherogenic potential, it was anticipated that the present oils would likewise be atherogenic, which has been confirmed by biological testing. However, there are certain differences in the triacylglycerol structures among these oils, which can be correlated with the variations in their atherogenic activity. The major differences reside in the linoleic/oleic acid ratios in the triacylglycerols, especially in the *sn*-2-position, and in the proportions in which these acids are combined with the long chain fatty acids. On the basis of the characteristic structures identified in the earlier analyzed atherogenic peanut oil, the peanut oil of South American origin would be judged to possess the greatest atherogenic potential and this has been borne out by biological testing.

Previous studies have suggested that the atherogenic potency of peanut oils is determined by their triacylglycerol structure (1). Randomized or racemic synthetic peanut oils were shown to possess marginal atherogenic activity when tested in animals (2). In an earlier analysis of peanut oil triacylglycerols, we demonstrated (3) that the native oil contains significantly greater proportions of certain triacylglycerol structures than rearranged or simulated synthetic oil. The specific triacylglycerols of the native oil were characterized by a specific confinement of the long chain saturated fatty acids to the *sn*-3-position and a much greater relative proponderance of linoleic acid in the *sn*-2-position, when compared to the artificial oils.

Recent assays of the atherogenic activity of certain other peanut oils, however, have shown variability in the atherogenic potential (4), and it was of interest to determine the triacylglycerol structure of the latter oils and compare the results to those obtained with the North American oil analyzed previously. This study shows marked similarities among all three peanut oils, as well as some differences between the North American and South American and African varieties. The theoretical significance of these differences is evaluated by comparing the determined and the calculated enantiomeric structures of these peanut oils.

MATERIALS AND METHODS

The native peanut oil of North American origin was as described by Kritchevsky et al. (1). It was a gift of Standard Brands, Wilton, CT. The African peanut oil was a gift of the Lesieur-Cotelle Co., Boulogne, France, and its origin was Senegal. The South American peanut oil was sent by Prof. H. Malmros, University of Lund, Sweden and was of Argentine origin. Synthetic *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diacylglycerols of the common saturated and unsaturated fatty acids were prepared in the laboratory (5).

Grignard Degradation of Triacylglycerols

The reaction was carried out as described by Myher and Kuksis (5) and the 1,2-(2,3)- and 1,3-diacylglycerols were isolated by TLC using borate-impregnated Silica Gel G and chloroform/acetone (96:4) as developing solvent.

Resolution of *sn*-1,2- and *sn*-2,3-diacylglycerols

The enantiometric diacylglycerols were

resolved by a stepwise stereospecific release of *sn*-1,2- and *sn*-2,3-diacylglycerols by phospholipase C following an intermediate preparation of *rac*-1,2-diacylphosphatidylcholines as described by Myher and Kuksis (5).

Determination of Molecular Species of Diacylglycerols

The molecular species of *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diacylglycerols were identified and quantitated as the t-BDMS ethers by gas chromatography with mass spectrometry via the relative intensities of the respective M-57 ions as described elsewhere (6-8). The t-BDMS ethers were analyzed using a Hewlett-Packard Model 5985B GC/MS system.

Thin Layer Chromatography

The triacylglycerols were purified by TLC on Silica Gel H (Merck and Co.) using heptane/isopropyl ether/acetic acid (60:40:4, v/v/v) as developing solvent (3).

Gas Liquid Chromatography

Intact triacylglycerols and diacylglycerol t-BDMS ethers were resolved according to molecular weight by GLC on 3% OV-1 (9), whereas fatty acid methyl esters were analyzed on columns prepared with 10% EGSS-X as previously described (10). The fatty acid methyl esters were obtained using 1 N sodium methoxide in methanol/benzene (60:10, v/v) as suggested by Glass (11).

Calculations

The positional distributions of the fatty acids in the acylglycerols were calculated by subtracting the fatty acid composition of the *sn*-2-monoacylglycerols from that of the corresponding *sn*-1,2-(*sn*-1-position) or *sn*-2,3-diacylglycerols (*sn*-3-position). The *sn*-2-position of the acylglycerols was obtained from the fatty acid composition of the *sn*-2-monoacylglycerols released by pancreatic lipase (3). Theoretical compositions of triacylglycerols and diacylglycerols were calculated on the basis of 1-random 2-random 3-random distribution (12), which assumes that the fatty acid composition of each position of the acylglycerol molecule is independent of the composition of the other two positions. The calculated carbon number profiles for triacylglycerols and for *sn*-1,2-; *sn*-2,3- and *sn*-1,3-diacylglycerols were obtained by multiplying the mole percent of fatty acid in each position and then summing the products of corresponding carbon number. The calculated distributions of triacylglycerols and diacylglycerols based on carbon number and degree of unsaturation were generated by appropriate further screening of the products of multiplication.

RESULTS

Analyses of Total Triacylglycerols

The composition and positional distribution of fatty acids in the two foreign and one North American variety of peanut oil are given in Table 1. The results for the North American oil agree well with previous analyses of the commercial oil of North American origin (3). The compositions of the fatty acids of the other two oils are significantly different; the South American oil had much less of the linoleic acid than the American oil, with opposite changes in the oleic acid content of these oils. The proportions of the long chain saturated fatty acids were essentially the same in all three oils. Furthermore, the long chain acids were confined exclusively to the *sn*-3-position of the triacylglycerols in all three oils. In all instances, the content of linoleic acid was highest in the *sn*-2-position, but the total amount of this acid was different for each of the oils.

Table 2 compares the quantitative estimates of the triacylglycerol carbon numbers. It is seen that the three oils possess closely similar GLC elution profiles and carbon number proportions. All three oils contain the bulk of the species in the form of C_{52}-C_{54} triacylglycerols, which reflect the presence of large amount of C_{18} and lesser amounts of C_{16} acids, along with smaller proportions of C_{56}-C_{60} triacylglycerols, which represent mainly combinations of two C_{18} and one long chain (C_{20}-C_{24}) fatty acid per triacylglycerol molecule. The triacylglycerols containing two long chain fatty acids per molecule appear to be mostly absent.

Analyses of Diacylglycerols

The positional distribution and molecular association of fatty acids in the triacylglycerol molecules was obtained by chromatographic and mass spectrometric examination of the diacylglycerols derived from the triacylglycerols by random degradation. The gas liquid elution patterns obtained for the *sn*-1,2- and *sn*-2,3-diacylglycerols of each oil are compared in Figure 1. It is seen that the *sn*-1,2-diacylglycerols of all the oils contain essentially species made up of C_{16} and C_{18} fatty acids (peaks 34 and 36). In contrast, the *sn*-2,3-diacylglycerols are characterized by a high proportion of species (peaks 38-42), which represent combinations of the C_{16} and C_{18} fatty acids with the long chain acids (C_{20}-C_{24}). Since the *sn*-2-position is common in the *sn*-1,2- and *sn*-2,3-diacylglycerols, it is obvious that the long chain

fatty acids are located exclusively in the *sn*-3-position of the triacylglycerol molecule of the peanut oils. This conclusion is further confirmed by the finding of high proportions of the long chain fatty acids in the *sn*-1,3-diacylglycerols (results not shown). These comparisons of the carbon number distribution of the diacylglycerols essentially exclude a significant presence of *sn*-2,3-diacylglycerols containing two long chain fatty acids per molecule. Furthermore, Tables 3 and 4 indicate that the three peanut oils contain closely similar proportions of the various molecular weight classes in the corresponding diacylglycerol types. This is due to the presence of closely similar compositions of fatty acids based on molecular weight.

The composition of the molecular species of the *sn*-1,2-diacylglycerol moieties of the peanut oil triacylglycerols is given in Table 3. The experimental values were obtained by GC/MS of the intact diacylglycerols, whereas the theoretical values were derived by 1-random 2-random 3-random calculation using the positional distribution of the fatty acids given in Table 1. It is seen that the modest differences in the molecular association of the fatty acids seen on the basis of carbon number

TABLE 1

Positional Distribution of Fatty Acids in Genetic Varieties of Peanut Oils of Varying Atherogenic Potential

Fatty acids	Pos. 1	Pos. 2	Pos. 3	Reconst.[a]	Total[b]
			North American		
			(mol %)		
16:0	17.1	1.78	11.4	10.1	9.64
16:1	0.14	0.56	0.18	0.33	0.14
18:0	5.0	0.37	6.32	3.89	3.72
18:1	43.8	57.7	50.2	50.7	49.5
18:2	32.38	39.32	11.26	27.7	28.3
20:0	–	–	4.91	1.64	1.80
20:1	1.50	0.28	2.95	1.48	1.57
22:0	–	–	7.95	2.65	3.25
22:1	–	–	–	–	0.24
24:0	–	–	4.80	1.60	1.74
			South American		
16:0	18.20	1.96	13.60	11.3	10.1
16:1	0.16	0.24	0.14	0.18	0.15
18:0	5.4	0.26	4.60	3.52	3.36
18:1	37.82	32.0	42.2	37.3	37.0
18:2	36.50	65.15	17.20	39.6	40.1
20:0	–	–	4.80	1.60	1.50
20:1	1.94	0.53	2.89	1.78	1.89
22:0	–	–	10.50	3.50	3.84
22:1	–	–	–	–	0.10
24:0	–	–	4.30	1.43	1.89
			African		
16:0	17.04	2.16	8.76	9.73	8.67
16:1	0.38	0.56	0.15	0.31	0.12
18:0	4.19	0.54	4.66	3.08	3.41
18:1	57.63	67.16	56.52	60.1	60.4
18:2	18.62	29.40	10.13	18.4	19.4
20:0	–	–	4.22	1.59	1.46
20:1	2.14	0.18	2.69	1.72	1.43
22:0	–	–	7.75	2.96	2.96
22:1	–	–	–	–	0.04
24:0	–	–	5.11	1.92	1.70

[a]Fatty acid composition obtained by summing the compositions of the *sn*-1-, *sn*-2-, and *sn*-3-positions.

[b]Fatty acid composition of original oil.

TABLE 2

Carbon Number Distribution of Triacylglycerols in Genetic Varieties of Peanut Oil of Varying Atherogenic Potential

Carbon number	North American		South American		African	
	Exp.	Calc.[a]	Exp.	Calc.	Exp.	Calc.
			(mol %)			
48	–	0.047	–	0.055	–	0.042
50	3.00	2.45	3.66	2.96	3.02	2.05
52	22.5	21.93	24.2	23.29	21.1	20.68
54	57.4	55.34	54.7	51.66	59.5	57.47
56	7.39	8.87	7.89	9.54	7.77	8.52
58	6.42	7.32	6.89	9.20	5.69	7.21
60	3.35	3.93	2.66	3.59	2.95	4.18
62	–	0.08	–	0.10	–	0.11

[a]Triacylglycerol carbon number calculated by summing appropriate molecular species, which were obtained by 1-random 2-random 3-random distribution.

distribution (Fig. 1, Tables 3 and 4) are considerably increased when the degree of unsaturation of the species within each carbon number is also considered. These differences arise mainly from the variable replacement of oleic by linoleic acid in the different oils. It was shown by calculation that the *sn*-1,2-diacylglycerols differ greatly in the content of the 18:1 18:1 species (36:2), which make up 39, 25 and 12% of the total in the African, North American and South American varieties of peanut oils, respectively. (The calculated values for these species are somewhat lower than the experimental values for 36:2, because the latter also include the contributions from 18:0 18:2 species.) The differences among the varieties are similarly striking for the 18:2 18:2 species (36:4), which make up 24, 13 and 5% of the

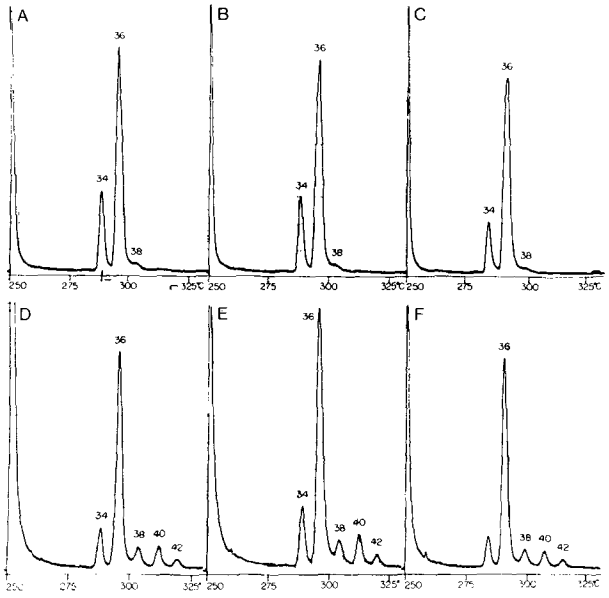

FIG. 1. The GLC profiles of the *sn*-1,2- and *sn*-2,3-diacylglycerol moieties of triacylglycerols from three genetic varieties of peanut oils of varying atherogenic potential. A, B and C represent *sn*-1,2-diacylglycerols and D, E and F represent *sn*-2,3-diacylglycerols of North American, South American and African peanut oils, respectively. Peaks 34-42 represent diacylglycerols with total number of acyl groups ranging from 34 to 42. Temperature program as shown. Other GLC conditions were as given in text. Sample: 1 μL of a 1% solution a t-BDMS ethers of diacylglycerols in petroleum ether.

TABLE 3

Molecular Species of *sn*-1,2-Diacylglycerol Moieties of Triacylglycerols in Genetic Varieties of Peanut Oils of Varying Atherogenic Potential

Molecular species	North American		South American		African	
	Exp.	Calc.[a]	Exp.	Calc.	Exp.	Calc.
			(mol %)			
32:0	0.65	0.30	0.89	0.36	0.62	0.37
32:1	0.44	0.10	0.26	0.05	0.44	0.10
32:2	0.22	0.001	0.08	0.004	0.12	0.002
	1.31	0.401	1.24	0.404	1.19	0.482
34:0	–	0.15	–	0.15	0.05	0.18
34:1	9.0	10.67	6.6	6.58	10.4	12.75
34:2	7.5	7.68	11.7	12.71	5.2	6.01
34:3	0.17	0.28	0.15	0.19	0.12	0.22
	16.67	18.785	18.45	19.64	15.74	19.154
36:0	–	0.02	–	0.01	–	0.02
36:1	2.3	3.11	1.5	1.96	3.3	2.90
36:2	32.7	27.35	16.6	15.72	43.7	40.16
36:3	32.0	35.90	33.5	36.32	25.2	29.53
36:4	11.7	12.73	25.3	23.78	7.0	5.49
	78.8	79.12	76.9	77.92	79.2	78.1
38:1	N.D.[b]	0.02	N.D.	0.034	N.D.	0.019
38:2	N.D.	0.99	N.D.	0.82	N.D.	1.54
38:3	N.D.	0.681	N.D.	1.46	N.D.	0.66
	3.19	1.689	3.45	2.312	3.84	2.23

[a]Molecular species of diacylglycerols were calculated as described in text.

[b]N.D. not determined.

South American, North American and African oils, respectively. The differences are somewhat smaller for the 18:2 18:1 and 18:1 18:2 diacylglycerols (36:3), which represent the next most abundant species in the *sn*-1,2-diacylglycerol moieties. The South American variety contained ca. 6% 16:0 18:1 (34:1) and 12% 16:0 18:2 (34:2), with the African and North American varieties containing these species in reverse proportions. There were no diacylglycerols containing C_{22} or C_{24} fatty acids among the *sn*-1,2-diacylglycerols. It should be noted that the experimental and calculated values are in rather good agreement except for the species representing the minor carbon numbers (C_{32} and C_{38}). However, some differences remain also among the major species and suggest that the placement of the fatty acids in any one position may not be completely independent of the composition of any other position, and that the 1-random 2-random calculation may not give the exact *sn*-1,2-diacylglycerol structure.

The molecular species of the *sn*-2,3-diacylglycerol moieties of the peanut oil triacylglycerols are given in Table 4. Again, the experimentally determined values have been compared to those calculated from the positional distribution of fatty acids given in Table 1. Interestingly, the content of 18:1 18:1 species (36:2) of the *sn*-2,3-diacylglycerols is very similar to that in the *sn*-1,2-diacylglycerols with the differences among the varieties maintained in identical proportions. The proportional similarity extends to the 18:1 18:2 species (36:4) of the *sn*-2,3-diacylglycerols but the overall level of these species in the *sn*-2,3-diacylglycerol is about two times lower than that in the *sn*-1,2-diacylglycerol moieties of the triacylglycerols. All the varieties contained about the same proportions of the 18:1 18:2 species (36:3) in the *sn*-2,3-diacylglycerols, unlike the *sn*-1,2-diacylglycerols, which contained a much higher relative proportion of this species in the South American variety as well as a nearly three times higher total proportion of this species in all varieties. The most characteristic feature of the composition of the *sn*-2,3-diacylglycerols is the presence of C_{20} to C_{24} saturated acids. The major species containing the latter acids are 18:1 20:0 (38:1) and 18:2 22:0 (40:2), which are present in similar proportions in all three oils; the proportion of the 18:1 20:0 species, however, is nearly two times lower in the South American than in the North American or African varieties. The 18:2 22:0 species was present in about two times greater relative

TABLE 4

Molecular Species of *sn*-2,3-Diacylglycerol Moieties of Triacylglycerols in Genetic Varieties of Peanut Oils of Varying Atherogenic Potential

Molecular species	North American		South American		African	
	Exp.	Calc.[a]	Exp.	Calc.	Exp.	Calc.
			(mol %)			
32:0	0.30	0.20	0.54	0.267	0.29	0.189
32:1	0.15	0.067	0.16	0.035	0.15	0.05
32:2	0.05	0.001	0.09	0.0003	0.05	0.0008
	0.50	0.28	0.79	0.302	0.49	0.24
34:0	–	0.153	0.07	0.126	0.01	0.15
34:1	6.1	7.51	5.3	5.2	7.0	7.14
34:2	4.4	5.07	7.1	9.34	3.0	3.21
34:3	0.22	0.134	0.17	0.13	0.15	0.10
	10.9	12.86	12.69	14.79	10.2	10.60
36:0	–	0.11	0.02	0.11	–	0.12
36:1	3.74	3.84	2.3	1.65	3.8	3.53
36:2	31.9	31.48	19.7	16.56	42.1	39.40
36:3	25.9	26.24	33.7	33.0	19.5	23.44
36:4	3.6	4.43	6.5	11.21	2.3	3.00
	65.4	66.14	62.2	62.59	68.0	69.46
38:0	0.04	0.16	0.25	0.22	0.14	0.19
38:1	3.3	2.9	2.3	1.6	3.5	2.9
38:2	3.4	3.77	4.3	4.73	3.7	3.15
38:3	1.3	1.19	2.6	1.97	1.1	0.97
	8.11	8.03	9.43	8.1	8.51	7.23
40:0	–	0.115	0.21	0.10	0.05	0.15
40:1	5.5	4.61	4.3	3.40	5.0	5.24
40:2	2.8	3.13	5.4	6.86	1.9	2.28
	8.3	7.87	9.88	10.36	6.96	7.67
42:0	0.20	0.02	0.18	0.011	0.28	0.03
42:1	2.4	2.8	2.1	1.43	3.4	3.45
42:2	2.0	1.89	1.8	2.80	0.6	1.50
	4.60	4.71	4.08	4.241	4.28	4.98
44:1	2.73	0.01	0.99	0.023	2.01	0.009

[a]Molecular species of diacylglycerols were calculated as described in text.

proportion in the South American oil than in either the North American or African varieties, but the 18:2 20:0 species (38:2) was present in about the same relative proportions in all oils. The 18:2 24:0 (42:2) species was present in higher amounts in the South American and North American than in African oil, whereas the 18:1 24:0 (42:1) was present in higher amounts in the American than in the other two peanut oil varieties. Again, there is a reasonably close agreement between the experimental and calculated values, especially for the major components. It is possible that there is a slightly better correspondence between the calculated and experimental values for *sn*-2,3- than for *sn*-1,2-diacylglycerols, which may be due to a truly more independent association of the fatty acids in the *sn*-2 and *sn*-3 positions.

The molecular species of the *sn*-1,3-diacylglycerol moieties of the peanut oil triacylglycerols showed only minor differences among the three varieties, except for the content of 18:1 18:1 (36:2) and 18:2 18:2 (36:4) species. The 18:1 18:1 species was found (results not shown) to represent 33, 22 and 16%, respectively, of the African, South American and North American varieties of the peanut oils. The 18:2 18:2 species was present in highest proportion in the South American (6%), lowest in the African (2%), with the North American oil falling in between (4%).

Reconstitution of Molecular Species of Triacylglycerols

Since the experimental and calculated distributions for the *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diacylglycerols agreed rather closely when compared on the basis of the proportions of the various unsaturation classes within each carbon number (many of which represented

single species), it was concluded that the 1-random 2-random 3-random calculation used to obtain them provided an acceptable approximation of the true composition of the various diacylglycerols. Hence, we assumed that in this specific instance the 1-random 2-random 3-random calculation would also give a reasonable approximation of the molecular species composition of the triacylglycerols. This assumption is experimentally verified in Table 2, which compares the experimental and calculated carbon number distributions of the triacylglycerols. The agreement is good. It is possible to extend this experimental verification further by comparing the distributions of the carbon number of the triacylglycerol fractions derived by argentation TLC and by calculation, but it was felt that this was superfluous for the present purpose. Table 5 gives the composition of the major molecular species of the peanut oil triacylglycerols derived by the 1-random 2-random 3-random calculation, which, as already noted, was experimentally justified at the diacylglycerol level. The molecular species are arranged in order of increasing carbon number and of increasing double bond number within each carbon number. Table 5 (Fig. 2) includes a total of 36 species, which accounts for about 80% of the peanut oils. Both differences and similarities in the distribution of the triacylglycerol species are seen among the three peanut oil varieties. There are marked differences even in the major triacylglycerol species. Thus, the African variety contains about four

TABLE 5

Molecular Species Differences of Triacylglycerols between Genetic Varieties of Peanut Oils of High and Low Atherogenic Potential[a]

Carbon number	Molecular species	Genetic variety of peanut oil		
		North American	South American	African
			(mol %)	
50	16:0 18:1 16:0	1.1246	-.3323	-.1221
50	16:0 18:2 16:0	.7665	.8461	-.3276
52	18:1 18:1 16:0	2.8806	-1.2341	.5099
52	16:0 18:1 18:1	4.9542	-2.4957	1.5140
52	18:2 18:1 16:0	2.1295	-.5405	-1.0341
52	18:1 18:2 16:0	1.9633	1.3877	-.4791
52	16:0 18:2 18:1	3.3767	1.6271	-.5451
52	16:0 18:1 18:2	1.1108	-.1088	.0485
52	18:2 18:2 16:0	1.4514	1.7826	-.9719
52	16:0 18:2 18:2	.7571	1.2824	-.2496
54	18:1 18:1 18:0	1.5970	-1.0401	.2027
54	18:0 18:1 18:1	1.4428	-.7134	.1477
54	18:2 18:1 18:0	1.1806	-.6431	-.5978
54	18:1 18:2 18:0	1.0884	.0450	-.3024
54	18:1 18:1 18:1	12.6897	-7.5809	9.1860
54	18:0 18:2 18:1	.9834	.5013	-.2871
54	18:2 18:2 18:0	.8047	.2892	-.5495
54	18:2 18:1 18:1	9.3811	-4.4506	-2.3132
54	18:1 18:2 18:1	8.6490	1.7490	.9273
54	18:1 18:1 18:2	2.8452	-.7629	1.0755
54	18:2 18:2 18:1	6.3939	3.6411	-3.2999
54	18:2 18:1 18:2	2.1034	-.0938	-.8366
54	18:1 18:2 18:2	1.9392	2.2988	-.2229
54	18:2 18:2 18:2	1.4336	2.6565	-.8791
56	18:1 18:1 20:0	1.2407	-.6596	.3927
56	16:0 18:2 22:0	.5339	.7112	-.1456
56	18:1 18:2 20:0	.8456	.3371	-.1306
56	18:1 18:1 20:1	.7454	-.3955	.2957
56	18:2 18:2 20:0	.6251	.5163	-.3941
58	18:1 18:1 22:0	2.0063	-.7351	.9933
58	18:2 18:1 22:0	1.4832	-.2564	-.5140
58	18:1 18:2 22:0	1.3674	1.2198	-.0543
58	18:2 18:2 22:0	1.0109	1.4860	-.5866
60	18:1 18:1 24:0	1.2129	-.6923	.7649
60	18:2 18:1 24:0	.8966	-.3942	-.2576
60	18:1 18:2 24:0	.8267	.2328	.0391
60	18:2 18:2 24:0	.6111	.4114	-.3314

[a]The molecular species of the North American oil have been subtracted from those of the South American and African oils.

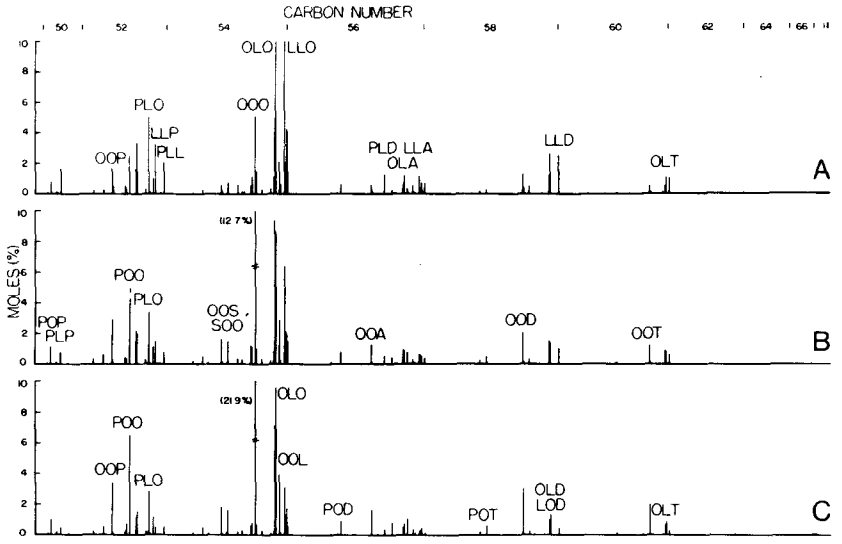

FIG. 2. Molecular species of triacylglycerols of three genetic varieties of peanut oils of varying atherogenic potential as obtained by 1-random 2-random 3-random calculation. A, South American; B, North American; C, African. The validity of the calculation was established at the *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diacylglycerol level by comparing experimental values with values obtained by 1-random 2-random 3-random calculation. O, oleic; P, palmitic; L, linoleic; S, stearic; A, arachidonic; D, behenic; T, tetracosanoic acid. The letters from left to right indicate the fatty acids in the *sn*-1-, *sn*-2- and *sn*-3-positions of the triacylglycerol molecule. Within each carbon number, the molecular species are arranged in order of increasing degree of unsaturation.

times and the North American variety two times as much 18:1 18:1 18:1 species than the South American variety. In contrast, the South American oil has about seven times and the North American oil about three times as much 18:2 18:2 18:2 than the African variety. On the other hand, the three oils contained comparable proportions of the 18:1 18:2 18:1 (8.6-10.4%), 18:1 18:1 18:2 (2.1-3.9%), 18:2 18:1 18:1 (5.0-9.5%) as well as the 16:0 18:1 16:0 (0.8-1.1%), 18:1 18:1 16:0 (1.6-3.4%), 18:1 18:1 18:0 (0.6-1.8%), 16:0 18:1 18:1 (2.5-6.5%) and 18:0 18:1 18:1 (0.7-1.6%). In most instances, the South American oil had the lower proportion and the African and North American oils higher and similar proportions of the above species. The major species 18:1 18:2 16:0 (1.5-3.4%), 18:1 18:2 18:0 (0.8-1.1%), 16:0 18:2 18:1 (2.8-5.0%), 18:2 18:2 16:0 (0.5-3.2%), 18:1 18:2 18:2 (1.7-4.2%) were present in the highest proportion in the South American oil with the African oil again having the lowest proportion and the North American oil being intermediate in its value. Likewise, the distribution of the triacylglycerols containing the long chain saturated fatty acids showed similarities and striking differences among the three peanut oil varieties. Thus, the South American oil contained relatively lower proportions of the long chain triacylglycerols containing oleic acid in the *sn*-2-position, with little regard for the composition of the *sn*-1-position, than either the African or the North American oils. In contrast, the South American variety contained relatively higher proportions of linoleic acid in the *sn*-2-position with little regard for the fatty acid composition of the *sn*-1-position. It should be noted, however, that the relative amounts of the total long chain triacylglycerols were similar in the three peanut oil varieties. The above distribution of triacylglycerol species is consistent with a comparable content of saturated medium and long chain fatty acids in all three peanut oil varieties and an essentially quantitative substitution of a part of the oleic acid in the African oil by linoleic acid in the North American and South American oils, or vice-versa.

Triacylglycerol Differences among Peanut Oils of Varying Atherogenic Potential

The atherogenicity of each oil was assessed in parallel experiments (4), in which rabbits were fed a diet containing 2% cholesterol and 6% peanut oil for 8 weeks. North American and

African peanut oils were compared in four experiments and South American peanut oil was used in three. Average serum cholesterol levels (mg/dL) and average atherosclerosis (arch plus thoracic/2) were: North American peanut oil, 2,366 and 1.59; African peanut oil, 2,473 and 1.75; South American peanut oil, 2,567 and 1.88; and corn oil, 1,922 and 1.36. Analysis of variance of the severity of atherosclerosis in the aortas of rabbits fed all four fats showed the differences in the arch (df 4; $F_{4,125} = 10.72$ and the thoracic aorta (df 4; $F_{4,125} = 6.93$) to be highly significant ($p<0.001$). Analysis of variance among the three types of peanut oil also indicated a significant difference in the arch (df 2; $F_{2,13} = 17.45$) ($p<0.001$) and thoracic aorta (df 2; $F_{2,73} = 4.17$) ($p<0.025$). The differences in the composition of the molecular species of the triacylglycerols among the three peanut oils were related to their atherogenic activity. It is seen that the peanut oils, when arranged according to increasing atherogenic activity (North American < African < South American), possess an increased relative proportion of the total linoleic acid in either *sn*-2-position of the triacylglycerol or in combination with the long chain saturated acids, regardless of the actual location of the linoleic acid and of the total number of its residues. The South American oil, which is the most atherogenic, clearly has the highest proportion of the long chain fatty acids in combination with linoleic acid. On the basis of this criterion, however, it would have been anticipated that the African oil would possess a significantly lower atherogenic activity than the American oil, yet the biological tests rated them in the opposite order. Therefore, in the case of African oil, the correlation must be made either to a relatively lower content of total linoleic acid or to the presence of other as yet unrecognized potentially atherogenic combinations of fatty acids in its triacylglycerol molecules.

DISCUSSION

Validity of Calculation of Molecular Species of Triacylglycerols

The calculation of the molecular species of triacylglycerols on the basis of the 1-random 2-random 3-random distribution is justified by the experimental demonstration of the existence of a noncorrelative distribution of the fatty acids in the *sn*-1,2-, *sn*-2,3- and *sn*-1,3-diacylglycerols. Although it does not follow with absolute certainty that the *sn*-1,2-diacylglycerol moieties are also combined with the fatty acids in the *sn*-3-position solely according to their molar proportions, it may be assumed to be correct as a first approximation for the present purposes in view of the absence of evidence to the contrary. A true experimental validation of this distribution would require the isolation and identification of at least some of the molecular species of triacylglycerols in pure state, which is not technically possibly at the present time. The 1-random 2-random 3-random distribution meets certain metabolic facts about triacylglycerol biosynthesis and secretion in animal and presumably plant systems. Thus, it is known that acyltransferases exist which are specific for the *sn*-1- and *sn*-2-positions of the *sn*-3-glycerol phosphate leading to the formation of phosphatidic acids with markedly different complements of fatty acids in the *sn*-1- and *sn*-2-positions (13). It is also known that the *sn*-3-position of the triacylglycerol molecule is synthesized last and that a fatty acid pool different from that utilized in the synthesis of the phosphatidic acids is utilized (14). There may also be differences in the subcellular sites involved in the synthesis of the different acylglycerol derivatives (15).

A Hypothesis of the Atherogenicity of Peanut Oils

Since the peanut oil triacylglycerols are presumably not absorbed intact, there is no need to search for any direct lethal effects of these oils. The atherogenic potential must be generated, therefore, during the metabolic utilization of the hydrolysis or resynthesis products of the peanut oil triacylglycerols. Since both atherogenic and nonatherogenic oil contain all their long chain fatty acids in the *sn*-3-position, they differ largely from each other only in the nature of the *sn*-1,2-diacylglycerol moieties. It is possible that the higher proportion of linoleic acid in the *sn*-1,2-diacylglycerol moieties of the more atherogenic oils may have contributed to a more efficient hydrolysis of these long chain triacylglycerols to the resulting *sn*-3-monoacylglycerols. These long chain monoacylglycerols could then become absorbed, reesterified and eventually transferred to any other tissues, where they again would be expected to at least partially resist hydrolysis by the tissue lipases (16). Should they accumulate, they could produce lesions as a result of their detergent-like activity. Previous work has shown (17) that triacylglycerols containing long chain saturated fatty acids (C_{22}) in combination with linoleic acid are readily hydrolyzed by pancreatic lipase, but less than 30% of the behenic acid in the *sn*-2-position was found in the lymph. The possibility that the structure of the triacylglycerol molecule is a determining factor in its absorb-

ability has been further substantiated by studies with isomeric dioleoylstearoylglycerols (18).

Recently, other laboratories have made attempts to determine the triacylglycerol structures of different varieties of peanut oils and have commented on their potentials (19-21). These analyses, however, involved only positional analyses of the fatty acids, without identifying any molecular species. On the basis of the present study, it would be possible to calculate the composition of the molecular species of these oils. From present detailed studies of the peanut oil varieties and from the assessment reported by others (19,20), it may be concluded that all natural peanut oils possess markedly nonrandom structures of highly asymmetric positional placement of the long chain saturated fatty acids.

ACKNOWLEDGMENTS

This work was supported in part by the Ontario Heart Foundation and the Medical Research Council of Canada (A.K., J.J.M. and F.M.) and by a grant (HL-03299) and a Research Career Award (HL-0734) from the National Institutes of Health, USPHS (D.K.).

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Transformation of Arachidonic Acid into Monohydroxy-Eicosatetraenoic Acids by Mouse Peritoneal Macrophages[1]

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ABSTRACT

Mouse peritoneal macrophages synthesize 6 monohydroxylated eicosatetraenoic acids when incubated with exogenous arachidonic acid. These compounds were identified by chromatographic techniques (high pressure liquid chromatography and high efficiency glass capillary column gas chromatography and mass spectrometry. The chromatographic and spectrometric data are presented. These results show that peritoneal macrophages constitute one of the best systems to study in evaluating the metabolism of oxygenated products of arachidonic acid.

INTRODUCTION

There are now many publications indicating that mononuclear phagocytes synthesize arachidonic acid oxygenation products (1-4). PGE_2 often appears as a major compound. In some cases, the metabolism of oxygenated products of arachidonic acid is dominated by the production of PGI_2. When mouse peritoneal macrophages were incubated with exogenous arachidonic acid, there resulted several products arising from the cyclooxygenase pathway; their distribution as determined by gas chromatography-mass spectrometry (GC-MS) has been established (Rigaud, personal communication). The most quantitatively important transformation of exogenous arachidonic acid by mouse peritoneal macrophages, however, results from the lipoxygenase activity of these cells. This activity has been reported in thrombocytes and many other mammalian cells (5-11), and also in mouse peritoneal macrophage cells (4).

Our work aimed to delineate the lipoxygenase metabolites of mouse peritoneal macrophage cells. When this cell population is incubated with exogenous arachidonic acid, the lipoxygenic activity leads to many compounds: monohydroxy, dihydroxy, trihydroxy polyunsaturated fatty acids and monohydroxy-epoxy compounds. In this publication, we describe the purification and the identification of six monohydroxypolyunsaturated fatty acids produced by resident mouse peritoneal macrophages when incubated with exogenous arachidonic acid: 5-, 8-, 9-, 11-, 12-, and 15-hydroxy-eicosatetraenoic acids (HETE). They were extracted from the incubation medium with organic solvent, and then separated by high pressure liquid chromatography (HPLC) on silica gel (μPorasil) using an isocratic eluent system. Each isolated fraction was submitted to gas liquid chromatography (GLC) on high efficiency glass capillary column prior to mass spectrometry coupled with a computer system. The recorded mass spectra of biological compounds were compared with those we synthesized ourselves.

MATERIALS AND METHODS

5,8,11,14-Eicosatetraenoic acid was obtained from Alltech Associates, Arlington Heights, IL [1-^{14}C] 5,8,11,14-Eicosatetraenoic acid (sp act: 56.5 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. Triphenylphosphine and N-methyl-N'-nitro-N-nitrosoguanidine (97%) were products from Aldrich-Europe. N,O-bis-(trimethylsilyl/trifluoroacetamide (BSTFA) and Platin-IV-oxid were obtained from Fluka AG, Buchs S.G. All the solvents were nanograde range Mallinckrodt and used without any further purification.

Chromatography and Mass Spectrometry Methods

HPLC was performed with a Waters Associates instrument (injector U6K, pump M600A, variable wavelength detector, Model UV 450S). A μPorasil column (3.9 mm id, 30 cm length) was used. Solvent was delivered under isocratic conditions.

GLC was performed on several high effi-

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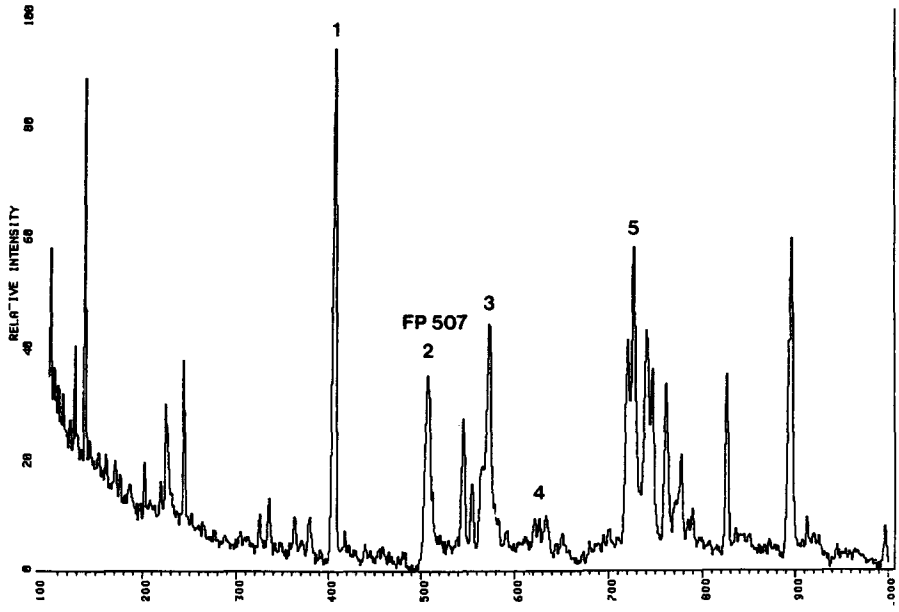

FIG. 1. Total ionization current of biological extract of incubation of arachidonic acid and pure mouse peritoneal macrophages obtained by gas liquid chromatography on high efficiency glass capillary column (OV-1—see text); organic solvent extract of the incubation medium was dried under nitrogen and derivatized as methyl ester, trimethyl silyl ethers. Peak 1: 12 hydroxystearic acid added as an internal standard; 2: 12-ho-20:4 and similar compounds (file position 507); 3: hydroxyepoxy compounds; 4: dihydroxy compounds; 5: trihydroxy compounds.

ciency glass capillary columns. They were homemade using methods previously described (12-15). The stationary phases were: OV-1, SE_{54}, FFAP and methylphenylsilicones (OV-17-like). The chromatographic characteristics of these columns were: (a) FFAP column: 40 m length, 0.3 mm id, 3,500 plates/meter. The purity of arachidonic acid used in our experiments was tested on this column which was able to separate octadeuterated 5,8,11,14-eicosatetraenoic acid (k'=9.46 at 190 C isothermal) from protonated homologous (k'= 9.58), with a resolution of 0.80. The carrier gas was helium (linear velocity 14.43 cm/sec); (b) OV-1 column: 25 m × 0.3 mm; 3,500 plates/meter for a capacity ratio 4.27 at 220 C at optimal flow rate; (c) OV-17 "like" column: 24 m × 0.3 mm; 2,000 plates/meter for k'=3.85 at 180 C. The stationary phase used for the coating of this column was synthesized as previously described (13). (d) SE_{54} column: 20 m × 0.3 mm; 2,100 plates/meter for k'=4.80 at 220 C.

Packard-Becker Model 417 and Carlo Erba 2900 chromatographs, with flame ionization detectors, were employed. An all-glass solventless injector was used. Details of GLC parameters are described elsewhere (16).

Gas Chromatography-Mass Spectrometry

A magnetic field mass spectrometer coupled with a glass capillary column and monitored by a computer system was used (LKB 2091-0.61). The mass spectra were recorded on electron impact at 20 eV with an accelerating voltage of 3.5 KV.

Preparation of Mouse Peritoneal Macrophages

Peritoneal cells from Swiss mice were collected directly by washing the peritoneal cavity with fresh sterile medium containing 10 units/mL heparin. Following collection, peritoneal cells were resuspended in minimum essential medium with Earle's salts supplemented with 1% nonessential amino acid mixture, 1% sodium pyruvate, 1% glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were then distributed into the wells of Falcon microtest plates and incubated at 37 C in a humidified atmosphere made up of 95% air/5% CO_2. After allowing macrophages to adhere for 1 hr, nonadherent cells were discarded and wells were washed with a stream of fresh medium. After allowing the cells to

adhere for a further 2-3 hr, the wells were again thoroughly washed three times, in order to ensure that all nonadherent cells had been eliminated. This method, as described by Dimitriu et al. (17) enables us to collect viable macrophages, 99% pure. The cell population is then subjected to microscopic studies in order to ensure that contamination with blood platelets has been avoided.

Incubation Reaction and Extraction

The adherent cells (3×10^6) (termed macrophages monolayer) were scraped using a rubber policeman and resuspended in 2 mL 0.05 M phosphate buffer containing acetylsalicyclic acid (2 mM). They were then incubated with a mixture of protonated (160 nmol) and ^{14}C-labeled (used as a tracer) arachidonic acid at 37 C for 30 min in air and shaken. The suspension was then acidified (HCl N) to pH 3 and extracted three times with diethyl ether. The organic phases were pooled and dried over anhydrous sodium sulfate, filtered and concentrated in a rotary evaporator under vacuum.

Preparation of Monohydroxy-eicosatetraenoic Acids

5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acids (HETE) were prepared from arachidonic acid by reaction with H_2O_2 in the presence of Cu^{++} ions, as described by Boeynaems et al. (18). Their method was slightly modified, the hydroperoxides being reduced with triphenylphosphine at 0 C for 30 min. The reaction products were submitted to HPLC using the solvent system of Porter et al. (19): acetic acid/ethanol/hexane (1:6:993 v/v). The flow rate was 3 mL/min and 1.5-mL fractions were collected. One aliquot of the fractions containing the separated HETE was dried under nitrogen and derivatized as methyl esters and trimethylsilylethers. Using this solvent, all the isomers, including 8 and 9 isomers, were resolved.

RESULTS AND DISCUSSION

When the derivatized biological extract was submitted to high efficiency glass capillary column GLC prior to MS, the total ionization current showed many peaks (Fig. 1). One of them at file position 507 (F.P. 507) exhibited the mass spectrum shown in Figure 2. This mass spectrum has a base peak at m/z = 295, suggesting the presence in the gas chromatographic peak of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12ho-20:4). When we considered this mass spectrum carefully, however, there were several fragments: m/z = 225, m/z = 255 and

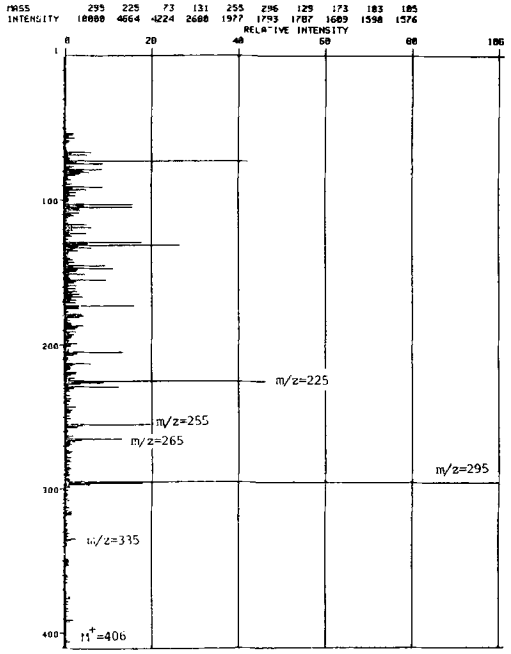

FIG. 2. Mass spectrum of the F.P. 507 peak from Fig. 1. The base peak is m/z = 295 (M^+-111, loss of $.CH_2$-CH=CH-$(CH_2)_4$-CH_3 from 12ho-20:4); peak at m/z = 225 has too high intensity to belong solely to 12ho-20:4. m/z = 255, m/z = 265 and m/z = 335 do not come within the 12-monohydroxylated fatty acid as compared with mass spectrum previously described (4).

m/z = 265, which are not arising from 12ho-20:4. This fact led us to consider this peak as a mixture of compounds so chemically similar that they were not resolved even by high efficiency GLC.

To support this hypothesis, the biological extract was submitted to multiple ion detection (MID), before and after catalytic hydrogenation. MID recording proved that the gas chromatographic peak was not one of a single compound (Fig. 3). On the graphs, one can see the enhanced resolution of the constituents of the mixture after catalytic hydrogenation. The full mass spectra of these hydrogenated compounds show fragmentations, suggesting to us that the F.P. 507 peak was a mixture of several monohydroxylated eicosatetraenoic acids. To clarify the chemical nature of this interesting gas chromatographic peak and to obtain conclusive arguments, we submitted the biological extract to HPLC. The elution profile obtained is depicted in Figure 4. This profile was compared with that of a chemically synthesized mixture of HETE. The capacity ratios of the

major peaks from both elution profiles were similar. The collected fractions corresponding to peaks from I to VI from both the biological extract and the chemical synthesis were dried under nitrogen and derivatized as methyl esters and TMS ethers. The gas chromatographic parameters (capacity ratio, methylene units on three stationary gas chromatographic phases OV-17, OV-1 and SE_{54}) and the mass spectra of each compound before and after catalytic hydrogenations enabled us to assign a chemical structure to each of them. Figure 5 shows the mass spectra and the gas chromatographic properties of these compounds. All these mass spectra present analogous fragmentation patterns. We detail here, as an example, the mass spectrum of compound III (11ho-20:4). It is characterized by ions at m/z 406 (M^+, relative intensity 0.46 %), 391 (M^+-15, relative intensity 0.82%), 375 (M^+-31, relative intensity

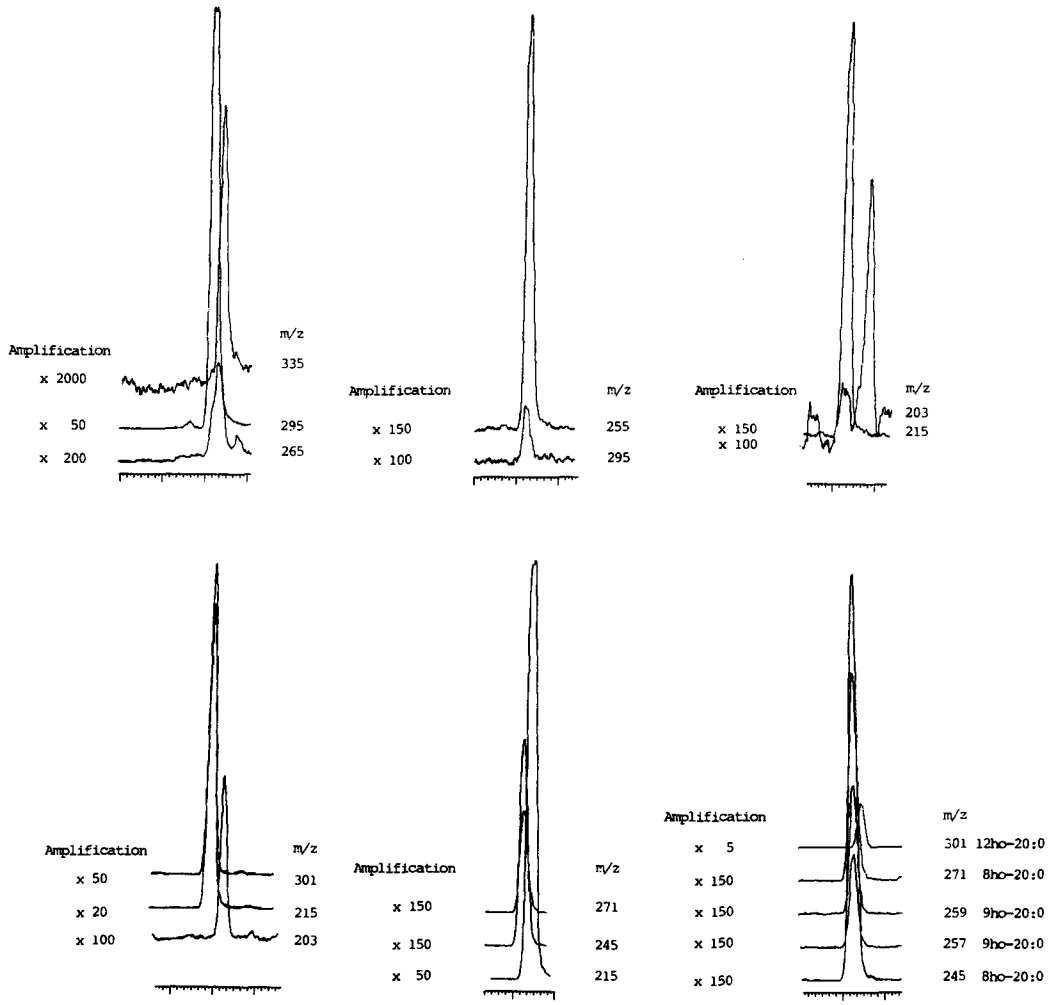

FIG. 3. 1–MID recording of F.P. 507 peak from Fig. 1. (A) m/z = 265, m/z = 295 (these m/z are, respectively, the base peak of 8ho-20:4, 12ho-20:4); m/z = 335 belongs to 15ho-20:4 as described by Boeynaems et al. (18). (B) m/z = 255 and m/z = 295. m/z = 255 is the base peak of 9ho-20:4. (C) m/z = 203 and m/z = 295. m/z = 203 belongs to 5ho-20:4. 2–MID recording of F.P. 507 peak after catalytic hydrogenation. The derivatized biological extract was dried under nitrogen. 500 μL methanol was added with 2 mg Platin-IV-oxid. H_2 was bubbled for 5 min, after which the platinium oxide was eliminated by filtration and the methanol evaporated under nitrogen. The residue was resuspended in 100 μL hexane (BSTFA (50:50, v/v)). (A) m/z = 203 arising from 5ho-20:4. m/z = 215 and m/z = 301 arising from hydrogenated 12ho-20:0. (B) m/z = 215. m/z = 245 and m/z = 271 are the two major fragments of 8ho-20:0. (C) m/z = 251 and m/z = 257 are the two major fragments of 9ho-20:0.

0.32%), 225, base peak (M^+-181, loss of ($^{\bullet}CH_2$-CH=CH-CH_2-CH=CH-$(CH_2)_3$-CO_2CH_3) 135 (225-90, relative intensity 3.84%). After catalytic hydrogenation, the mass spectrum was typical of the methyl ester, trimethylsilyl ether of a monohydroxylated saturated fatty acid, with two major fragmentations occurring on each side of the trimethylsilyl ether substituted carbon. Our spectra data are in full agreement with those of Boeynaems et al. (18).

These data, HPLC and gas chromatography retention parameters, recordings of full mass spectra of each compound, before and after saturation of the double bonds, allowed us to conclude that mouse peritoneal macrophages are able to produce, from exogenous arachidonic acid, six monohydroxylated eicosatetraenoic acids, i.e., 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12ho-20:4), 15-hydroxy-5,8,-11,13-eicosatetraenoic acid (15ho-20:4), 11-hydroxy-5,8,12,14-eicosatetraenoic acid (11ho-20:4), 8-hydroxy-5,9,11,14-eicosatetraenoic acid (8ho-20:4), 9-hydroxy-5,7,11,14-eicosatetraenoic acid (9ho-20:4), and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5ho-20:4). The configuration and the position of the conjugated diene of the monohydroxyeicosatetraenoic acid has been established by Porter et al. (19). The close agreement between their retention parameters and ours suggests the following structure: *cis-trans*-12; *cis-trans*-15; *cis-trans*-11; *cis-trans*-9; *cis-trans*-8 and *cis-trans*-5. These liquid chromatographic properties are not absolute proof of the diene configuration. To delineate it more precisely, we are performing ultraviolet and infrared investigations. The chirality center of these compounds is being studied.

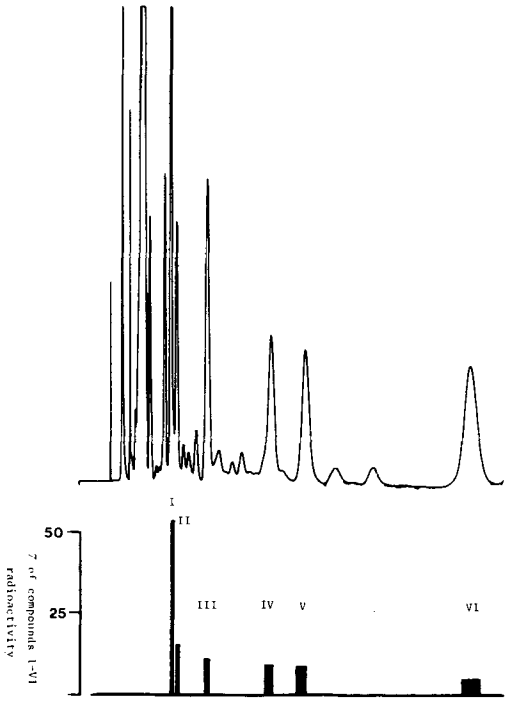

FIG. 4. High pressure liquid chromatogram of biological extract. Each bar depicts the radioactivity in each 1.5-mL fraction. The upper trace is the UV recording (234 nm) of the products of the reaction between arachidonic acid, Cu^{++} and H_2O_2 after extraction and reduction by triphenylphosphine. Full scale deflection 0.2 od.

In our experimental conditions (inhibition of the cyclooxygenase pathway by aspirin; this inhibition by various concentrations of aspirin allowed us to rule out any role of this enzyme for the generation of the C11 hydroxylated eicosatetraenoic acid), the distribution of the six lipoxygenase metabolites (measured by isotopic dilution) is the following: 12ho-20:4 52%, 15ho-20:4 15%, 11ho-20:4 10%, 8ho-20:4 8.5%, 9ho-20:4 8.5% and 5ho-20:4 6.8%. Twenty-seven percent of the exogenous arachidonic acid was transformed via this lipoxygenase pathway.

To assess the enzymatic origin of these HETE, some experiments were carried out under different conditions. When boiled macrophages were incubated with exogenous arachidonic acid, HETE were not produced. The enzymic activity was inhibited by eicosatetraynoic acid (60 μg/mL of incubation medium). The activity of the enzyme was not dependent on the glucose medium concentration. The lipoxygenase activity was not restricted to arachidonic acid, as other polyenoic acids such as dihomo-γ-linolenic acid led to several monohydroxylated compounds.

CONCLUSION

The evidence (as shown in this paper) that mouse peritoneal macrophages synthesize monohydroxylated compounds when incubated with arachidonic acid suggests to us some main points: first, high efficiency glass capillary columns GLC alone failed to resolve completely all the HETE, even on polar stationary phases like OV-17 (M.U. of HETE ranging from 25.76 to 25.90) or SE_{54} (M.U. ranging from 23.34 to 23.40). Used after HPLC, however, glass capillary columns were effective as an analytical tool because the MID detection limit with glass capillary columns exceeds those reported for packed columns (16). This fact allows the quantification of minute amounts of HETE produced by macrophages, under phagocytosis stimulation, more specifically and precisely

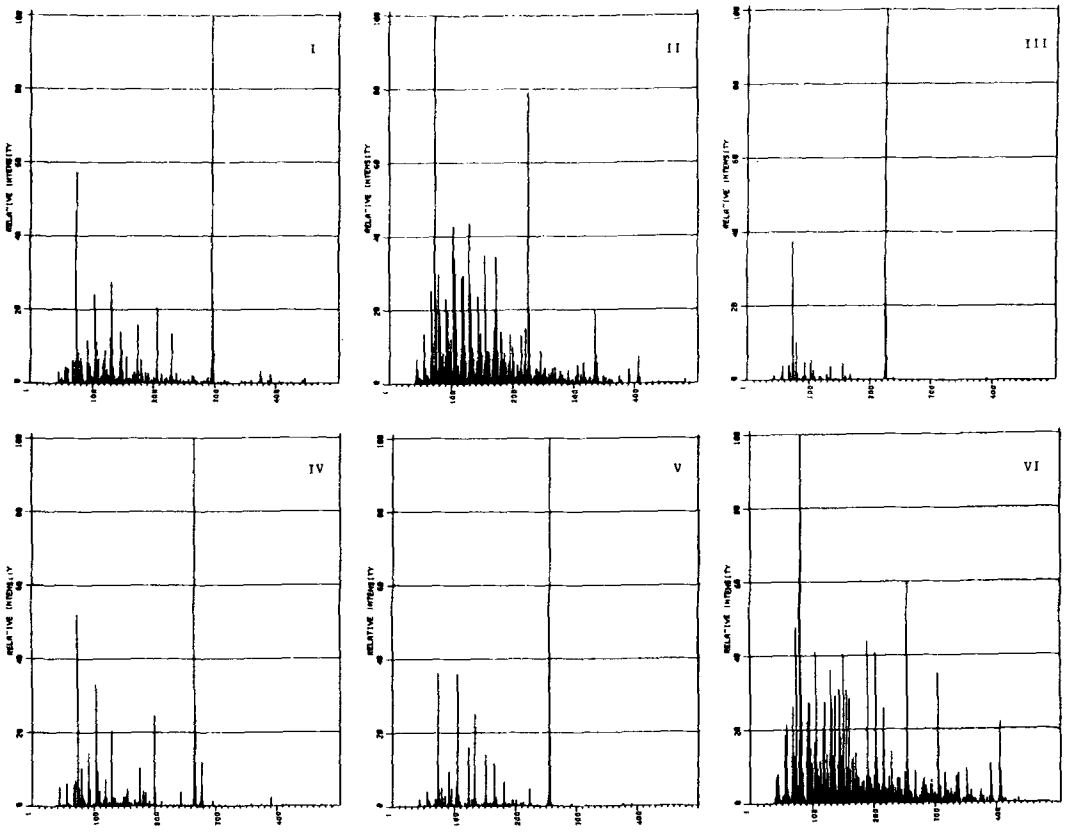

FIG. 5. a–Mass spectra of the compounds I to VI (see Fig. 5b, next page).

than do other analytical methods. Second, the monohydroxylated compounds of macrophages arise very likely from hydroperoxides, one of them (5-hydroperoxyeicosatetraenoic) being the precursor of compounds possessing slow reactive substance activity (20). That 5-lipoxygenase activity was found in these cells would suggest the capacity of these cells to produce leukotrienes. Moreover, these cells might be the site of synthesis of other similar compounds originating from 8 or 15 hydroperoxyeicosatetraenoic acids.

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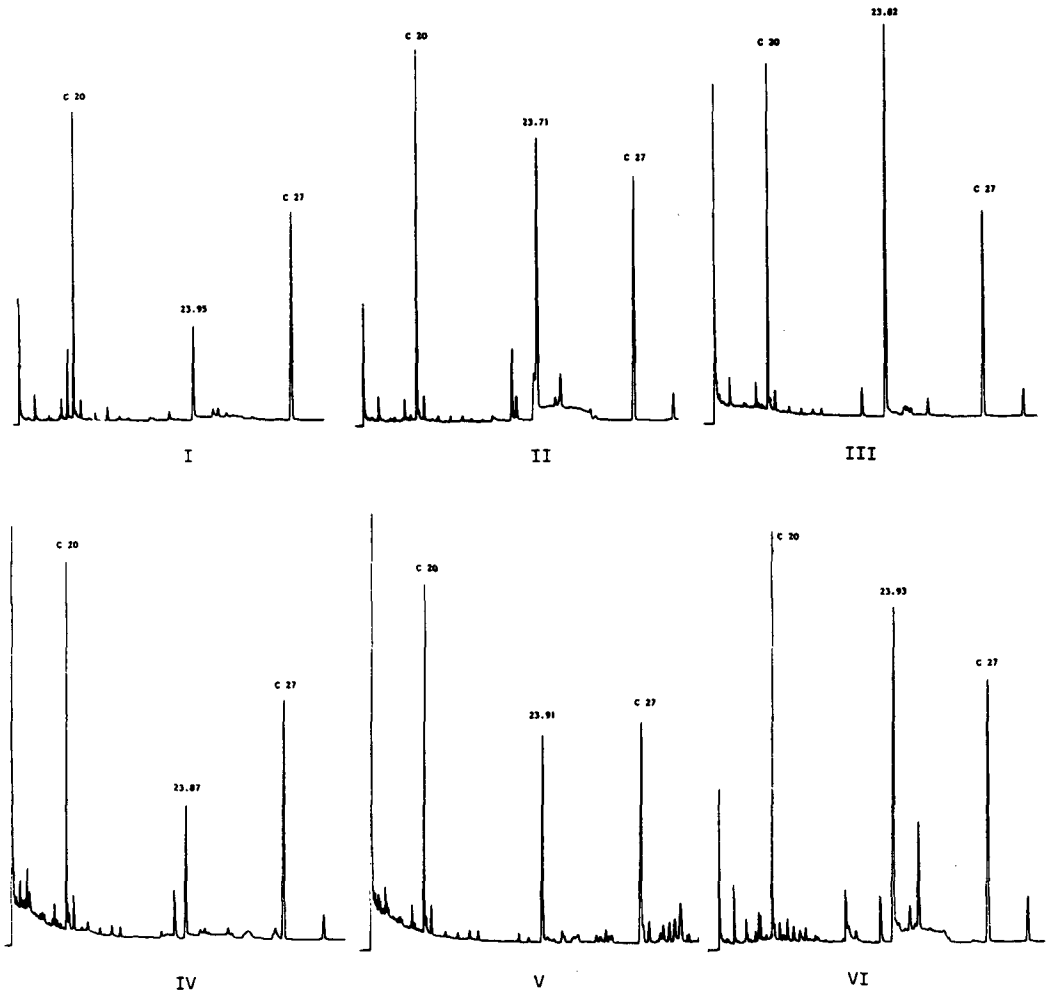

FIG. 5. b–Gas chromatographic behavior of monohydroxylated eicosatetraenoic acids as ME TMS ethers on glass capillary column 20 m × 0.3 mm; stationary phase: OV-1 (2%). Carrier gas was helium at the optimal flow rate. Flame ionization detection. Attenuation: $2.5\text{-}10^{-12}$ Amp. × 16. Chart rate: 0.5 cm/min. Oven temperature programmed from 180 to 240 C at 2 C/min. Methylene units were calculated using the formula: MU = n × (t_RHETE-T_RC_{29}/t_RC_{27}-T_RC_{29}) + 20. 5ho-20:4:23.93; 9ho-20:4: 23.91; 8ho-20:4: 23.87; 11 ho-20:4: 23.82; 12ho-20:4: 23.95; 15ho-20:4: 23.71.

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Effect of Diabetes and Insulin Replacement on the Lipid Properties of Hepatic Smooth Endoplasmic Reticulum

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ABSTRACT

This study is a characterization of the lipid properties of the smooth and rough endoplasmic reticulum (SER, RER) of liver from streptozotocin-induced diabetic rats. A significant decrease in membrane microviscosity was observed in the SER but not the RER of diabetic rats when compared to that of normal controls. This decrease in SER membrane microviscosity correlated with a decrease in cholesterol/phospholipid ratio of these membranes that could be accounted for solely by a change in the membrane cholesterol content. Changes in phospholipid fatty acyl chain composition were also observed in the SER membranes but these changes were small when compared to the large change in cholesterol content observed. Insulin treatment for only one day did not significantly alter the microviscosity of the SER but after 2, 4 and 6 days of treatment both membrane microviscosity and membrane cholesterol content were restored to values similar to those for normal animals. No significant changes in the RER lipid composition were observed. It is well known that increases in glucose-6-Pase (G-6-Pase) activity of liver ER membranes are associated with diabetic onset. An increase in the specific activity of G-6-Pase was observed in both SER and RER membrane preparations, although the observed increase in the SER membrane is higher. The changes in the G-6-Pase activity of the SER membranes were correlated with the alterations in the microviscosity and lipid composition of these membranes. It is postulated that lipid properties of the SER membranes may contribute to the regulation of G-6-Pase activity in that membrane.

INTRODUCTION

Significant proliferation of hepatic smooth endoplasmic reticulum (SER) from streptozotocin-diabetic rats, especially apparent in periportal hepatocytes of the liver lobule, has been previously reported (1). In addition, it is well known that there is a decrease in the content and a disruption of the form of the hepatic rough endoplasmic reticulum (RER) of diabetic animals (2). The ultrastructural changes in SER were correlated with significant changes in glycogen distribution and an increase in glucose-6-phosphatase (3.1.39 glucose-6-Pase) activity in these cells. Insulin treatment of the diabetic rats reversed both the ultrasturctural alterations and the increased glucose-6-Pase (G-6-Pase) activity observed in the diabetic rat preparation. We report here the further characterization of hepatic SER membranes from diabetic rats.

The fluid nature of biological membranes is well documented (3) and a considerable variety of methods has been employed to determine membrane fluidity. The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), has been used extensively in studies of membrane fluidity by fluorescence polarization (4,5). The probe is distributed evenly among the hydrocarbon chains of the lipid layer of the membrane and the derived microviscosity is therefore a weight average of all lipid domains.

Prominent determinants of membrane microviscosity are the cholesterol/phospholipid ratio, the degree of phospholipid acyl chain unsaturation, and the phospholipid polar head group compositions (5-7). It has been shown that enrichment or depletion of membrane cholesterol content in a variety of biological systems is correlated with changes in microviscosity determinations (6-8). The role of acyl chain saturation and membrane microviscosity in affecting membrane functional parameters has also been studied in both model membranes and biological systems (7,9-13).

It has been clearly demonstrated that physical characteristics of membrane lipids, including membrane microviscosity, have modulating effects on transport functions, enzyme activity and hormone-receptor interactions (8-14). Recently, the role of insulin on cell membrane has been examined. Insulin activates hexose uptake in adipocyte plasma membranes and studies indicate that the physical state of the lipids can modify this action (15,16). The microviscosity of liver plasma membranes is increased in the presence of insulin when physiological concentrations of the hormone are used (17). Concomitant to the increased

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microviscosity, the overall degree of insertion of membrane protein appears to be substantially increased upon insulin binding.

This study is a characterization of lipid properties of the liver membranes from diabetic rats. Lipid composition as well as membrane microviscosity have been measured and the effect of insulin on these membranes determined. The role of lipid composition in the regulation of the membrane-bound enzyme G-6-Pase is also discussed.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200-250 g were purchased from Hill Top Laboratory (Scottsdale, PA). Animals were kept in wire cages under a 12:12 hr light-dark cycle and fed ad libitum with Charles River rat-mouse formula (22% protein, 5% fat, 4% fiber, 5% ash, the remainder carbohydrate) and water. Rats to be rendered diabetic were fasted overnight and were intraperitoneally administered streptozotocin (Sigma) freshly prepared in 50 mM sodium citrate adjusted to pH 4.0 at doses of 75 mg/kg body weight. Control animals were fasted overnight and injected with saline sodium citrate. The animals were given food and water ad libitum after the drug injection and were killed for these studies by decapitation from 8:00 a.m. to 10:00 a.m. 2 weeks later. Plasma glucose levels were determined on an autoanalyzer by glucose-oxidase-peroxidase as previously described (1) and diabetic animals with > 400 mg glucose/dL were selected for this study. Insulin levels in these animals were determined as previously described (1) and these levels were < 10 μU/mL. Each group consisted of 6 animals. Insulin-treated animals were administered 10 U of NPH illetin (Isophane insulin, Eli Lilly) for 2-6 days, 2 weeks after streptozotocin-induced diabetes. The insulin was injected subcutaneously at 5:00 p.m., shortly before the onset of the dark phase of their light-dark cycle, when the rats normally start their most active feeding.

Preparation of Liver Homogenate and Microsomes

From the decapitated animals, 3 g of liver was rapidly excised and placed in preweighed beakers containing sufficient ice-cold 0.25 M sucrose to give a 20% w:v liver-to-sucrose concentration. The liver was minced and homogenized with 4 strokes of a motor-driven Teflon pestle in a glass homogenizer. The homogenate was filtered through a double layer of cheesecloth.

To prepare microsomes, the homogenate was centrifuged twice at 10,000 G for 20 min, which removed mitochondria, unbroken cells, and other cellular debris. Microsomes were sedimented by centrifuging the postmitochondrial supernatant (PMS) at 386,000 G for 60 min. The microsomal pellets were quickly frozen for subsequent resuspension and assay in 0.25 M sucrose.

RER and SER fractions were prepared according to Dallner (18) with slight modifications as previously described (1). Membranes were subjected to a washing procedure designed to remove adsorbed proteins, ribosomes and lipids as well as lumenal contents (12). The SER and RER preparations were rapidly frozen in liquid nitrogen, and stored at -80 C for biochemical assay.

Enzyme Assay

G-6-Pase enzyme activity was determined as previously described (1). The incubation medium for G-6-Pase contained 40 mM sodium cacodylate buffer (pH 6.5), 60 mM G-6-Pase, and homogenate or microsomes to make a final vol of 0.2 mL. The release of inorganic phosphate determined by the Fiske and Subbarow method (19) was linear for the reaction conditions used (30 C for 15 min). Protein was determined according to Lowry et al. (20) using serum albumin as the standard.

Microviscosity Measurements

Microviscosity was determined according to the method of Shinitzky and Barenholz (6), using the lipid-soluble fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. All measurements were taken at 37 C in a Hitachi Perkin-Elmer spectrofluorimeter Model MPF-3. Microviscosity values ($\overline{\eta}$) are calculated from the Perrin equation $r_o/r = 1 + C(r)\tau/\overline{\eta}$, where r_o = limiting anisotropy of the probe, taken as 0.362, r = measured anisotropy, defined as $(I_{/\!/}/I_\perp - 1)/(I_{/\!/}/I_\perp + 2)$. $I_{/\!/}$ and $I_\perp$ are the experimentally determined fluorescence intensities parallel and perpendicular, respectively, to the direction of polarization of the excitation beam. $C(r)$, a function of the molecular shape of the probe, is taken as 8.6×10^5 poise deg^{-1}; T = absolute temperature; τ = excited state lifetime, taken at 6 nsec at 37 C, from studies with lecithin liposomes (6). Light-scattering was less than 3% and fluorescence values were corrected accordingly. The phospholipid:diphenylhexatriene ratio was always maintained at more than 200:1 (mol/mol) in order to minimize probe-probe interactions and/or perturbations of the membrane bilayer.

Phospholipid Phosphorous

Phospholipid was extracted by the method of Folch et al. (21) and phospholipid phosphorous was determined by the method of Bartlett (22).

Fatty Acid Analysis

RER or SER (0.10 mg) was diluted to 0.5 mL in water and 50 μg of pentadecanoic acid was added as an internal standard. To each tube was added 0.2 mL 10% ethanolic KOH and the tubes were heated at 100 C for 20 min. The extracts were acidified with 0.2 mL 4 N H_2SO_4 and the liberated fatty acids were extracted 3 times with pentane. The combined pentane extract was taken to dryness in a stream of nitrogen and the residue was methylated with 3 drops of an ethereal solution of diazomethane (an excess). The residual diazomethane was removed in a stream of nitrogen.

The methyl esters were dissolved in 10 μL of Lipopure hexane (Applied Sciences Labs, Inc., State College, PA), and 1-μL aliquots were analyzed by gas liquid chromatography (GLC).

Gas Liquid Chromatography

A Shimadzu GC-FID-4c gas chromatograph fitted with a flame ionization detector was used for the quantitative analysis of mixtures of fatty acid methyl esters. Stainless steel columns (3 mm id, 4 mm od) 3 m long were packed with 10% Silar 10 C, 100/120 mesh on Gas Chrom Q support (Applied Sciences Labs., Inc., State College, PA). The column was operated with temperature program from 160-210 C at a rate of 2 C/min with a helium gas flow of 50 mL/min. Peaks were quantitated relative to an internal standard with a Shimadzu Chromtopac E1A integrator.

Cholesterol Analysis

SER and RER (0.10 mg) were extracted by the method of Bligh and Dyer (23), with 50 μg β-sitosterol added to the membranes as an internal standard prior to extraction. Free cholesterol was quantitated using GLC as just described, using 3-m glass columns packed with 3% OV-17, 100-120 mesh, Gas-Chrom Q support (Applied Science Laboratories, State College, PA) at 360 C.

RESULTS

Membrane microviscosity was determined on washed RER and SER fractions prepared from normal, diabetic, and diabetic rats treated with insulin. Diphenylhexatriene was added to the membranes and fluorescence depolarization measured at 37 C as described in Materials and Methods. Microviscosity values were calculated from the Perrin equation and the results are presented in Figure 1. As indicated, the microviscosity values for SER and RER of normal rats are similar within the limits of these measurements. In the diabetic rat, there is no change in the microviscosity of the RER compared to the same fraction from normal rats; however, a significant decrease in the microviscosity of the hepatic SER from the diabetic rats is observed. Upon treatment with insulin, the reduced SER microviscosity from the diabetic animals returns to a value similar to that of hepatic SER from normal rats. This rise

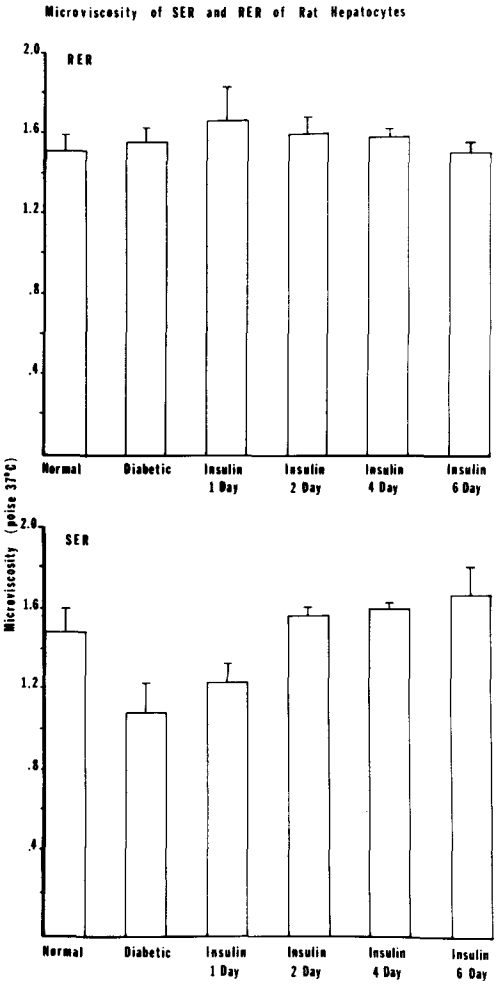

FIG. 1. Each membrane microviscosity value is for duplicate samples determined from 8 separate animals and are represented as ± SEM. Differences for membranes from control animals are $p < 0.01$ (Fisher r-test).

in microviscosity does not occur immediately upon insulin replacement, but follows a lag of at least 2 days following insulin treatment.

Changes in microviscosity values calculated from fluorescence depolarization measurements of diphenylhexatriene are associated with changes in the lipid environment of the probe, in both animal and model membrane systems. It was important, therefore, to analyze the lipid composition of the SER and RER of these animals to determine if changes in the microviscosity of these membranes were associated with significant shifts in their lipid composition. These results are presented in Table 1. The phospholipid content of these membranes was determined as total lipid P. No significant change in this value for SER and RER was observed, although an overall decrease in these values upon insulin treatment was observed. Phospholipid species were not characterized in these studies.

The cholesterol content of the SER was measured and a significant decrease in the content of cholesterol was observed in SER of diabetic rats compared to that of normal animals. Diabetic rats treated with insulin for one day exhibited the level of cholesterol/phospholipid seen in the untreated diabetic rats. After two days of insulin treatment, the cholesterol content of the SER was returned to near normal values. Importantly, the change in cholesterol content of the membranes paralleled the microviscosity changes observed in these same membranes. By contrast, the extensive shift in cholesterol content observed in the SER membrane from diabetic animals was not detected in membranes of the RER.

The first few days following streptozotocin treatment and the induction of diabetes, a significant proliferation is observed in the liver SER membrane fraction (1). Our experiments on the lipid composition of the SER membrane are performed after 2 weeks, a time sufficient for this proliferation to be complete. The decrease in the cholesterol content of the SER membrane from diabetic rats may be related to an inadequate supply of cholesterol for insertion into the newly synthesized SER. No accumulation of new RER appears with the induction of diabetes (1) and the membrane cholesterol content of this fraction should remain intact and, in fact, does so.

The lipid composition of the SER and RER membranes was further characterized by analyzing the fatty acid composition of the constituent phospholipids present. Small but significant shifts in the SER fatty acid composition were observed and they are reported in Table 2. No change in the ratio of moles of saturated fatty acid to moles of unsaturated fatty acid was observed. A decrease in palmitic acid and an increase in stearic acid resulted but the total number of saturated fatty acid moles was not changed. A decrease in oleic acid was observed that appeared to be balanced by an increase in linoleic acid. A decrease in arachidonic acid is also observed along with an increase in the level of docasahexaneoic acid. No significant change in RER membrane fatty acid composition was observed.

Membrane microviscosity changes are often associated with the modulation of membrane

TABLE 1

Lipid Composition of SER and RER Membranes of Rat

	Phospholipid[a] content	Cholesterol[a] content	Cholesterol/phospholipid
	μmol/mg protein	μmol/mg protein	Molar ratio
SER			
Normal	0.63 ± 0.03 (10)	0.223 ± 0.003 (4)	0.35
Diabetic	0.68 ± 0.03 (10)	0.128 ± 0.001 (4)[b]	0.18
Insulin 1 day	0.63 ± 0.13 (10)	0.129 ± 0.002 (4)[b]	0.20
Insulin 2 day	0.61 ± 0.11 (10)	0.254 ± 0.002 (4)	0.42
Insulin 4 day	0.61 ± 0.07 (10)	0.216 ± 0.002 (4)	0.35
Insulin 6 day	0.56 ± 0.03 (10)	0.220 ± 0.001 (4)	0.39
RER			
Normal	0.50 ± 0.06 (10)	0.085 ± 0.001 (2)	0.17
Diabetic	0.48 ± 0.09 (10)	0.085 ± 0.001 (4)	0.18
Insulin 1 day	0.47 ± 0.06 (10)	0.079 ± 0.001 (4)	0.17
Insulin 2 day	0.51 ± 0.08 (10)	0.071 ± 0.001 (4)	0.14
Insulin 4 day	0.41 ± 0.04 (10)	0.680 ± 0.001 (4)	0.17
Insulin 6 day	0.44 ± 0.09 (10)	0.061 ± 0.001 (4)	0.14

[a]All values are mean ± SEM. Numbers in parentheses = number of animals.

[b]Significantly different from normal and from insulin-treated $p < .01$. (Fisher τ-test).

TABLE 2

Fatty Acid Composition of SER and RER of Diabetic and Normal Rat Liver

Fatty acid	SER		RER	
	Normal[a] (mol %)	Diabetic[a] (mol %)	Normal[a] (mol %)	Diabetic[a] (mol %)
16:0	30.1	26.9	24.9	23.8
16:1	0.9	0.6	0.3	0.6
18:0	21.5	26.2	28.0	28.0
18:1	13.8	11.1	10.8	11.7
18:2	14.4	18.7	15.8	16.5
20:4	16.8	11.9	16.5	15.4
22:6	1.9	3.7	2.5	5.2

[a]Values are for mol % fatty acid present and represent the mean of 3 determinations that agreed within 10%.

activities. Hepatic G-6-Pase is a membrane-bound enzyme for which the activity is changed in the diabetic state (24,25). The G-6-Pase sp act, expressed on the basis of membrane phospholipid activity of the hepatic SER and RER of normal, diabetic and insulin-treated diabetic rats, is shown in Table 3. The level of G-6-Pase activity is elevated in both RER and SER of the diabetic animals. However, the relative increase in activity of each fraction compared to the same fraction from normal rats is nearly 25% greater for the SER. Treatment of the diabetic animals with insulin for 2 days or longer produces a return of microsomal G-6-Pase activity to enzyme levels observed in RER and SER from normal rats.

The lipid dependence of G-6-Pase activity has been recently reinvestigated (10,11) and specific phospholipid requirements established for the transport unit and the catalytic moiety of this system. In our experiments, the observed fluctuations in G-6-Pase activity correlate well with the fluctuations in lipid composition and membrane microviscosity in the SER but not in the RER membrane.

DISCUSSION

Lipid properties of liver SER and RER membranes from streptozotocin diabetic rats have been studied and a clear difference in SER and RER lipid properties established. Previous observations made on this system using ultrastructure measurements identified the SER population of the hepatocytes as increasing in the diabetic state but no increase in RER was observed (1). We have observed a decrease in cholesterol content, a change in lipid ester unsaturated fatty acid composition and a decrease in microviscosity values in the SER but not RER of diabetic rats.

The change in SER lipid composition observed may reflect a change in the lipid pool available for membrane synthesis in the liver of the diabetic rat. Changes in pool size of both cholesterol and fatty acid synthetic enzymes (26) have been observed previously in the liver of the diabetic rat and these would directly affect the pool size of these compounds. The second factor may be the availability of substrates for de novo synthesis of lipids as the liver switches from an anabolic to a catabolic state in diabetes.

Unsaturated fatty acid composition changes observed in the SER are consistent with the known effect of diabetes on enzymes of fatty acid biosyntheses. Inhibition of the Δ9 and Δ6 fatty acid acyl CoA desaturase activities (1.14.99.5) and an increase of cytochrome b_5 are associated with diabetes (27-29). A decrease

TABLE 3

G-6-Pase of Liver RER and SER
(μmol Pi/min/mg Phospholipid)

SER	
Normal	0.481 ± 0.03
Diabetic	1.234 ± 0.05
Insulin	
1 day	1.266 ± 0.10
2 day	0.892 ± 0.07
4 day	0.413 ± 0.05
6 day	0.514 ± 0.04
RER	
Normal	0.862 ± 0.04
Diabetic	1.670 ± 0.06
Insulin	
1 day	1.554 ± 0.13
2 day	1.555 ± 0.05
4 day	0.944 ± 0.04
6 day	0.967 ± 0.08

Values represent mean ± SEM of 10-17 animals for each group.

in a series of unsaturated fatty acids available for phospholipid biosynthesis could result from a decrease in these enzymes. The elongation of palmitic acid to stearic acid may increase with more cytochrome b_5 available, as elongation of fatty acids requires cytochrome b_5 for activity (30). Upon insulin treatment for 48 hr, both fatty acid desaturases and cytochrome b_5 levels return to levels comparable to those for normal rats (29). The replacement of oleic acid by linoleic acid could occur easily since linoleic acid is present in the rat diet. The decrease in arachidonic acid and oleic acid in the liver lipids of the diabetic rats has been previously observed (28) and the increase in docasahexanoic acid has been previously reported (31). The complex series of enzymatic reactions responsible for polyunsaturated fatty acid biosynthesis has been recently reviewed (32) and the key role of the $\Delta 6$ desaturase in regulating this process discussed (33) but no data are available at this time that could adequately explain the observed increase in docosahexanoic acid in the diabetic animal.

Pool size of unsaturated fatty acids may be directly affected not only by changes in the enzymes of unsaturated fatty acid metabolism but also by changes in the availability of saturated fatty acids as substrates for these enzymes. Although the flux of fatty acids through the liver is high in the diabetic animal, oxidation of these fatty acids is their predominant metabolic fate. Synthesis of fatty acids is inhibited in the liver of the diabetic rat and the central role of malonyl CoA in controlling the levels of fatty acid oxidation, and synthesis has been described (34).

Specific decreases in cholesterogenic enzymes have been observed in the liver of diabetic rats. 3-Hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase (1.1.1.34) is significantly reduced in these animals and a return to normal levels of the enzyme occurs after insulin treatment (26). A decrease in the conversion of acetate to cholesterol was also observed in these animals, with a corresponding return to normal levels after insulin treatment. The increase in cholesterogenic enzyme activity in response to insulin treatment was observed to occur in a matter of hours after treatment. In our experiments, even after 24 hr, no increase in membrane cholesterol content was observed.

Local changes in the pool of cholesterol available for membrane synthesis cannot necessarily be viewed as simply correlated with biosynthetic enzymes or with overall tissue content of this substance. The cholesterol content of whole livers from diabetic rats has been measured and compared to the level of HMG-CoA reductase present in this tissue (35). Although a 3-fold decrease in enzyme was measured, no significant change in total liver cholesterol content was observed. Upon cholestryramine feeding, the HMG-CoA reductase was stimulated 13-fold in the diabetic rat but no significant difference in total liver cholesterol was observed. Specialized pools of cholesterol in the endoplasmic reticulum have been implicated in controlling HMG-CoA reductase activity (36). This enzyme, however, can be stimulated by insulin in a matter of 2-4 hr whereas our membrane cholesterol content requires over 24 hr of insulin treatment to return to normal levels.

The dynamic lipid changes observed in the SER of liver of diabetic rats are associated with significant changes in the physical properties of this membrane. A decrease in the cholesterol content of the membrane was observed to correlate with a decrease in membrane microviscosity in the diabetic rat. Insulin treatment restored both the sterol content and the microviscosity values to control levels. Phospholipid content and composition may also significantly influence microviscosity values. No phospholipid head group measurements have been made at this time but in our experiments no change in total lipid phosphorous was observed. Fatty acyl composition of the constituent phospholipids was measured only as total fatty acid composition. Small changes in the fatty acid composition were observed but there are no data available from model membrane systems at this time that would predict what effect small changes in phospholipid fatty acyl residues would have on microviscosity values. Changes in both microviscosity values and cholesterol/phospholipid ratios have been observed previously in proliferating ER membrane systems (37). In our experiments, streptozotocin-induced SER proliferation is complete (1) as all our experimental animals are used 2 weeks after streptozotocin treatment. However, the hepatic cholesterol pools available for membrane synthesis may be rate-limiting in the diabetic rat, resulting in maintenance of a membrane with both a lower sterol content and a decreased membrane microviscosity.

Membrane functions, including transport, enzyme activities and hormone receptor activities all have been shown to be modulated by the physical characteristics of the membrane lipids. G-6-Pase is a membrane-bound enzyme that is known to change in diabetes. Is the change in enzyme activity that is observed in diabetes a result of or influenced by the lipid changes observed? An increase in sp act of G-6-Pase is observed in both the RER and SER

membranes, but the increase is greater in the SER of diabetic animals. After insulin treatment for 4 to 6 days, the sp act of the G-6-Pase of both the RER and SER drops significantly and returns to normal levels. The lipid compositional changes appear, however, to reside predominantly in the SER fraction.

In comparing G-6-Pase content of whole liver homogenates, and in estimating what fraction of the activity is attributable to SER, it is clear that the major change is an increase in SER G-6-Pase activity. The small increase in overall RER does not contribute significantly to the total increase in G-6-Pase activity observed.

Arion and coworkers have proposed that the functional G-6-Pase is a system composed of a specific G-6-P transporter and a nonspecific phosphohydrolase-phosphotransferase (38). They postulate that, in the diabetic rat liver, an increase occurs in the phosphohydrolase portion of the enzyme and that the transport component of the enzyme thereby becomes limiting. This postulate was based on the observation that a significant increase in the latency of the G-6-Pase activity occurs in the diabetic rat (39). Further, a higher latency of G-6-Pase has been observed in the SER of rat liver when compared to RER in both normal and diabetic rats (40). These data indicate that the SER and RER contain different forms of G-6-Pase and that in diabetes, where the SER has been shown to proliferate, a transport-deficient form of G-6-Pase is the predominant one induced.

The lipid dependence of G-6-Pase of rat liver and rat hepatoma has been recently studied. The data of Eletr et la. (11), using lipophilic spin-labeled molecular probes and enzyme measurements, indicate that G-6-Pase activity is dependent on the physical state of membrane lipids. The result of studies using protein-mediated lipid exchange and G-6-Pase activity indicate that there are different phospholipid requirements for the transport unit and for the phosphohydrolase unit of the enzyme (10). Our results suggest that membrane fluidity, sterol content of the membrane and G-6-Pase activity are all regulated coordinately. We have no direct latency data at this time to indicate that a change in the transport component of the enzyme correlated with the lipid changes observed. Furthermore, we have no data concerning specific phospholipid changes in these membranes and previous work indicates these changes may provide significant control of this enzyme. Future experiments will focus on the role of cholesterol and phospholipids in regulation of the transport and the catalytic moiety of G-6-Pase.

A change in membrane microviscosity has been observed for the SER in the diabetic rat and this may have a functional significance in the regulation of blood glucose levels during diabetes. Homeoviscous adaptation of membranes has been observed in some plasma membrane systems and this concept argues in favor of membranes adapting their lipid composition in order to maintain conformational mobility at a constant level (41). The SER subcellular membrane of the liver may specifically utilize a variation in membrane microviscosity as one method of regulating some of its membrane functions. The regulation of G-6-Pase activity in the RER membrane does not appear to involve a change in lipid composition and it is possible that the large increase in G-6-Pase activity in this membrane may be regulated solely by an increase in protein synthesis of the enzyme.

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Microsomal Phosphatidylethanolamine Methyltransferase: Some Physical and Kinetic Properties

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ABSTRACT

Some physical and kinetic properties of the microsomal enzyme(s) that convert phosphatidylethanolamine to phosphatidylcholine in rat and guinea pig livers have been investigated. The pH optima of the reactions were 9.8, 9.3 and 9.5 for the first, second and third methylation reactions, respectively. Incomplete heat denaturation of the protein catalyzing the first reaction contrasts with inactivation at 60 C of the enzymes catalyzing the second and third methylations. The maximal velocity of the first reaction of the guinea pig liver enzyme is 48 pmol/min/mg protein, substantially less than exhibited rate-limiting reaction of the three step methylation sequence in rat liver, 114 pmol/min/mg. The affinity of the microsomal enzyme for S-adenosylmethionine is greater in rat liver (Km = 18.2 μM) than in guinea pig liver (Km = 302 μM).

Hepatic phosphatidylcholine (PC) is synthesized by two major pathways in the rat. The Kennedy pathway (1), utilizing choline phosphotransferase (EC 2.7.8.2), may be responsible for the majority of PC synthesized in rat liver; however, the importance of the methylation pathway in membrane structure (2) and chemotaxis (3,4) has recently become evident. This pathway has been estimated to contribute from 15 to 40% of the total PC synthesized in rat liver (5-8) or from 10 to 15% in human liver (9). Greenberg and colleagues (10,11) were the first to report the stepwise methylation of phosphatidylethanolamine (PE) to form PC via PE methyltransferase (EC 2.1.1.17). S-Adenosylmethionine donates each methyl group in three reactions to successively form monomethylphosphatidylethanolamine, dimethylphosphatidylethanolamine and finally PC. The presence of one (12) or two (13,14) enzymes that catalyze the three-step reaction in rat liver is undecided; however, it is generally agreed that the first methylation is rate-limiting (12-17).

The substantial difference in the levels of polyunsaturated fatty acid containing PC in rat and guinea pig liver microsomes (18) may result from the significant differences in the activities inthe PC biosynthetic enzymes present in these two species (15,19). In the assay of the last reaction of rat liver PE methyltransferase, the specific activity (sp act) was nine times greater than that in guinea pig liver (19). Thus, it appeared profitable to investigate the PE methyltransferase reactions in further detail since this enzyme synthesizes the more highly unsaturated fatty acid PC species (8,20-22).

It is the purpose of this paper to report the temperature denaturation, pH, and detergent characteristics of the three methylation reactions in rat liver and compare the kinetic properties of the microsomal enzymes from rat and guinea pig livers.

MATERIALS AND METHODS

Chemicals

S-Adenosyl-L-[methyl-^{14}C] methionine (45.9 mCi/mmol) was purchased from New England Nuclear and diluted to 0.1 mCi/mmol with unlabeled AdoMet purchased from Sigma Chemical Co. Triton X-100, Tween 20, sodium cholate, sodium deoxycholate, Tris base, ethanolamine, and 3-*sn*-phosphatidylcholine were obtained from Sigma. Egg PE, phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine were obtained from Avanti Polar Lipids, Birmingham, AL.

Animals and Tissue Preparation

Female albino rats of the Sprague-Dawley strain, 70-110 days old, weighing 150-200 g, were used in all experiments. Female guinea pigs (15 weeks old) were purchased from Gopher State Caviary, Minneapolis, MN. Animals were maintained on Purina Chow ad libitum and were kept in individual cages with a dark period from 8:00 p.m. to 8:00 a.m. The animals were killed by decapitation, and the tissue was removed, rinsed with cold water, blotted, weighed, and homogenized in ice-cold 0.25 M sucrose/1.0 mM $MgCl_2$ with a Teflon-plug homogenizer. The subcellular fractions were isolated by differential centrifugation (23). The nuclear and mitochondrial fractions were separated from the homogenate by centrifugation at 14,500 G for 10 min. The supernatant fluid was centrifuged at 78,670 G for 60 min to sediment the microsomal pellet which was then suspended in 0.25 M sucrose/

1.0 mM $MgCl_2$ for enzyme assay. Protein was determined by a modified Biuret method (24).

PE Methyltransferase Assays— First Methylation Reaction

Routine analysis of the methylation of endogenous microsomal PE to PC was measured by a modified method of Bremer and Greenberg (16,17). The reaction mixture contained 2 mg egg PC (solubilized by sonification in detergent/buffer), 0.9 mM sodium deoxycholate, 0.3 M Tris/HCl buffer (pH 8.6), 0.2 mM S-adenosyl-L-[methyl-^{14}C]methionine (0.1 mCi/mmol) and microsomes (1-3 mg protein) in a final vol of 1.4 mL. The assay was initiated by addition of microsomes, reaction time was 15 min at 37 C, and the reaction was terminated with 0.14 mL concentrated HCl. Extraction of the ^{14}C-labeled reaction product, PC, was according to the procedure of Bligh and Dyer (25). The phospholipid was isolated by addition of 4.25 mL of 2.5:1 methanol/chloroform, mixing, addition of 1.50 mL water and 1.50 mL chloroform to effect the biphasic separation, aspiration of the upper aqueous phase and removal of an aliquot of chloroform for determination of radioactivity in a Packard liquid scintillation spectrometer. To determine the amount of interfering radioactivity, parallel incubations, in duplicate, were run with HCl added to the mixture before the addition of microsomes. The use of 15 min and 1-3 mg microsomal protein in the reaction conditions were chosen following previous studies by Cornatzer and colleagues (19,26). The two-dimensional thin layer chromatography method of Katyal and Lombardi (27) was used to identify reaction products. Following three separate chromatographic analyses of reaction products, a total of 88% of the chloroform-soluble radioactivity was recovered from the spots migrating with authentic methylated derivatives. Of this fraction, 95, 1 and 4% of the ^{14}C-methyl label cochromatographed with PC, phosphatidylmonomethylethanolamine, and phosphatidyldimethylethanolamine, respectively. Chromatography of the phospholipid products subsequent to modification of the reaction conditions resulted in similar findings.

Second and Third Methylation Reactions

Assay conditions for these reactions were essentially the same as those of Rehbinder and Greenberg (14). Assay of the second methylation utilized 1.0 mM phosphatidylmonomethylethanolamine as substrate and 3.0 mM phosphatidyldimethylethanolamine for measurement of the last reaction. The assay medium contained lipid substrate (solubilized by sonification in detergent/buffer) 6.3 mM sodium deoxycholate, 0.3 M Tris/HCl buffer (pH 8.6), 0.35 mM S-adenosyl-L-[methyl-^{14}C]methionine (0.1 mCi/mmol) and microsomes (1-3 mg protein) in a final vol of 1.15 mL. The assay was initiated by addition of microsomes. Reaction time was 15 min at 37 C and terminated with 0.10 mL concentrated HCl. The labeled reaction product was isolated and analyzed as already described using 3.30 mL 2.5:1 methanol/chloroform, and 1.16-mL vol of water and chloroform.

Analysis of the reaction products of the second reaction revealed an 89% conversion of phosphatidylmonomethylethanolamine to PC. This is greater than has been reported previously for this reaction (12,28); however, the larger quantity of emulsifier may make more substrate available for completion of the reaction sequence. Radioactivity was solely incorporated into PC, the last methylation reaction. In some experiments, the incubation time of the reactions was adjusted so that no more than 14% of either substrate was used in the experiment.

Treatment of Data

Data are expressed as sp act (pmol or nmol PC formed/min/mg/microsomal protein). A correction for the number of methyl groups required to produce PC has been made by division of the sp act by 3, 2, or 1 for the first, second, or third reactions, respectively. Data were analyzed by the statistical methods of Zar (29), or by the method of Florini and Vestling (30) for analysis of multisubstrate kinetics.

RESULTS AND DISCUSSION

Detergent Effects on Enzyme(s)

The reaction mixture used by Bremer and Greenberg (15,16) to assay PE methyltransferase contained additional compounds, used as cofactors, emulsifiers or modifiers of enzymatic activity. Addition of various concentrations of glutathione, $MgCl_2$ or KCl did not significantly influence the enzymatic activity (data not shown). Addition of the emulsifiers Tween 20, Triton X-100 or sodium cholate showed no significant stimulation of sp act of the first methylation and were inhibitory at higher concentrations (Fig. 1). Supplementation of the nitrogenous base and precursor of PE was without effect on the rate of PC synthesis. Sodium deoxycholate stimulated enzyme activity at low concentrations (Fig. 1) but exhibited considerable inhibition at 2.5-3.0 mg/mL, the concentration which provides maximal activities of the second and third methylation reactions. At 6-7 μM sodium deoxycholate

(2.5-3.0 mg/mL), a 10-fold and a 4-fold increase in the enzymatic activity over control was found for the second and third methylation reactions. Addition of PC or PE, up to 4 mg/mL, did not significantly affect the activities of the second or third reactions.

Product inhibition was anticipated when egg PC was added to the reaction mixture; however, a 25% stimulation in activity of the first methylation reaction was observed. The emulsifying action of PC on the membrane of the endoplasmic reticulum may well explain the action. Combination of the two emulsifiers, sodium deoxycholate and PC, resulted in a 50-55% increase in activity of the first methylation reaction. Addition of the substrate, PE (synthetic, dipalmitoyl; isolated from egg yolk or rat liver), did not substantially increase the sp act (Fig. 1) and was not additive with sodium deoxycholate (data not shown).

pH Optima of Enzyme(s)

Previous studies by Bremer and Greenberg had demonstrated a pH optimum of the first methylation reaction to be 10.4 (15). To substantiate this finding and to determine the pH optima of the second and third methylations was the objective of this experiment. Under the experimental conditions used here, the pH curves for the three reactions were broad with apparent optima at pH 9.8, 9.3 and 9.5 for the first, second and third methylations, respectively (Fig. 2). One factor which could contribute to the pH optimum of the first reaction high above the physiological norm may be a high pKa for the ethanolamine of 9.50 (31) to 10.5 (32). The pKa values for phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine are both lower, 9.27 and 9.34, respectively (31). Presumably, the primary amine group of PE is the most difficult to methylate, which would explain its rate-limiting action. Second, the solubilizing effect of a high pH on the enzyme environment of microsomal phospholipids may also contribute to the high pH optima. The pH of 8.6, chosen for routine enzyme assay, compromises the low activity at physiological pH with the degradative effects on S-adenosylmethionine at the higher pH.

Effect of Temperature on Enzyme(s) Activity

Upon heating the microsomal suspension for determination of residual enzyme activity in routine assays, it was observed that 10-15% of the activity of the first reaction of the PE methyltransferase remained. The enzymatic activity that remains following heat denaturation at various temperatures and at pH 7.2, 8.6 and 10.0 is presented in Figure 3. Denaturation was virtually complete at 60 C for the second and third methylations, whereas 15-30% of the activity remains for the first methylation reaction. This could also be due to renaturation

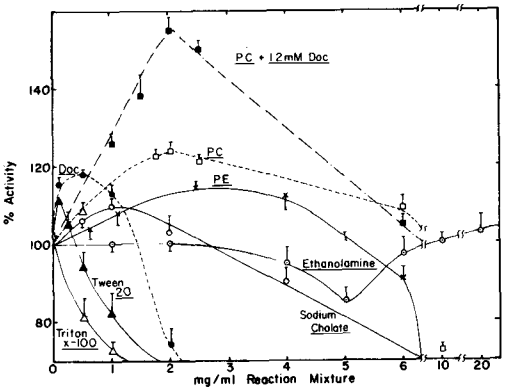

FIG. 1. Effect of detergents on the first methylation reaction of rat liver microsomal phosphatidylethanolamine methyltransferase. Reaction conditions were as outlined in Materials and Methods except for addition of indicated detergents. A sp act of 68 pmol phosphatidylcholine formed/min/mg protein with no exogenous lipid substrate or emulsifiers was set at 100%. Each point is the mean of duplicate determinations from 3 experiments. Bars indicate standard deviations at each point. The compounds added were Triton X-100 (△—△), Tween 20 (▲—▲); ethanolamine (⊙—⊙); sodium cholate (○—○); phosphatidylethanolamine (PE) (X—X); phosphatidylcholine (PC) (□—□); sodium deoxycholate (Doc) (●—●); and 1.2 mM sodium deoxycholate with varied PC (■-·-·-■).

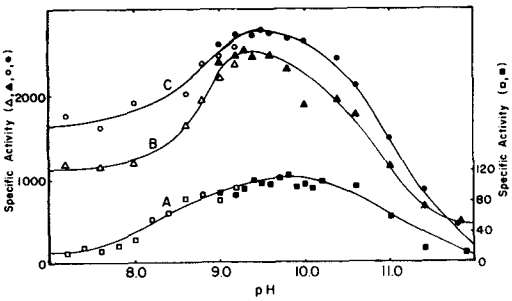

FIG. 2. pH profile of first, second, and third methylation reactions of rat liver microsomal phosphatidylethanolamine methyltransferase. Reaction conditions are the same as given in Materials and Methods except for the use of glycine/KOH buffer at high pH range (■, ▲, ●). Tris/HCl buffer was used between pH 7.2 and 9.2 (□, △, ○). All points are means of triplicate determinations. Substrates used for each assay were (A) endogenous phosphatidylethanolamine (□—□, ■—■); (B) phosphatidylmonomethylethanolamine (△—△, ▲—▲) and (C) phosphatidyldimethylethanolamine (○—○, ●—●).

of the enzyme at the incubation temperature of 37 C. These results may indicate that two enzymes exist for the complete methylation of PE to PC or may reflect the different environments throughout the matrix of the membrane. It has been suggested that the enzyme or enzymes are situated in the membrane such that the first reaction occurs on the cytoplasmic side, the second methylation is internal and the last reaction takes place on the cisternal side of the endoplasmic reticulum where PC is deposited (2,33).

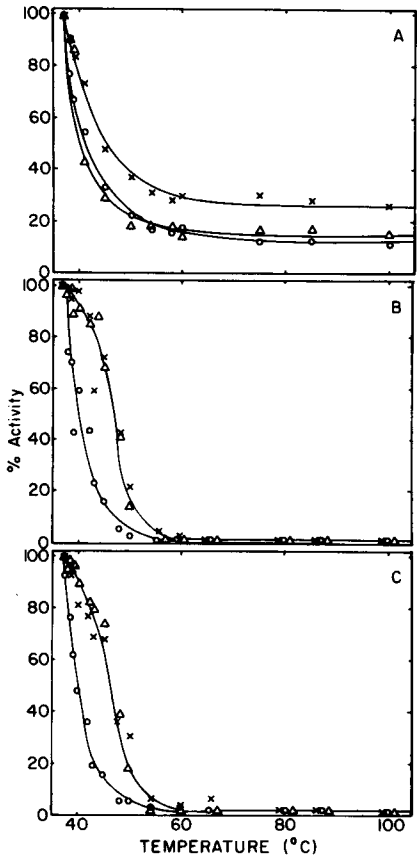

FIG. 3. Effect of heat denaturation on the reaction of phosphatidylethanolamine methyltransferase(s). Rat liver microsomal suspensions were incubated for 1 hr prior to use for enzyme assay. Parallel incubations, in duplicate, to which HCl was added before the microsomal suspension, were run to determine residual radioactivity in the chloroform layer. Assay conditions were as given in Materials and Methods. One hundred percent activity of the first methylation (A) corresponds to 20 pmol/min/mg at pH 7.2 (X—X), 98 pmol/min/mg at 8.6 (o—o) and 112 pmol/min/mg at pH 10.0 (Δ—Δ). One hundred percent activity of the second methylation (B) corresponds to 1110 pmol/min/mg at pH 7.2 (X—X), 1490 pmol/min/mg at pH 8.6 (o—o), and 1820 pmol/min/mg at pH 10.0 (Δ—Δ). One hundred percent activity of the last methylation (C) corresponds to 1080 pmol/min/mg at pH 7.2 (X—X), 1740 pmol/min/mg at pH 8.6 (o—o), and 2110 pmol/min/mg microsomal protein at pH 10.0 (Δ—Δ).

Kinetics of the Liver Microsomal Enzyme

The kinetic parameters of the three methylation reactions were investigated as a possible means of differentiating between the presence of one or two enzymes in rat liver. Utilization of endogenous phospholipid substrate for assay of the first methylation precludes determination of the kinetic properties for this substrate. Saturating levels of PE are present in the microsomes (17,34). The affinity of the rat liver enzyme (Km) for AdoMet has been determined to be 18.2 μM and the maximal velocity was 114 pmol PC formed/min/mg protein as determined by double-reciprocal plots (see Fig. 4 and Table 1). Four- to 8-min incubations with varied concentrations of one substrate at fixed concentrations of the other substrate were used to determine the kinetic properties of the second and third methylation reactions. Replots of the maximal velocities according to Florini and Vestling (30) yielded true Km and maximal velocity values for each of the multisubstrate reactions. The Michaelis constant for S-adenosylmethionine in the second methylation was 295 M (Fig. 5A) and 733 M for phosphatidylmonomethylethanol-

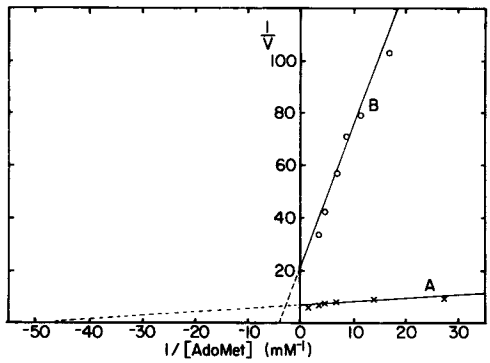

FIG. 4. Typical double reciprocal plot of initial velocity pattern with endogenous phosphatidylethanolamine as substrate. Assay conditions are the same as detailed in Materials and Methods except for the varied S-adenosylmethionine concentrations. Assay of the first methylation reaction is shown for (A) rat liver or (B) guinea pig liver microsomes. The initial reaction velocity (v) is expressed as nmol phosphatidylcholine formed/min/mg protein. Each point is the mean of duplicate determinations.

TABLE 1

Summation of the Kinetic Paramters for Microsomal Phosphatidylethanolamine Methyltransferases from Rat and Guinea Pig Livers

Reaction	Km		Maximal velocity	
	S-adenosylmethionine	Lipid	S-adenosylmethionine	Lipid
	(μM)		(pmol/min/mg protein)	
First				
Rat (N = 10)	18.2 ± 7.1	–	114 ± 35	–
Guinea pig (N = 3)	302 ± 18	–	48 ± 6	–
Second				
Rat*	295	733	3340	3330
Guinea pig*	49.7	516	549	447
Third				
Rat*	87.0	2770	5720	5750
Guinea pig*	1.8	134	95	95

Values (followed by standard deviations) are the means of duplicate determinations from either *N* number of analyses or (*) from experiments where 5 S-adenosylmethionine and 5 lipid concentrations were used (see Figs. 5-6). The results were replotted according to Florini and Vestling (30) to obtain true Km values and maximal velocities. Lipid substrate for the first methylation reaction was endogenous microsomal phosphatidylethanolamine; the second reaction used egg phosphatidylmonomethylethanolamine and the last reaction utilized egg phosphatidyldimethylethanolamine as substrate. Details for each reaction are given in figure legends and in Materials and Methods.

amine (Fig. 5B). The true maximal velocity for the second reaction was 3330-3340 pmol/min/mg microsomal protein. The true Km value of the enzyme(s) for S-adenosylmethionine was 87 μM (Fig. 5C) and 2770 μM phosphatidyldimethylethanolamine (Fig. 5D) for the last methylation reaction. Maximal velocity of the last reaction was 5720-5750 pmol/min/mg. Removal of the enzyme(s) from the lipid environment of the microsomes significantly alters the kinetic properties of the three reactions. The Km values of the enzyme(s) are proportionately greater in the microsomal suspension than in the partially purified states; however, markedly reduced velocities are reported for the partially purified states (12). It was anticipated that, if the last two reactions were catalyzed by one enzyme, they would exhibit similar kinetic properties. The Km values of these two reactions are quite dissimilar; however, the maximal velocities are of the same order of magnitude. Kinetic analysis of the three reactions in rat liver microsomes is insufficient to determine the presence of one, two or three enzymes, nor has partial purification of the enzyme proved definitive (12). The majority of evidence for the existence of two methylating enzymes comes from the work of Axelrod and colleagues (2,13,35). A requirement for magnesium ion was found from the first methylation reaction in adrenal medulla tissue (13). However, no magnesium ion dependency was found for this reaction in rat liver microsomes prepared in the presence of 1 mM EDTA, washed and suspended in 0.25 M sucrose (data not presented).

Various dietary treatments affect the first and last methylation reactions similarly in rat liver microsomes (36). Similarities in the responses of the two reactions may result from their close proximity in the membranes (2) or may be indicative of a single enzyme catalyzing the reactions.

Previous investigations have indicated that the sp act of the first and last methylation reactions of PC biosynthesis in guinea pig liver were considerably less than that in rat liver (15,19). In addition, Miller and Cornatzer (18) have shown that the concentration of PC containing polyunsaturated fatty acids in guinea pig liver microsomes is about one-fourth that in rat liver, further indicating the presence of a less active methylation pathway. A series of kinetic studies on the three methylation reactions in guinea pig liver were carried out as a comparison with the rat liver emzyme(s). Assay of the first methylation yields an apparent Km value for S-adenosylmethionine of 302 μM and a maximal velocity of 48 pmol PC formed/min/mg microsomal protein (Fig. 4). The lesser affinity for AdoMet of the guinea pig enzyme than the rat liver enzyme may sufficiently explain the differences in the methylation pathways of the two species. However, further experiments revealed lower Km values for both substrates of the second and third methylation reactions (Table 1, Fig. 6 A-D) in the guinea pig compared to rat. In rat liver, the rate of the final methylation reaction of phosphatidylmethylethanolamine to PC has been

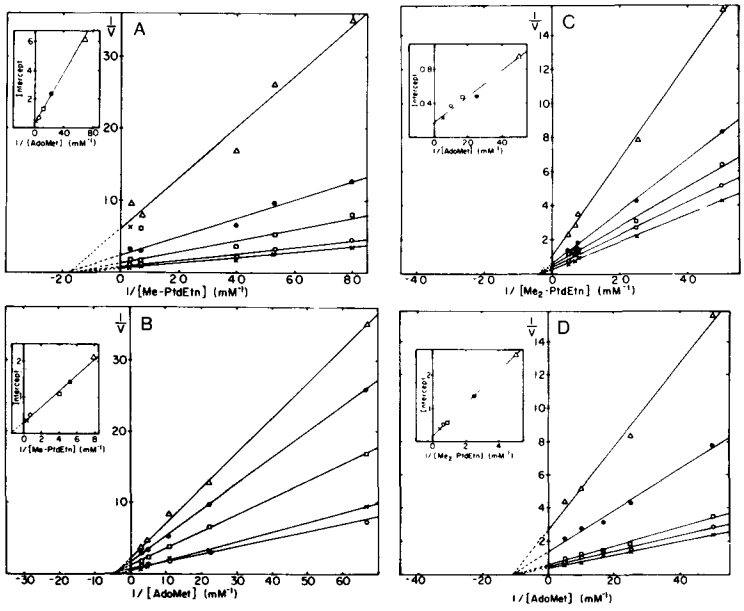

FIG. 5. (A) Double reciprocal plot of initial velocity of phosphatidylcholine (PC) biosynthesis in rat liver with phosphatidylmonomethylethanolamine (MePtdEtn) as lipid substrate. MePtdEtn concentrations were varied at the mixed concentrations of AdoMet of 0.3 mM (X—X), 0.2 mM (○—○), 0.09 mM (□—□), 0.045 mM (●—●), and 0.015 mM (△—△). Assay conditions are as given in Materials and Methods except for the indicated variation in substrate concentrations and incubation times of 4 min. Each point is the mean of duplicate determinations. *Inset:* Double reciprocal plot of maximal velocities (expressed as nmol/min/mg vs fixed concentration of S-adenosylmethionine (AdoMet). (B) Double reciprocal plot of initial velocity of PC biosynthesis in rat liver with MePtdEtn as lipid substrate. The fixed concentrations of MePtdEtn were 3 mM (X—X), 1.43 mM (○—○), 0.25 mM (□—□), 0.199 mM (●—●), and 0.125 mM (△—△). *Inset:* Double reciprocal plot of maximal velocities vs fixed concentrations of MePtdEtn. (C) Double reciprocal plot of initial velocity of PC biosynthesis in rat liver with phosphatidyldimethylethanolamine (Me_2PtdEtn) as lipid substrate. The fixed concentrations of AdoMet were 0.2 mM (X—X), 0.1 mM (○—○), 0.06 mM (□—□), 0.04 mM (●—●), and 0.02 mM (△—△). *Inset:* Double reciprocal plot of maximal velocities vs fixed concentrations of AdoMet. (D) Double reciprocal plot of initial velocity of PC biosynthesis in rat liver with Me_2PtdEtn as lipid substrate. The fixed concentrations of Me_2PtdEtn were 2 mM (X—X), 1.45 mM (○—○), 1.25 mM (□—□), 0.4 mM (●—●) and 0.2 mM (△—△). Double reciprocal plot of maximal velocities vs fixed concentrations of Me_2PtdEtn.

reported to be highly dependent on the degree of unsaturation of the fatty acids in the dimethyl derivative; the more unsaturated moieties enhance the activity of the reaction (37). This property is difficult to ascertain for the first methylation since it does not readily utilize exogenous substrate. However, if the highly unsaturated fatty acid species of PE is the preferred substrate, then the level of these compounds in rat and guinea pig liver may significantly influence the kinetics of the methylation reactions. The level of the dienoic classes of fatty acids in PE in guinea pig liver is considerably greater than that in rat liver; however, the quantities of the polyunsaturated classes of fatty acids in PE in the guinea pig is lower (18,38,39).

The importance of the methylation pathway in overall PC biosynthesis in rat liver has been estimated to range from 15 to 40% (4-8). In the guinea pig and human livers, ca. 10 to 15% of the total PC is synthesized by the methylation pathway and ca. 90% is synthesized by the Kennedy route (8,18). The sp act of choline phosphotransferase (19), PE methyltransferase (19, Table 1) and choline oxidase (40) in guinea pig liver are significantly reduced when compared with rat liver. These observations are in accordance with a regulatory mechanism for cellular choline, such that when the PC biosynthetic routes (choline phosphotransferase and PE methyltransferase) are modified, a

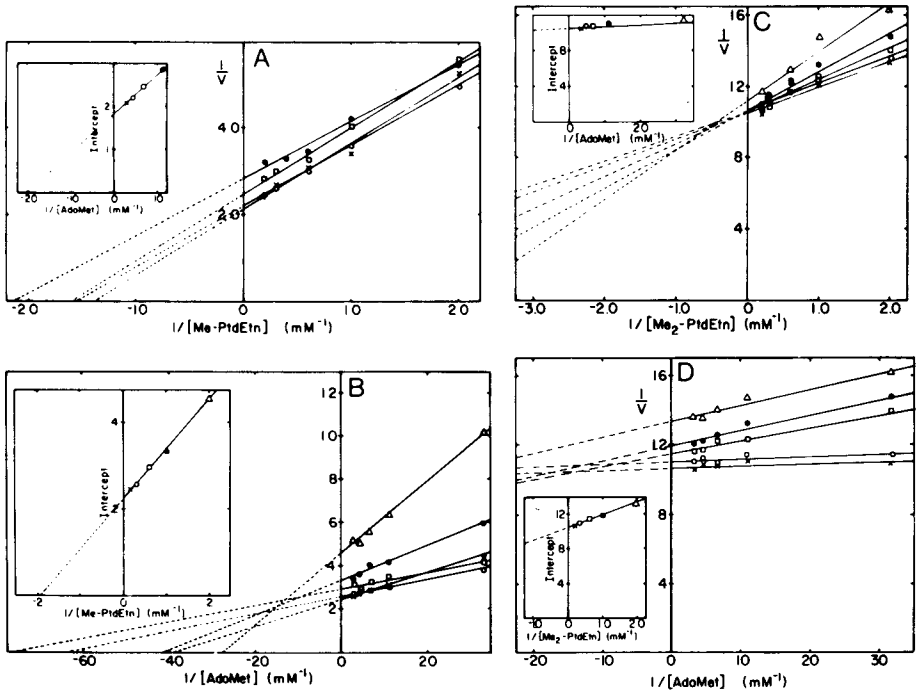

FIG. 6. (A) Double reciprocal plot of initial velocity of phosphatidylcholine (PC) biosynthesis in guinea pig liver with phosphatidylmonomethylethanolamine (MePtdEtn) as lipid substrate. The MePtdEtn concentrations were varied at the fixed concentrations of S-adenosylmethionine (AdoMet) of 0.3 mM (X—X), 0.225 (○—○), 0.15 mM (□—□), 0.09 mM (●—●), and 0.03 mM (△—△). Assay conditions are as given in Materials and Methods except for the variation in substrate concentrations and incubation times of 8 min. Each point is the mean of duplicate determinations. *Inset:* Double reciprocal plot of maximal velocities (expressed as nmol/min/mg) vs fixed concentrations of AdoMet. (B) Double reciprocal plot of initial velocity of PC biosynthesis in quinea pig liver with MePtdEtn as lipid substrate. The fixed concentrations of MePtdEtn were 5 mM (X—X), 3.33 mM (○—○), 1.67 mM (□—□), 1 mM (●—●), and 0.5 mM (△—△). *Inset:* Double reciprocal plot of maximal velocities vs fixed concentrations of MePtdEtn. (C) Double reciprocal plot of initial velocity of PC biosynthesis in guinea pig liver with phosphatidyldimethyldethanolamine (Me_2PtdEtn) as lipid substrate. The fixed concentrations of AdoMet were 0.3 mM (X—X), 0.225 mM (○—○), 0.15 mM (□—□), 0.09 mM (●—●), and 0.03 mM (△—△). *Inset:* Double reciprocal plot of maximal velocities vs the fixed concentrations of AdoMet. (D) Double reciprocal plot of initial velocity of PC biosynthesis in guinea pig liver with Me_2PtdEtn as lipid substrate. The fixed concentrations of Me_2PtdEtn were 5 mM (X—X), 3.33 mM (○—○), 1.67 mM (□—□), 1 mM (●—●), and 0.5 mM (△—△). *Inset:* Double reciprocal plot of maximal velocities vs fixed concentrations of Me_2PtdEtn.

similar change in the choline degradative pathway (choline oxidase) also occurs (19,40). Since the Kennedy pathway is dependent on dietary choline, PA methyltransferase thus serves as the sole source of de novo choline in the cell. Furthermore, Wise and Elwyn (8) report that the methylation pathway is capable of providing 13 μmol choline/day/g liver or the equivalent of the normal dietary intake of choline in the rat.

The methylating enzymes of rat and guinea pig liver do possess different kinetic characteristics; however, the first methylation reaction in both species is rate-limiting. Purification of the enzyme(s) to homogeneity would be desirable but removal from its native lipid environment may result in markedly different kinetic properties.

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METHODS

Analysis of the Phospholipid of the Nuclear Envelope and Endoplasmic Reticulum of Liver Cells by High Pressure Liquid Chromatography

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ABSTRACT

A method is described for the separation and analysis of phospholipids from rat-liver nuclear envelope and endoplasmic reticulum. The procedure employs a liquid environment, to which antioxidants can be added, and results in separation of NL, PE, PI, PS, and PC in 99% purity in 12 min; analytical columns and a radial compression system may be employed. The procedure results in phospholipids with a large proportion of highly unsaturated fatty acids; some differences in fatty acid distributions were found when nuclear envelope phospholipid fractions were compared with the corresponding fractions from endoplasmic reticulum.

There is increasing evidence to suggest that the lipid microenvironment influences the function of membranes, including their enzymatic components; specific demonstrations of lipid modulation of enzymatic activities include mitochondrial (1) and erythrocyte membranes (2), and the ER of liver (3,4). In the liver cell ER, modulation of lipids (phospholipids) has been shown to alter the level of mixed-function oxidases (4). Also of interest is the interplay between intracellular membranous systems; analysis of such interplay requires not only separation of the subcellular systems but also a detailed examination of them. We addressed the problem of separating the phospholipids of the ER and those of the NE reproducibly, simply, and in high yield; further, we wished to undertake these procedures in a liquid environment and to use antioxidants to reduce endogenous peroxidation (5). To do so, we modified a system designed to separate phospholipids using HPLC (6). Using this modified technique, we demonstrate subtle differences in fatty acid composition of NE and ER, with a notable preservation of highly unsaturated fatty acids.

Abbreviations: NE, nuclear envelope; ER, endoplasmic reticulum; PL, phospholipid; NL, neutral lipid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Membrane Isolation

Male rats were obtained from Charles River Breeding Laboratories (Wilmington, MA), maintained on a 12/12 hr light/dark regimen, fed Purina Lab Chow (Ralston Purina, Richmond, IN), and were used with body weights of 250 g. The animals were sacrificed under ether narcosis.

Rat livers were perfused in situ with 0.9% saline and were homogenized in 3.4 vol of 100 mM KPO_4 buffer (pH 7.4) with or without 1% butylated hydroxytoluene. The resulting brei was centrifuged at 12,000 G for 10 min at 4 C in an SS34 rotor and Sorvall centrifuge; microsomes were harvested from the supernatant fluid by centrifugation at 105,000 G for 1 hr at 4 C in a Beckman ultracentrifuge. The pellets were resuspended in 20 vol of 50 mM Tris-HCl buffer (pH 7.4) containing 175 mM KCl and were recentrifuged as before.

Rat liver nuclei were isolated by the method of Blobel and Potter (7). NE was purified by a method derived from that described by Monneron (8). Isolated nuclei were homogenized in 5 mL of 50% sucrose-TM buffer (50 mM Tris-HCl, pH 7.6; 500 mM $MgCl_2$). This viscous suspension was overlayered with a 10-mL cushion of 40% sucrose-TM buffer and a linear

40-20% sucrose-TM gradient, and centrifugation and harvesting were as described. We obtained similar results with the technique of Harris and Milne (9); Monneron's procedure is the most suitable for enzymatic analyses whereas that of Harris and Milne results in higher yields.

Extraction of Lipid

The final sediment (microsomes or NE) was removed from the plastic tubes with stainless steel hexane-washed spatulas and transferred to thick-walled glass centrifuge tubes. The pellets were extracted in 20 vol of distilled-in-glass hexane/isopropranol (Burdick and Jackson Laboratories, Muskegon, MI) (3:2). The mixture was sonicated with a Branson Sonifier at a voltage setting of 50 W and a 2 mm horn for 30 sec at 5 sec bursts. The resulting brei was centrifuged at 770 G for 10 min at 4 C (10). The organic phase was removed and washed with one-half vol of saline. (the aqueous layer was reextracted with 20 vol of hexane/isopropanol (7:3); however, we subsequently found this step to be unnecessary, as had been previously suggested [10]). Aliquots of the final extract were taken for quantitation of phospholipid phosphorus (11) and purification of the phospholipid components.

Chromatographic Separation of Phospholipids

A portion of the extract, generally containing 2-4 mg of phospholipid was reduced in volume by evaporation under N_2 to 100 μL; ca. 50 μL of this concentrated fraction was injected into a Hewlett-Packard 1084A high pressure liquid chromatograph (Hewlett-Packard, Palo Alto, CA) through a Rheodyne injection port (Rheodyne, Berkeley, CA). We used either a Hewlett-Packard Si100 10-μm column or a Waters radial compression module RCM-100 (Waters Associates, Milford, MA) equipped with a silica cartridge under 135 kg/cm^2 pressure. The stainless steel columns could be used without any conditioning. The Waters cartridge was initially washed with 15 mL pure hexane, followed by the elution solvent A (hexane/propanol/water, 6:8:0.75) (total volume was 30 mL).

Phospholipid elution was accomplished with a continuous gradient from 100% solvent A (hexane/propanol/water, 6:8:0.75) at zero time to 100% solvent B (hexane/propanol/water, 6:8:1.5) at 5 min for the Hewlett-Packard columns. The elution was modified for the Waters system; 90% solvent B was reached at 4.5 min and 100% solvent B was attained at 9 min. The flow rate was 1.5 mL/min. Elution was monitored with a Varian spectrophotometer (Varian, Los Altos, CA) at 206 nm. Samples were generally collected manually.

Identification of specific lipids was made by comparison to the elution times and by TLC comparison to known standards purchased commercially (or donated by the Cancer Research Institute, UCSF by Dr. S. Shohet). Standards were purchased from Alltech Associates (Arlington Heights, IL) and included PE, PI, PS, PC, neutral lipid, dipalmitoylglyceride, and glycolipid (cerebroside). Each individual standard was also used as a reference in TLC analysis. Radioactively labeled phospholipids (PC, PE, PS) were purchased from Amersham Searle (Arlington Heights, IL), New England Nuclear (Boston, MA), or ICN (Cleveland, OH).

Thin layer separations were carried out using heat-activated silica plates (Brinkmann Instruments, Westbury, NY) which were heated to 120 C for 1 hr prior to use. Lipids were placed on the plates in chloroform or hexane, either as spots or streaks. The volume used for spotting was chosen to yield 60-120 μg lipid phosphorus. Separation was achieved by ascending chromatography with chloroform/methanol/acetic acid/water (40:25:3:7, v/v) or diisobutyl ketone/acetic acid/water (40:25:5, v/v) at room temperature. The plates were dried in a fume hood.

Spots were detected as described (5), using: a 5% ethanolic solution of H_2SO_4 with heating to 180 C for 30 min; a 0.01% Rhodamine G6 solution in water, with visualization under ultraviolet light; I_2 vapor exposure of the plate with subsequent viewing under visible and ultraviolet light; a 2',7'-dichlorofluorescein solution in 0.2% alcohol, applied as a spray, and the plate examined in ultraviolet light; a ninhydrin spray for detection of free amino groups; PAS stain for glycolipids; and a 0.5% α-naphthol solution in water/methanol for glycolipids.

Following column separation, the phospholipid fractions were dried to a small volume under N_2; fatty acid methyl esters were obtained by reacting the phospholipid residue with methanolic base reagent (Supelco, Bellefonte, PA) at 80 C for 15 min. The result esters were then analyzed on a Hewlett-Packard 5830A gas chromatograph using a cyanosilicone column (SP-2330 on 100-120 Chromosorb WAW from Supelco). Fatty acids were identified by comparison to known standards and their elution times.

Column efficiency was restored by passing 20 mL of a regeneration solvent (obtained from Alltech Associates) through the column, followed by 30 mL of methylene chloride. The column was then purged with 3 runs of the

complete elution solvent gradient or until the absorption at 206 nm became constant, indicating no contamination of the eluting solvents with the regenerating solvents.

RESULTS

The yield of phospholipid from ER was 0.606-0.620 mg PL/mg protein, and was 0.253-0.326 for NE, as determined from phosphorus quantitation and using a conversion factor of 25 (11). These values are comparable to those that have been reported (8,9,12).

Addition of radioactively labeled PC to microsomal or NE suspensions before extraction revealed a 97% recovery by the technique employed. When radioactive tracers (PE, PS, PC) were applied to the column, recovery of radioactivity in the effluent varied between 92 and 95% in all cases. Additional experiments were done in which recovery of lipid phosphorus was assayed; 91.7 ± 1.7% of the lipid phosphorus injected into the apparatus appeared in phospholipid fractions of the column eluate. For this reason, we were confident that losses during chromatography were minimal and that no differential loss of phospholipid occurred.

Samples of extracted lipid (1-3 mg phospholipid) were separated using the HPLC technique described. The order of elution of lipids in both systems was: 1) NL and glycolipids (as a single peak), 2) PE, 3) PI, 4) PS, and 5) PC (Fig. 1 shows a typical elution profile). Sphingomyelin either occurred as a tail on the PC peak or as a small peak at 14-16 min (not shown), and phosphatidic acid eluted at ca. 3 min with the Si100 column. The elution pattern for the radial compression chamber was very similar to that obtained with the Si100 columns; an additional small peak of unknown material eluted at 8.7 min, and phosphatidic acid eluted at 2.1 min. The radial compression module was very responsive to changes in the elution program and was capable of handling 2-3 times the amount of added PL with only minor program changes (the larger quantities subjected to analysis may account for the smaller peaks becoming visible). The radial column can be reversed and the structure has the added advantage that particulate material not separated by precolumns and filters can be washed from the structure.

Samples of ER and NE were obtained as described, and the phospholipids were fractioned. The proportions of phospholipid classes obtained from the total lipid extracts were reproducible in both systems (Table 1) and agreed with previously published data, both in proportion and in mg PL extracted/mg protein (12,13).

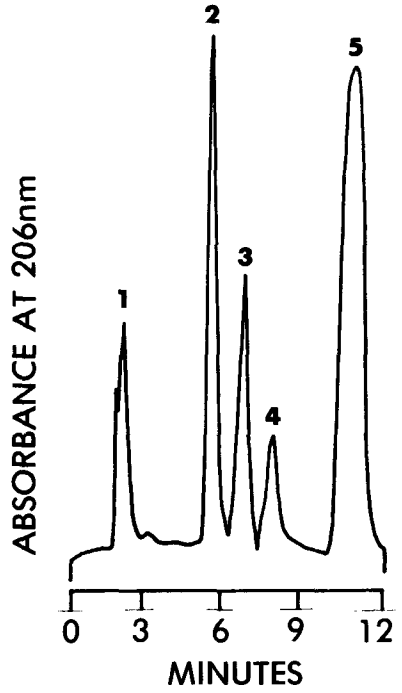

FIG. 1. Elution of phospholipids from a total lipid extract of microsomes. A Hewlett-Packard Si100 column with 10-μm particle size was employed. Approximately 2 mg phospholipid was injected onto the column in a 50-100 μL aliquot of solvent A (hexane/propanol/water, 6:8:0.75). A linear gradient from 100% solvent A at zero time to 100% solvent B (hexane/propanol/water, 6:8:1.5) at 5 min was employed. Flow rate was constant at 1.5 mL/min. The fractions (from left to right) are 1) neutral lipid and glycolipid, 2) phosphatidylethanolamine, 3) phosphatidylinositol, 4) phosphatidylserine, and 5) phosphatidylcholine.

Analysis of the eluted peaks by TLC showed the phospholipid fractions to be pure (>99%) with the exception of PC. This phospholipid may be contaminated by as much as 5% of its total lipid phosphorus content by sphingomyelin. The first peak (void volume) was shown to contain both neutral lipids (both by comparison to standards and its reactivity to I_2 vapor, Rhodamine G6, and charring) and glycolipids (by comparison to standards, and staining reaction with PAS and α-naphthol). No staining with PAS or α-naphthol was detected in PL spots. When baseline separation was not achieved due to decreased column efficiencies, the largest cross-contamination was 5% of PI in the PS fraction (with the Si100 column) and a 5-8% contamination of PE in the PI fraction

(with the radial compression module). Regeneration restored lost efficiency in both systems equally well and the columns remain functional in our hands through a large number of regenerations. Since ca. 20 runs could be obtained between regenerations, the life expectancy of the columns is extremely long (one of our columns has been in service for 4 years).

The separated phospholipids were then analyzed for fatty acid composition. Following transesterification, gas chromatographic analysis (Table 2) revealed an unusually high yield of polyunsaturated fatty acids (arachidonic and docosahexenoic, especially the latter) in PL fractions from ER and NE. Subtle differences were found when fatty acids from nuclear envelope PL fractions were compared with those of the corresponding ER fractions, especially in the PS fraction (Table 2).

Experiments in which the lipid extract was evaporated to dryness often showed only 50% as much docosahexenoic and 25% less arachidonic acid, consistent with peroxidation or destruction (data not shown). Determination of diene conjugation revealed that higher A_{232}/mg lipid phosphorus ratios were obtained with dried preparations as compared with samples which were maintained in a small volume of solvent. This underscores the relative protection from peroxidation afforded by our method.

DISCUSSION

The rapidity, reproducibility, and use of a liquid environment, coupled with a high pressure chromatographic separation of the several phospholipid species, permits analytical comparison of relative concentrations of the phospholipids in the membranes we have studied. More importantly, the technique described yields phospholipids with low levels of diene conjugation and high proportions of arachidonic and docosahexenoic acids, as demonstrated by transesterification and subsequent gas chromatographic analysis (Table 2). This

TABLE 1

Phospholipid Composition of Nuclear Envelope and Endoplasmic Reticulum

Sample	Phospholipid composition of lipid extracts (% lipid phosphorus)			
	PE	PI	PS	PC
NE	25.3 ± 4.2	10.8 ± 3.1	3.6 ± 0.6	60.5 ± 6.7
ER	22.3 ± 2.5	11.2 ± 1.5	3.7 ± 0.7	62.5 ± 4.4

Total lipid extracts in hexane/isopropanol/water (6:8:0.75) containing 2-4 mg PL (or less) were separated using HPLC. Each fraction was collected and phosphorus quantitated. Values represent the means ± standard deviations from 4 or more experiments.

TABLE 2

Fatty Acid Composition of Nuclear Envelope and Endoplasmic Reticulum Phospholipid Fractions

Sample		Fatty acid composition (% ot total)					
		16:0	18:0	18:1	18:2	20:4	22:6
NL	ER	22.5 ± 2.7	5.0 ± 0.7	22.8 ± 1.8	26.7 ± 3.2	8.6 ± 1.8	4.1 ± 0.6
	NE	19.4	9.8	21.8	29.9	7.1	5.3
PE	ER	15.9 ± 1.0	28.2 ± 1.2	5.8 ± 1.6	7.2 ± 0.9	30.5 ± 1.3	11.2 ± 0.8
	NE	18.9 ± 3.0	28.3 ± 1.8	4.1 ± 1.3	8.0 ± 1.2	26.0 ± 3.1	13.9 ± 0.8
PI	ER	2.4 ± 0.7	45.2 ± 2.3	1.9 ± 0.7	1.2 ± 0.8	38.2 ± 3.5	2.4 ± 0.9
	NE	7.7 ± 2.2	45.2 ± 4.8	1.0 ± 1.1	2.3 ± 0.6	39.7 ± 5.0	3.9 ± 1.5
PS	ER	5.6 ± 1.5	39.7 ± 3.8	5.1 ± 0.5	4.0 ± 0.4	29.7 ± 1.8	13.9 ± 1.0
	NE	10.6 ± 8.4	35.3 ± 4.7	7.0 ± 3.3	10.6 ± 3.4	23.2 ± 1.4	13.3 ± 1.7
PC	ER	17.7 ± 1.5	25.6 ± 1.1	6.0 ± 0.6	11.7 ± 0.8	29.3 ± 1.6	6.8 ± 1.0
	NE	20.8 ± 2.0	25.6 ± 1.1	7.1 ± 1.5	13.8 ± 3.1	25.2 ± 4.5	7.0 ± 3.0

Phospholipid fractions from nuclear envelope and endoplasmic reticulum were isolated, fatty acids were transesterified, and the methyl esters were analyzed using gas chromatography (as described). Values represent means ± standard deviations from many experiments (NL values for NE represent means from 2 preparations).

indicates greater preservation of sensitive unsaturations during the separation.

In comparing NE and ER from rat liver, some differences were found in the compositions of individual phospholipid fractions. It therefore appears that this technique is suitable for investigating the interrelationships between the many subcellular membrane systems. There is compelling evidence to indicate the participation of NE and ER fractions (especially ER) in the processes of cell injury and carcinogenesis, with peroxidation of fatty acids suggested as a mechanism of injury (14). For this reason, attention to techniques for reduction of further endogenous peroxidation was critical.

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Occurrence of Mixtures of Geometrical Isomers of Conjugated Octadecatrienoic Acids in Some Seed Oils: Analysis by Open-Tubular Gas Liquid Chromatography and High Performance Liquid Chromatography

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ABSTRACT

Analytical methods to obtain the detailed compositons of the fatty acids in oils containing more than one conjugated octadecatrienoic acid by open-tubular gas liquid chromatography (GLC) and by reversed-phase high performance liquid chromatography (HPLC) were established. Effective GLC separations of *cis,trans,trans*-9,11,13-octadecatrienoic acid (*ctt*-9,11,13-18:3), *ctc*-9,11,13-18:3, *ttc*-9,11,13-18:3, *ttt*-9,11,13-18:3, *ttc*-8,10,12-18:3, and *ttt*-8,10,12-18:3 were obtained with an open-tubular column coated with the nonpolar liquid phase OV-1 using an instrument having all-glass carrier gas pathways. The HPLC method also gave satisfactory separations for the isomeric conjugated octadecatrienoates on the basis of number of the *cis* and *trans* double bonds. Two or three minor conjugated trienoic acids were found along with the principal conjugated trienoic acid in tung oil, and seed oils of cherry, *Prunus sp.*, *Momordica charantia*, *Trichosanthes anguina*, *Punica granatum*, *Catalpa ovata*, and *Calendula officinalis*. The mechanism for the formation of the conjugated trienoic acid mixtures in the seed oils is discussed. The *C. ovata* seed oil also contained *ct* and *tt*-9,12-octadecadienoic acids. The *tt* isomer is presumed to be a precursor of *ttc*-9,11,13-18:3, the main conjugated trienoic acid in this oil.

INTRODUCTION

Various geometrical isomers of conjugated octadecatrienoic acids are known: α-eleostearic acid (*cis,trans,trans*-9,11,13-octadecatrienoic acid (*ctt*-9,11,13-18:3)), punicic (*ctc*-9,11,13-18:3), catalpic (*ttc*-9,11,13-18:3), calendic (*ttc*-8,10,12-18:3), and jacaric (*ctc*-8,10,12-18:3) acids have been reported as single conjugated trienoic components in some seed oils (1-3). Recently, a ^{13}C NMR method for determining the composition of seed oils which contain more than one conjugated trienoic acid was presented by Tulloch and Bergter (4), and it was shown that the fatty acids of *Fevillea triobata* contained 30% punicic acid and 9% α-eleostearic acid (4). This seems to be the only paper which reports the occurrence of a geometrical isomer mixture of conjugated trienoic acids in the fatty acids of a natural seed oil, although it has been shown that in tung oil, β-eleostearic acid is an alteration product of and coexists with α-eleostearic acid (5).

In this study, the geometrical isomers of conjugated octadecatrienoic acids have been successfully separated by reversed-phse high performance liquid chromatography (HPLC) and open-tubular gas liquid chromatography (GLC), and practical methods for determining the compositions of fatty oils which contain more than one conjugated trienoic acid have been established. These methods were applied to the fatty acid analysis of some seed oils containing conjugated octadecatrienoic acids, and minor geometrical isomers of conjugated trienes have been satisfactorily determined.

MATERIALS AND METHODS

Materials

Air-dried seeds of *Trichosanthes anguina* (snake gourd), *Calendula officinalis* (pot marigold), *Momordica charantia* (litchi, Japanese name Futo-reishi) were the products of a nursery company (Sakata Shubyō Co., Ltd., Yokohama). Intact air-dried pods of *Catalpa ovata* (Japanese name Ki-sasage) were obtained at a pharmacy for Chinese drugs. Fresh cherry kernels were obtained from cherries *Prunus* sp. labeled as Hood River Cherries, Weyerhaeuser Co., Yakima, WA USA, and *Punica granatum* seeds were obtained from the fruits labeled as California pomegranate, USA. Tung oil was a bottled commercial product. The seeds were ground to a powder with an electric mill, and extracted with hexane 3 times at room temperature. Each extraction consisted of standing with occasional shaking for about 3 hr. After drying with anhydrous sodium sulfate, hexane was evaporated below 30 C in a rotary evaporator. The residual oil was directly converted to methyl esters by addition of 0.5 M sodium methoxide/methanol reagent. The mixture was

allowed to stand overnight at room temperature and the methyl esters were recovered by hexane extraction. All these procedures were done in the dark under nitrogen.

Preparation of α-eleostearate (94% purity) was carried out with an urea adduct method from the mixture of monoenoates and conjugated octadecatrienoates separated by $AgNO_3$-silica gel column chromatography of tung oil methyl esters. Eleostearate was extracted from the nonurea-adduct fraction after acidification with dilute hydrochloric acid (6). Geometrical isomerization of tung oil and *C. officinalis* oil methyl esters was effected by addition of a trace amount of iodine to a CS_2 solution of the methyl esters (7), and keeping for 10 hr at room temperature. Palmitic acid (99% purity) was a product of P.L. Biochemicals, Inc., Milwaukee, WI.

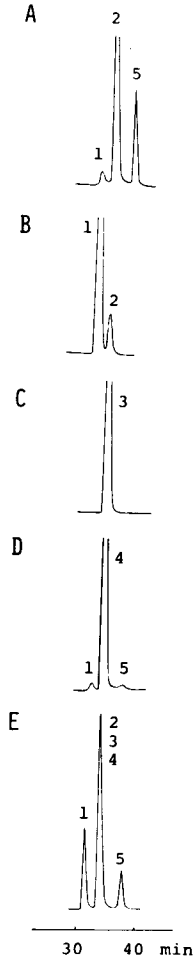

FIG. 1. HPLC resolution of conjugated octadecatrienoates on a Zorbax ODS column. (1) *ctc*-9,11,13-18:3; (2) *ctt*-9,11,13-18:3; (3) *ttc*-8,10,12-18:3; (4) *ttc*-9,11,13-18:3; (5) *ttt*-9,11,13-18:3. (A) Tung oil; (B) *T. anguina*; (C) *C. officinalis*; (D) *C. ovata*; (E) A mixture of A, B, C, D and *trans* isomerized A. HPLC conditions are given in Materials and Methods.

HPLC Analysis

A mixture of the conjugated octadecatrienoates was separated according to the configuration of the conjugated double bonds by a HPLC instrument, Shimadzu-Du Pont LC-1 (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan), including a PCP-1 Model constant pressure liquid pump, a vacuum degassing-nitrogen sealing attachment, a GRE-1 solvent programmer for gradient elution, and a SPD-1 spectrophotometric detector for detection in wavelength from 190 to 750 nm. HPLC analysis was done on a 25 cm × 4.6 mm Zorbax ODS column connected to a Permaphase ODS precolumn (5 cm × 2 mm) by elution with acetonitrile/H_2O (4:1, v/v) at a flow rate of 1.4 mL/min and a pressure 50 kg/cm^2. The column temperature was 42 C. Usually 0.5 μL of 1% (w/v) sample solution of methyl esters in hexane was injected at sensitivity 0.32 AUFS.

GLC Analysis

Open-tubular GLC of the methyl esters was done with a Shimadzu GC-6AM instrument equipped with a dual FID detector on a wall-coated open-tubular (WCOT) glass column, 54 m × 0.28 mm, coated with OV-1 (Nippon Kuromato Kōgyō Co. Ltd., Tokyo). The carrier gas was N_2 at a flow rate of 0.4 mL/min and split ratio 1/155. The column temperature was 190 C, and injector and detector were 200 C. All carrier gas pathways including the splitter consisted of glass tubes, and the vaporized sample was kept completely out of contact with any metal surface. Usually, 1 μL of 1% (w/v) sample solution in hexane was injected.

RESULTS AND DISCUSSION

HPLC Separation

In HPLC, under the conditions of this study, the conjugated octadecatrienoate peaks emerged in the order (*cis,trans,cis*), (*cis,trans, trans*, and *trans,trans,cis*), and (*trans,trans, trans*). Each peak was completely separated from the others. Three positional isomers, catalpic (*ttc*-9,11,13), calendic (*ttc*-8,10,12) and α-eleostearic (*ctt*-9,11,13) acids could not be separated to any degree (Fig. 1). The methyl esters obtained from the seed oils showed, as expected, a symmetrical large peak for the main conjugated trienoate component, and most of them additionally showed small peaks for

minor components, thought to be geometrical isomers of the conjugated trienoates. The recorder chart area ratios of the peaks were dependent on the wavelength of the detector, since each conjugated trienoate has absorption maxima of different intensity and wavelengths: α-eleostearic acid nm ($E^{1\%}_{1\ cm}$) 272 (1766) in cyclohexane, β-eleostearic acid 268 (2190) in ethanol, and punicic acid 275 (1694) in cyclohexane (3). In this study, the detector was operated at 284 nm for quantitative anlayses. Since the absorption of α- and β-eleostearic acids and punicic acid shows almost the same intensities at 284 nm (8), the area percent of each peak is essentially equal to mole or weight percent. The deviations of the peak area percents relative to the wavelength selection for the detector are shown in Table 1. The ratio of α- and β-eleostearic acids in tung oil has been calculated indirectly from the absorption of UV spectra at the 3 wavelengths using an equation for the calibration (9). In the method, small instrumental deviations have a large influence on the accuracy of the results, since the interval of α- and β-eleostearate peak maxima is only about 4 nm, and most parts of the 2 peaks overlap. The deviation can be corrected with pure standard reference samples uncontaminated with other isomers. However, preparation of such specimens is very difficult. The HPLC method presented in this paper enables the separation of more than one geometrical isomer of the conjugated trienoates and it gives more accurate results directly without any pure reference specimen. Determination of conjugated trienoates is possible with an HPLC instrument having only a simple UV detector for an absorption at a fixed wavelength (e.g., 254 nm) using calibration factors.

Recrystallization of the esters or the acids has frequently been used for the refining and preparation of conjugated trienoates (4), but the efficiency of this procedure is poor, judging by the results of HPLC and GLC analyses of the recrystallized products in our experiments. This study showed that the HPLC method gave better results in the preparation and purification of the conjugated trienoates on a small scale. The separated sample showed only one peak in HPLC, and the results of the open-tubular GLC also gave the evidence for the effective separation.

GLC Separation

GLC analysis of the methyl esters of conjugated trienoic acids has been generally recognized as giving unsatisfactory results because of decomposition and isomerization (4,10,11). Thus, there have been no reports of identification of particular conjugated trienoate isomers by GLC, though the total contents of the conjugated octadecatrienoate isomers obtained by GLC were reported to be in fair agreement with the contents obtained by UV analysis (12), and conjugated dienoates were determined by GLC without isomerization (13).

In this study, open-tubular GLC effectively separated the geometrical isomers of conjugated octadecatrienoates, and an almost pure single peak was observed for the conjugated trienoates in the open-tubular GLC of C and D in Figure 2 and for cherry methyl esters. The compositions of the conjugated trienoates determined by GLC were in accord with those obtained from the HPLC analysis (Table 2). These results show that isomerization and decomposition of the conjugated trienoates were not caused by GLC under the conditions of this experiment. The conjugated trienoate peaks were sharp and their theoretical plate numbers were nearly equal to those of the ordinary fatty acid methyl esters. This supports the view that no decomposition

TABLE 1

Comparison of Composition of Conjugated Octadecatrienoic Acid Isomers Obtained by GLC and HPLC (Peak Area %)[a]

Isomer[b]	GLC	HPLC (detector, wavelength nm)					
		254	273	283	284	285	290
ctc	32.14 ± 0.28[c]	25.11	27.92	31.22 ± 0.65	31.31 ± 0.33	31.48 ± 0.70	43.64
ctt + *ttc*[d]	38.49 ± 0.36	38.49	39.36	37.93 ± 0.22	38.89 ± 0.26	39.96 ± 0.21	41.73
ttt	28.58 ± 0.15	36.40	32.71	30.86 ± 0.54	29.80 ± 0.11	28.56 ± 0.64	16.54

[a]A mixture of methyl esters of *T. anguina* seed oil, tung oil and *trans* isomerized tung oil was used. GLC and HPLC conditions are given in Materials and Methods.

[b]*C* and *t* denote *cis* and *trans* double bonds in order in the 9-, 11- and 13-positions in octadecatrienoic acids.

[c]Mean ± SD of 5 analyses.

[d]Source of *ttc* isomer is tung oil, see Table 3.

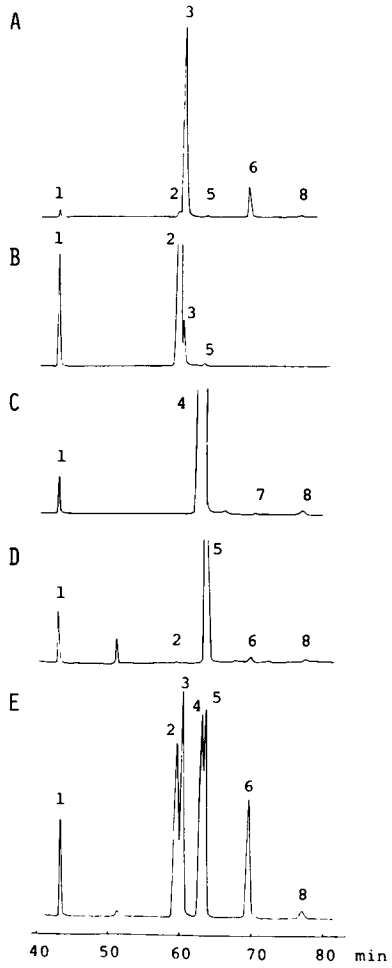

FIG. 2. GLC resolution of conjugated octadecatrienoates on a WCOT OV-1 column. (1) 16:0; (2) *ctc*-9,11,13-18:3; (3) *ctt*-9,11,13-18:3; (4) *ttc*-8,10,12-18:3; (5) *ttc*-9,11,13-18:3; (6) *ttt*-9,11,13-18:3; (7) *ttt*-8,10,12-18:3; (8) 20:1. (A) Tung oil; (B) *T. anguina*; (C) *C. officinalis*; (D) *C. ovata*; (E) A mixture of A, B, C, D and *trans* isomerized A. GLC conditions are given in Materials and Methods.

and no isomerization occur on the conjugated trienoates in the GLC. GLC of the mixtures of methyl palmitate and α-eleostearate in known weight ratios gave a weight response factor of 1.06 for the eleostearate relative to palmitate. The respective response factors for oleate, linoleate, linolenate and docosahexaenoate were 0.99, 0.99, 1.01 and 1.07 under the same conditions.

To establish the degree of loss of the conjugated octadecatrienoates in the esterification procedure, the methyl esters of tung oil were treated with the reagents CH_3ONa-CH_3OH, BF_3-CH_3OH, and HCl-CH_3OH under various conditions, and the products were analyzed by GLC before and after the treatment. The CH_3ONa-CH_3OH reagent gave reproducible results without the loss of the conjugated octadecatrienoates as shown in Table 3. To avoid the reported loss of conjugated trienoates (14), the preparation of the methyl esters for this study was carried out with 0.5 M CH_3ONa-CH_3OH for 10-12 hr at room temperature under nitrogen for the analysis of the seed oils containing the conjugated trienoates.

The peaks of punicate and α-eleostearate were completely coincident in GLC on Silar 5CP and 10C open-tubular columns (67 and 56 m × 0.28 mm, respectively), but they were effectively separated on the OV-1 open-tubular column (54 m × 0.28 mm) with a peak separation 80% in the analysis of 1:1 (w/w) mixture. The open-tubular GLC on OV-1 generally showed the much better separation than HPLC on Zorbax ODS, except for the separation between punicate and α-eleostearate which gave 100% peak separation in the HPLC.

In the GLC on OV-1, the peaks appeared in the following order, as shown in Figure 2 and Table 4, *ctc*-9,11,13; *ctt*-9,11,13; *ttc*-8,10,12; *ttc*-9,11,13; *ttt*-9,11,13; *ttt*-8,10,12. Under the conditions of this analysis, the two peaks having nearly the same height and a difference of 0.01 in the ECL were separated to the extent

TABLE 2

Comparison of Compositions of Conjugated Octadecatrienoic Acids in Some Seed Oils by GLC and HPLC (Peak Area %)[a]

	Tung oil		*Prunus* sp.		*M. charantia*		*T. anguina*		*P. granatum*		*C. ovata*		*C. officinalis*	
Isomer[b]	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC
ctc	1.7	2.3	–	–	0.9	1.2	92.7	94.1	96.1	96.5	0.1	2.5	–	–
ctt + *ttc*	84.3	83.8	99.8	99.8	98.6	98.6	7.3	5.9	3.9	3.5	98.6	96.3	99.6	99.8
ttt	14.0	13.9	0.2	0.2	0.5	0.2	–	–	–	–	1.3	1.2	0.4	0.2

[a]GLC and HPLC conditions are given in Materials and Methods.

[b]*C* and *t* denote *cis* and *trans* double bonds in order in the 9-, 11- and 13- (8-, 10- and 12- for *C. officinalis*) positions in octadecatrienoic acids.

of about 30-40% (e.g., the *ttc*-9,11,13 and *ttc*-8,10,12-18:3 peaks).

Composition of Conjugated Trienoates

The fatty acid compositions of the seed oils reported in this paper have been reported in a previous paper (12), but the data were obtained by packed column GLC. The occurrence of *ctt*-9,11,13-18:3 in the fatty acids of cherry kernel oil has been reported with UV spectrophotometric and GLC data (15,16). In this study, the fatty acid composition of some seed oils was obtained in more detail using open-tubular GLC (Table 4). The percentages of main components are generally in harmony with those described in the previous paper (12).

It is notable that *ttc*-9,11,13-18:3 was found in the fatty acids from tung oil, *T. angina* oil, and *P. granatum* oil, and *ctc*-9,11,13-18:3 was found in the fatty acids from tung oil, *M. charantia* oil, and *C. ovata* oil, all as minor components.

A considerable amount of *ttt*-9,11,13-18:3 was found in the fatty acids of tung oil, *M. charantia* oil, and *C. ovata* oil, but it was only a trace in the methyl esters from fresh cherry kernels (Table 4). The presence of the *ttt* isomer in these oils has been recognized due to isomerization with light and other factors after extraction. The isomerization of the conjugated trienoates in the seeds may be possible, since various amounts of the *trans* isomers of the main conjugated trienoates have been found in the seed oils as minor components, though the extraction of the seeds was done carefully in the dark under nitrogen. It may be safely said that *ttt*-9,11,13-18:3 and *ttt*-8,10,12-18:3 are both natural fatty acids.

It is plausible that the biosynthetic pathway

TABLE 3

Influence of the Esterification Procedures on the Compositions of Conjugated Trienoates[a]

Isomer[b]	Control	CH_3ONa-CH_3OH 0.05 M	CH_3ONa-CH_3OH 0.5 M
ctc	1.4	1.4	1.4
ctt	67.8	68.4	67.4
ttt	11.3	11.1	11.2
Usual acids[c]	19.5	19.1	20.0

[a]Peak area % by GLC (conditions are given in Materials and Methods). Tung oil methyl esters (control) were treated with the reagents for 10 hr at 20-28 C.

[b]*C* and *t* denote *cis* and *trans* double bonds in order in the 9-, 11- and 13-positions in octadecatrienoic acids.

[c]Sum of usual fatty acid components.

TABLE 4

Fatty Acid Compositions of Some Seed Oils Containing Conjugated Octadecatrienoic Acids by GLC (Peak Area %)

Acid	ECL[a]	Tung oil	*Prunus* sp.	*M. charantia*	*T. anguina*	*P. granatum*	*C. ovata*	*C. officinalis*
14:0		tr	0.05	tr	tr	0.04	tr	0.05
15:0		–	0.03	tr	tr	tr	tr	tr
16:1	15.79	–	0.49	tr	tr	0.06	0.04	tr
16:0		2.61	7.56	1.46	5.31	2.25	2.77	2.40
17:1	16.71	–	0.09	–	–	–	tr	–
17:0		0.06	0.09	0.09	0.09	–	tr	tr
18:2ω6	17.62	7.07	38.01	8.60	17.22	4.23	39.95[b]	27.92
18:3ω3	17.66	0.11	tr	tr	tr	tr	0.56	0.70
18:1ω9	17.72	6.57	38.56	14.64	16.40	3.98	7.71	3.95
ω7	17.76	0.36	0.71	0.11	0.50	0.35	0.97	0.47
18:0		2.13	2.48	17.40	8.15	1.77	2.65	1.22
18:3 *ctc*-9,11,13[c]	18.95	1.33	–	0.50	48.48	82.99	tr	–
ctt-9,11,13	18.99	67.69	10.63	56.24	3.43	3.16	–	–
ttc-8,10,12	19.09	–	–	–	–	–	–	62.17
ttc-9,11,13	19.10	0.17	–	–	0.41	0.20	42.25	–
ttt-9,11,13	19.36	11.27	tr	0.32	–	–	0.55	–
ttt-8,10,12	19.39	–	–	–	–	–	–	0.24
20:1	19.63	0.54	0.32	0.34	tr	0.54	0.42	0.33
20:0		tr	0.99	0.29	–	0.42	0.23	0.24
Others		0.15	–	–	–	–	1.89	0.77

[a]ECL = equivalent chain length. GLC conditions are given in Materials and Methods.

[b]Includes 9,12-18:2 isomers: *cc* 70.8%, *ct* 3.2%, and *tt* 26.0% (ECL *cc* 19.43, *ct* 19.20, *tc* 19.28, and *tt* 19.02 on Silar 10C open-tubular column (56 m × 0.28 mm) at 170 C.

[c]18:3 *ctc*-9,11,13 = *cis,trans,cis*-9,11,13-octadecatrienoic acid.

of the conjugated trienoates could include a mechanism for elimination of the hydroperoxide group from linoleate hydroperoxide formed by peroxidase, rather than the dehydration of conjugated hydroxy dienoates presented previously by Gunstone and coworkers (17,18). Thus, *ctt*- and *ctc*-9,11,13-18:3 found in the seeds might be formed from linoleate through specific oxidation at position 13 (which is frequently observed in enzymatic oxidation [19]).

Occurrence of *ttc*-9,11,13-18:3 in *C. ovata* indicated in Table 4 is similarly explainable by the peroxidation of *tt*-9,12-18:2 at position 13. In practice, the presence of *tt*-9,12-18:2 in *C. ovata* seeds was established by coinjection with reference material prepared by *trans* isomerization of linoleate with nitrous acid (20). The ECL values of the *trans* isomers are in harmony with those reported previously (21). The results of the GLC analysis are shown in Table 4 (footnote b gives the retention data). The *tt*-9,12-18:2 acid has been also found in seed oil of *Chilopsis linearis*, which contains *ttc*-9,11,13-18:3 (22). Enzymatically specific conversion of the *tt*-9,12-18:2 component of the 9,12-18:2 isomers to *ttc*-9,11,13-18:3 will occur in both of these seeds. Formation of *ttc*-8,10,12-18:3 in *C. officinalis* will occur through 9-oxidized-*tc*-10,12-18:2 formed from linoleate by the similar mechanism. In conclusion, the pathway to the conjugated trienoates shown in Table 4 could include the following sequence. The underlined compounds are main components.

T. anguina and *P. granatum*
cc-9,12-18:2 → 13-oxidized-*ct*-9,11-18:2 →
ctc-9,11,13-18:3 and *ctt*-9,11,13-18:3
ctc-9,11,13-18:3 → *ttc*-9,11,13-18:3

M. charantia
cc-9,12-18:2 → 13-oxidized-*ct*-9,11-18:2 →
ctt-9,11,13-18:3 and *ctc*-9,11,13-18:3
ctt-9,11,13-18:3 → *ttt*-9,11,13-18:3

C. ovata
tt-9,12-18:2 → 13-oxidized-*tt*-9,11-18:2 →
ttc-9,11,13-18:3 and *ttt*-9,11,13-18:3

C. officinalis
cc-9,12-18:2 → 9-oxidized-*tc*-10,12-18:2 →
ttc-8,10,12-18:3
ttc-8,10,12-18:3 → *ttt*-8,10,12-18:3

Further study on the component fatty acids in the natural lipids containing conjugated fatty acids by GLC and HPLC methods proposed in this paper can be expected to give the detailed information which will be useful in understanding the mechansim for the biosynthesis of conjugated fatty acids in plants.

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COMMUNICATIONS

Different Thermal Behavior of Neonatal Hepatic and Cerebral 3-Hydroxy-3-methylglutaryl-CoA Reductase

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ABSTRACT

The Arrhenius plots of hepatic and cerebral 3-hydroxy-3-methylglutaryl-CoA reductase activity were studied in neonatal chicks fed with a standard diet. Supplementation of the diet with 2% cholesterol from hatching has no effect on the thermal characteristics of the brain enzyme. The Arrhenius plot of brain reductase was practically similar to that found in control chicks. However, hepatic reductase was inhibited by cholesterol feeding. Dietary cholesterol increased the cholesterol/lipidic phosphorus molar ratio in liver microsomes, whereas no significant differences were observed in brain microsomes. These results are in agreement with the hypothesis that activity of hepatic reductase is regulated by the fluidity of microsomal membrane and show that cholesterol feeding does not alter the fluidity of microsomal membranes in neonatal chick brain having, thus, no effect on the thermal behavior of cerebral reductase.

INTRODUCTION

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (mevalonate:NADP oxidoreductase (CoA-acylating), EC 1.1.1.34) is the major regulatory site in cholesterol synthesis. The hepatic enzyme was found to be depressed by a high cholesterol diet (1). Cholesterol feeding lowers HMG-CoA reductase activity by two mechanisms, an immediate inactivation of preformed enzyme and a longer term reduction of enzyme synthesis (2,3). Since the reductase is membrane-bound, recently it was proposed that another mechanism responsible for the regulation of its activity would be an alteration in the fluidity of the membrane, induced by dietary cholesterol (4). In recent years, interest has grown in understanding the influence of lipids on membrane enzymes. Many laboratories have shown that Arrhenius plots of membrane-bound enzymes show discontinuities with a higher Arrhenius activation energy below the transition temperature.

In this paper, we have studied for the first time the thermal behavior of neonatal HMG-CoA reductase activity in liver and brain from chicks fed standard diets or diets supplemented with cholesterol, as well as the changes in cholesterol/lipidic phosphorus molar ratio in microsomes in response to cholesterol feeding.

MATERIAL AND METHODS

White Leghorn male chicks (14-day-old) were used. Newborn animals were obtained from a commercial hatchery and fed ad libitum on a commercial diet or a diet supplemented with 2% cholesterol in a chamber with a light cycle from 0700 to 1900 hr and controlled temperature. The chicks were killed by decapitation at the same hour (9 a.m.) The tissues were rapidly removed, weighed, minced and then homogenized with a motor-driven all glass Potter-Elvehjem homogenizer in 3 vol of 50 mM phosphate buffer, pH 7.4, containing 30 mM EDTA, 250 mM NaCl and 1 mM DTT. The broken cell preparation was centrifuged at 5,000 G for 15 min. The resulting supernatant fraction was centrifuged at 15,000 G for 15 min to sediment the mitochondrial fraction. Microsomes were obtained by centrifuging the 15,000 G supernatant fraction for 60 min at 105,000 G. The pellet which contained the microsomes was resuspended in 1 mL of the same buffer. All operations were done at 4 C. Protein concentration was determined by the method of Lowry et al. (5), using albumin as a standard.

HMG-CoA reductase activity was measured essentially as described by Shapiro et al. (6). This method measured the formation of radioactive mevalonate from [^{14}C] HMG-CoA, using [^{3}H] mevalonate as an internal standard. Reductase activity was expressed as pmol of mevalonic acid synthesized/min/mg protein. Lipids were extracted from microsomes by the method of Santiago et al. (7). Cholesterol was

determined according to Carr and Drekter (8) and Martensson (9). Lipidic phosphorus was determined by the method of Bartlett (10).

RESULTS AND DISCUSSION

Temperature-dependent changes in hepatic and cerebral HMG-CoA reductase activity were studied in 14-day-old chicks fed a standard diet ad libitum. The Arrhenius plots of the reductase activity of such preparations (Fig. 1) displayed transition temperatures of 26.1 C for the hepatic enzyme and 22.7 C for the cerebral enzyme. The correlation coefficients for all slopes were 0.99. Discontinuities in the Arrhenius plots had been described to be due to phase changes in the lipid systems (11). Thus, differences in the transition temperatures for the reductase from chick liver and brain could indicate a different microsome lipid composition. The activation energies below and above the transition temperatures are summarized in Table 1. The values obtained for chick liver and brain enzymes were lower than those described for hepatic reductase from rat (12). Nevertheless, it is important to note that below the break temperature, the activation energies in hepatic and cerebral chick reductase were different (11.83 and 14.62 kcal.mol^{-1}, respectively), whereas above this point, both activation energies were similar (8.45 and 8.08 kcal.mol^{-1}, respectively), thus suggesting a lower environmental dependence of the enzyme when the transition temperature has been exceeded.

It has been known for some time that the cholesterol content of many membranes can be changed experimentally, and that when this is done there are accompanying changes in both in membrane fluidity (13) and in various enzyme and transport systems (14). This effect is probably associated with a general reduction of fluidity within the bilayer matrix caused by incorporation of cholesterol. With this in mind, we have studied the effect of cholesterol feeding on the thermal behavior of hepatic and

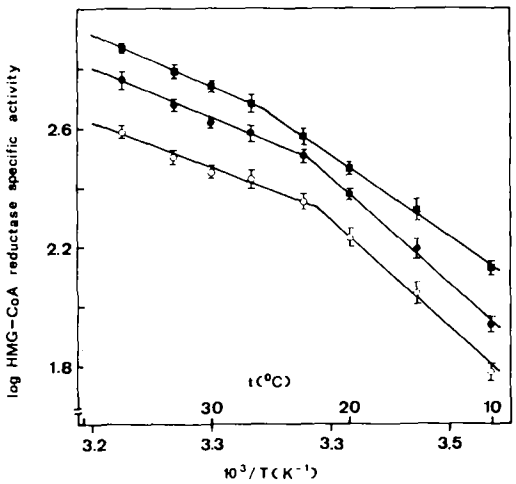

FIG. 1. Arrhenius plots of HMG-CoA reductase activity. (■), hepatic microsomes from neonatal chicks fed with standard diet. (●), cerebral microsomes from neonatal chicks fed with standard diet. (○), cerebral microsomes from neonatal chicks fed with diet supplemented with 2% cholesterol. Each point represents mean ± SEM of three experiments with pools of 5 chicks.

cerebral HMG-CoA reductase from neonatal chicks, as well as its possible influence on the cholesterol/lipidic phosphorus molar ratio in both liver and brain microsomes. Cholesterol feeding has no effect on the thermal characteristics of brain reductase. The Arrhenius plot of this activity gave a transition temperature of 22.2 C (Fig. 1), similar to that found for the cerebral enzyme in control chicks. The activation energies below and above this transition temperature were also similar to those observed in control brains (Table 1).

Dietary cholesterol produced a clear inhibition of hepatic HMG-CoA reductase. At 37 C, a sp act of 18.3 ± 1.5 pmol/mg/min was observed in cholesterol-fed chicks, while a sp act of 739.4 ± 26.4 pmol/mg/min was found in

TABLE 1

Activation Energies and Transition Temperatures of HMG-CoA Reductase from Neonatal Chick Liver and Brain

Tissue	Treatment	E_{A1}^a (Kcal.mol^{-1})	E_{A2}^a (Kcal.mol^{-1})	Transition temperature (C)
Liver	None	11.83	8.45	26.1
Brain	None	14.62	8.08	22.7
Brain	+ 2% cholesterol	14.62	7.46	22.2

[a]E_{A1} and E_{A2} are the activation energies at lower and higher temperatures respectively, than transition temperature.

TABLE 2

Effect of Cholesterol Feeding on Cholesterol/Lipidic Phosphorus Molar Ratio in Chick Liver and Brain Microsomes

	Cholesterol/lipidic phosphorus molar ratio[a]	
	Control	Cholesterol-fed
Brain	0.314	0.310
Liver	0.265	0.730

[a]Results are given as means of 2 determinations with pools of 5 chicks.

normal chicks. At lower temperatures, the levels of HMG-CoA reductase were less than the minimum detectable activity. Thus, it was not possible to obtain the Arrhenius plot in these conditions. However, Mitropoulos et al. (12,15) found two breaks, one at 28 C and the other at 19 C, in Arrhenius plots of hepatic microsomal HMG-CoA reductase from rats fed standard diets or diets supplemented with cholesterylamine, but only a questionable discontinuity at 19 C in material from animals fed cholesterol.

On the other hand, the cholesterol/lipidic phosphorus molar ratio has been studied as an index of membrane fluidity in chick liver and brain microsomes. Table 2 shows that cholesterol feeding clearly increased this molar ratio in liver microsomes, whereas no significant differences were observed in brain microsomes. These results show that changes in cholesterol/lipidic phosphorus molar ratio in response to dietary cholesterol are analogous to those observed in the thermal behavior of hepatic and cerebral HMG-CoA reductase and corroborate the hypothesis of Sabine and James (4) that "the activity of hepatic HMG-CoA reductase is critically regulated by the fluidity of its supporting microsomal membrane." Likewise, our results seem to suggest that the lack of influence of cholesterol feeding on the cholesterol/lipidic phosphorus molar ratio in brain microsomes could explain the non-effect of this treatment on the thermal behavior of cerebral reductase.

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Molecular Packing and Stability in the Gel Phase of Curved Phosphatidylcholine Vesicles

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ABSTRACT

The instability of small unilamellar phosphatidylcholine vesicles in the gel phase can be understood as the effect of gel phase structural parameters on the intrinsic packing asymmetry of the vesicles.

Small unilamellar vesicles (1) have been used for many years as model systems for biological membranes, and, more recently, as drug carriers in in vivo and in vitro experiments (2), so their properties are of continuing interest. When the vesicles are prepared from saturated fatty acid phosphatidylcholines, the lipids undergo, at a characteristic temperature above 0 C, a phase transition from the liquid crystalline phase, in which they are prepared, to the gel phase. The stability of these vesicles has been a matter of some contention. Gradually, however, it has become clear that the small vesicles undergo a spontaneous size transformation to larger vesicles via fusion or lipid transfer mechanisms at temperatures below the phase transition temperature (T_m) (3-7). Unlike other properties such as permeability (8) or fatty acid induced fusion (9), the relatively slow (half times of hours or days) rate of size transformation does not maximize at T_m, but instead appears, at least for distearoyl phosphatidylcholine vesicles, to increase as the incubation temperature is decreased below T_m (5). In this report, we will attempt to show that vesicle instability below T_m can be understood as the effect of the structural changes that occur upon cooling to the gel state on the geometric constraints imposed by the low radius of curvature.

For planar bilayers, the basic structure of both the gel and liquid crystalline phases has been well characterized by X-ray diffraction methods (10-12). For dimyristoyl and dipalmitoyl phosphatidylcholine, the bilayer hydrocarbon thickness, calculated from the long spacing, increases from 35 Å in the liquid crystalline phase for both lipids to 45 Å for dimyristroyl and 49 Å for dipalmitoyl phosphatidylcholine in the gel phase. The area/molecule decreases from 62 $Å^2$ for dimyristoyl and 70 $Å^2$ for dipalmitoyl phosphatidylcholine in the liquid crystalline phase to 47.5 $Å^2$ for both lipids in the gel phase.

Small unilamellar vesicles have been well characterized in the liquid crystalline phase by hydrodynamic methods (13-16), and asymmetric packing parameters have been determined for dipalmitoyl (15) and egg (16) phosphatidylcholine vesicles. It is difficult to compare in detail parameters derived from hydrodynamics with those from X-ray diffraction, because of the packing asymmetry of small vesicles, and, more fundamentally, because of the difficulties in determining and compartmentalizing the water present in the system. Cornell et al. (17) have approached these problems, and have made the reasonable suggestion that bilayer thicknesses and areas/molecule in the two systems can be compared at the hydrocarbon-polar region interface, if the packing assymmetry is known. Extending this idea to the gel phase, we have assumed that the dipalmitoyl phosphatidylcholine planar bilayer hydrocarbon thickness and area/molecule are reasonable first approximations for dipalmitoyl phosphatidylcholine vesicles in the gel phase, where asymmetric packing parameters have not been determined. This is supported by the results of Watts et al. (14) for dimyristoyl phosphatidylcholine vesicles. Although they did not determine the packing asymmetry, they found gel phase thickness increases and area/molecule decreases roughly comparable to those found for planar bilayers. It should also be noted that rather large changes can be made in the gel phase thicknesses and areas chosen without affecting qualitatively the results of the hypothetical calculations outlined in the next paragraph.

The question is, then, what is the effect of a decrease in area/molecule from 70 $Å^2$ to about 48 $Å^2$ in the gel phase, and an increase in hydrocarbon thickness from 35 Å to around 49 Å on the highly curved small vesicles. When the vesicles are cooled below T_m, the decrease in area/molecule means that both the outside and inside radii must decrease. In addition, the increase in bilayer thickness means that the inside radius must decrease 10-15 Å more than that of the outside. This implies that the inside monolayer area/molecule will be the limiting

factor in the shrinking, thickening process. If, in addition, spherical geometry is assumed for gel phase vesicles, then an outside radius and area/molecule can be calculated from the liquid crystalline radius. For each monolayer, in the absence of any change in the number of molecules upon going into the gel state (18-20), the ratio of the interfacial areas/molecule in the two phases will be

$$\frac{A_{I,l}}{A_{I,g}} = \frac{(R_{0,l} - t_l)^2}{(R_{0,g} - t_g)^2} \quad [1]$$

where $A_{I,l}$ (70 Å^2) and $A_{I,g}$ (47.5 Å^2) are the inside monolayer areas/molecule in the liquid crystalline and gel phase, respectively; t_l (35 Å) and t_g (49 Å) are the liquid crystalline and gel phase bilayer thickness, respectively; and $R_{0,l}$ and $R_{0,g}$ are the outside liquid crystalline and gel phase radii, respectively. For any given liquid crystalline phase radius, then, a gel phase radius can be calculated and used in a similar formula for the outside monolayer to calculate an outside gel phase area/molecule $A_{0,g}$. This quantity, normalized to the gel phase planar bilayer area/molecule, so that the numbers should apply equally well to dimyristoyl or distearoyl phosphatidylcholine, is plotted as a function of the liquid crystalline phase vesicle radius in Figure 1.

Inspection of Figure 1 reveals that for vesicles with radii less than about 200 Å, the calculated area/molecule is closer to the liquid crystalline phase value than the gel phase value. The obvious conclusion is that the assumption of spherical shell geometry is not valid; the simple packing formulas used above T_m no longer apply. It should therefore be emphasized that $A_{0,g}$ is not a predicted area/molecule ratio, but an approximate measure of how far the spherical geometry value is from the real value, and thus, we feel, a measure of the instability of the vesicles. For vesicles with radii larger than about 400-500 Å, the calculated gel phase area/molecule approaches the planar bilayer value. A recent study (21) has indicated that vesicles larger than about 400 Å do not undergo size transformation to any significant extent, which supports our analysis.

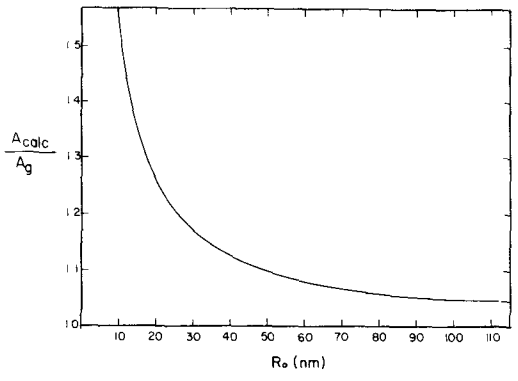

FIG. 1. The ratio of the calculated vesicle outside monolayer gel phase head group area/molecule to the gel phase planar bilayer area/molecule as a function of the liquid crystalline phase outside vesicle radius.

Watts et al. (14) concluded, based on hydrodynamic and freeze fracture electron microscopy data, that the gross morphology of small dimyristoyl phosphatidylcholine vesicles below T_m is spherical, in apparent conflict with our analysis. There is, however, a more subtle explanation for the failure of the spherical packing formulas, which is provided by the structure, based on X-ray diffraction data, proposed by Blaurock and Gamble (22) for dipalmitoyl phosphatidylcholine vesicles below T_m. They interpret their results as indicating that the vesicles are composed of areas of planar packing, and are thus faceted or polygonal. The fatty acid chains within a facet would be aligned in a nonradial fashion. Such an arrangement provides one possible way of partially relieving the packing strains caused by simultaneously decreasing the total area and increasing the thickness of a liquid crystalline phase vesicle while preserving the spherical morphology. Features consistent with a faceted vesicle structure have also been observed by freeze etch electron microscopy (D. Papahadjopoulos, personal communication). It should be noted that the "average" molecular packing in a small vesicle must be different from that of planar bilayers, since the transition temperature is about 4 C less for vesicles (3,4). Further, although the two monolayers of dipalmitoyl phosphatidylcholine vesicles appear to melt independently, they do so at approximately the same temperature (19).

There have been several studies (9,14,18-20) on small, sonicated vesicles which indicate that it is possible to do experiments on small vesicles below T_m without complications due to size transformation, if the experiments are of relatively short duration, and the temperature close to T_m. Our interpretation of this is that the faceted vesicle structure relieves some, but not all, of the curvature-induced gel phase packing strain, so the vesicles transform only slowly to larger, more planar species. In addition, we think that packing arguments can be used to understand qualitatively why the transformation proceeds faster as the temperature is lowered below T_m (5). For planar bilayers, X-ray (10-12), Raman (23-25), infrared (26) and nuclear magnetic resonance

(27,28) studies have shown that the gel phase is not totally ordered. Although the exact nature of the disorder is not clear at this point, all of the studies cited indicate that the amount of disorder gradually decreases as the temperature is decreased below the pretransition temperature. For vesicles in the gel phase, Gaber and Peticolas (24) have shown using Raman spectroscopy that both the amount of disorder and the amount of change with temperature are larger for small vesicles than for planar bilayers. If the lipid molecules are considered rigid rods (or conical sections) relative to the more fluid molecules above T_m and the degree of rigidity increases as the temperature is decreased, then the distortions necessary to create planar packing areas within a vesicle will become larger with decreasing temperature and the vesicles less stable. In addition, the gel phase partial specific volumes for the two monolayers of a vesicle may have different temperature coefficients (18), due to the different radii of curvature, which might create further instabilities.

In summary, we feel that the faceted vesicles plus the asymmetric packing analysis provide a means of understanding vesicle instability below the phase transition temperature. Furthermore, the presence of areas of planar packing and/or the packing irregularities in the boundaries between the facets will presumably affect other vesicle properties, such as their tendency to aggregate (22), which may be a necessary precursor to size transformation (7). We also note that Hwang et al. (29) have provided evidence that insulin binds only to small vesicles below T_m, not above, nor to large multilammellar vesicles either above or below T_m, which may again be attributed to easier binding to boundaries between facets than to planar bilayers.

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Diplocyclos palmatus L.: A New Seed Source of Punicic Acid

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ABSTRACT

The seeds of *Diplocyclos palmatus* L. (Cucurbitaceae) contained 23% oil and 15% protein. The UV, IR, ^{1}H-NMR and ^{13}C-NMR spectrometry of the oil, and oxidation, reduction and gas liquid chromatography (GLC) of the methyl ester of conjugated fatty acid isolated by preparative thin layer chromatography (TLC) showed the presence of punicic (octadeca-*cis*-9,*trans*-11,*cis*-13-trienoic) acid. The fatty acid composition (wt %), as determined by GLC, is: punicic, 38.2; 18:2, 43.9; 16:0, 8.1; 18:0, 4.9 and 18:1, 4.9.

INTRODUCTION

Over 40 conjugated fatty acids have been identified in seed oils (1). However, punicic acid, octadeca-*cis*-9,*trans*-11,*cis*-13-trienoic acid, is restricted to a few species in two plant families, Punicaceae and Cucurbitaceae. In Cucurbitaceae, this acid occurs in Momordica, Cayaponia, Trichosanthes, Cucurbita, Apodanthera and Fevillea (1-3). This report presents evidence for the presence of punicic acid in *Diplocyclos palmatus* (L.) C. Jaffrey, syns. *Bryonopsis laciniosa* (L.) Naud and *Bryonia laciniosa*, L., another species belonging to the Cucurbitaceae family. The plant is a creeper and yields red globose, subessile fruits (1.3-2.5 cm diam) with vertical stripes. The seeds (5-6 mm long) are yellowish brown and resemble grape seeds.

MATERIALS AND METHODS

The crushed seeds were repeatedly extracted in a conical flask at room temperature (~30 C) with *n*-hexane. Ultraviolet (UV) spectrum of the oil was recorded in cyclohexane in a Unicam SP-700 spectrophotometer. Infrared (IR) spectrum of the oil was taken as a thin film in a Perkin-Elmer 221 unit. ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectrum was obtained in a $CDCl_3$ solution of oil in a Varian A-60A instrument.

Natural abundance ^{13}C-NMR spectrum was obtained on a Jeol FX-90Q spectrometer in the FT mode at 22.5 MHz with proton noise decoupling and 16 K data memory. The number of data points was 8,192, spectral width was 5 KHz and acquisition time was 0.8192 sec. The spectrum (1,000 accumulations, 45° pulses, pulse repetition time, 3 sec) was taken in a $CDCl_3$ solution of oil. Chemical shifts were given in ppm downfield from the internal TMS-^{13}C signal.

Methyl esters were prepared by shaking the oil sample at room temperature with a 1% solution of sodium methoxide in anhydrous methanol. The conjugated trienoate (ca. 95% pure) was isolated from total methyl esters by thin layer chromatography (TLC) on 1-mm silica gel layer containing calcium sulfate using *n*-hexane/diethyl ether (93:7, v/v). Periodate-permanganate oxidation of the conjugated trienoate was done in 60% *tert*-butanol at reflux temperature of the reaction mixture, following the procedure described by Youngs (4). Hydrogenation was done in a Paar unit using Pd/C in cyclohexane at room temperature for 4.5 hr at a hydrogen pressure of 2 atm. Gas liquid chromatography (GLC) was done in a Toshniwal unit equipped with dual column and hydrogen flame ionization detectors. Stainless steel columns, 6′ × 1/8″ packed with 15% EGSS-X on Gas Chrom Q (100/120 mesh) and 4′ × 1/8″ packed with 5% SE-30 on Chromosorb W AW DMCS (80/100 mesh) were used. Isothermal operations were done at 200 C with injection ports and detectors maintained at 240 C. For analysis of the oxidation products, the column temperature was programmed from 110 to 200 C at 6 C/min. The flow rate of carrier gas (nitrogen) was 30 mL/min. Peak areas were measured by multiplying peak height with width at half-height.

RESULTS AND DISCUSSION

The seeds contained 23% oil and 15% protein (5) on a moisture-free basis. The oil in

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TABLE 1

Comparison of the ^{13}C-NMR Chemical Shifts[a] (ppm) of *Diplocyclos palmatus* Oil with Those of *Trichosanthes anguina* Oil and *Fevillea trilobata* Methyl Esters

Carbon	*D. palmatus*	*T. anguina*	*F. trilobata* (ref. 3)
2	34.08	34.03	34.14
3	24.92	24.87	24.99
4	29.20	29.09	29.15
5	29.20	29.09	29.15
6	29.20	29.09	29.10
7	29.74	29.69	29.65
8	27.85	27.79	27.88
9	128.84	128.89	128.92
10	132.42	132.31	132.45
11	127.98	127.81	127.87
12	127.98	127.92	127.99
13	132.63	132.58	132.63
14	128.84	128.79	128.83
15	27.63	27.58	27.63
16	31.97	31.91	31.92
17	22.38	22.32	22.37
18	14.14	14.09	13.97

[a]Shifts are tabulated only with respect to methyl octadeca-*cis*-9,*trans*-11,*cis*-13-trienoate.

cyclohexane gave λ max at 265, 275 and 287 nm, indicating *cis*, *trans*, *cis* conjugation (1). Bands at 975 and 925 cm^{-1} in the IR spectrum indicated conjugated unsaturation involving a *trans* double bond (1). The conjugated acid was separated as methyl ester by preparative TLC on silica gel. The isolated methyl ester had the same retention time on the SE-30 column as did methyl punicate or α-eleostearate. The ester was subjected to periodate-permanganate oxidation. Azelaic acid half-ester and valeric acid were identified in the oxidation products by GLC. The GLC of the hydrogenated ester of the conjugated acid showed that it is a C_{18} acid.

The oil was also analyzed by ^{1}H- and ^{13}C-NMR spectrometry. The ^{1}H-NMR spectrum gave the following signals (τ): 9.13 (terminal CH_3); 8.7 (-$[CH_2]_n$-); 7.7 (-$C\underline{H}_2$-$COOCH_3$); 7.3 (=CH-$C\underline{H}_2$-CH=); 5.8 (CH_2O- of glyceride); 4.75 (CHO- of glyceride) and ca. 3.7 (-CH=CH-CH=CH-CH=CH-) (2). Tulloch and Bergter (3) recently studied the chemical shift parameters of the component conjugated trienoic acids of *Fevillea trilobata* oil in ^{13}C-NMR spectrum. Their assignment of the chemical shifts for the methyl ester of 9, 11, 13-*cis*,*trans*,*cis*-octadecatrienoic acid is compared in Table 1 with the shifts of *Trichosanthes anguina* seed oil, known to contain the same acid (1) as well as those of the *D. palmatus* oil under examination. Oils, as well as their methyl esters, give essentially the same spectra for the fatty acid carbons (3). The data confirm that the component conjugated fatty acid in *D. palmatus* seed oil is octadeca-*cis*-9,*trans*-11,*cis*-13-trienoic acid (punicic). The percentage (wt) composition of fatty acids was determined by GLC using both SE-30 and EGSS-X columns (Kaimal et al., unpublished). On the SE-30 column, conjugated trienoic acids are well separated from other C_{18} acids, do not undergo alterations as on polyester columns (6) and are estimated quantitatively. Since the separation achieved on this column is only according to chain length, analysis on a polar (EGSS-X) column was also necessary for determination of detailed fatty acid composition. The composition of *D. palmatus* determined thus is as follows: punicic, 38.2; 16:0, 8.1; 18:0, 4.9; 18:1, 4.9 and 18:2, 43.9. *D. palmatus* seed is therefore a potential source of a drying oil.

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Microsomal Phosphatidylethanolamine Methyltransferase: Inhibition by S-Adenosylhomocysteine

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ABSTRACT

Inhibition by S-adenosylhomocysteine (AdoHcy) of the three reactions of phosphatidylethanolamine methyltransferase which catalyzes the production of phosphatidylcholine from phosphatidylethanolamine in guinea pig and rat liver microsomes has been evaluated. Five of the six methylation reactions in these two species exhibit greater affinity for inhibitor, AdoHcy, than for substrate, S-adenosylmethionine (AdoMet). The Ki values for the rate-limiting reactions were 3.8 μM and 68 μM in rat and guinea pig livers, respectively. An AdoMet:AdoHcy ratio of 12:1 in developing liver was found to decline to a constant value in the adult of 5:1. The concentration of AdoHcy in rat and guinea pig liver increases markedly following death of the animal. A concomitant decrease in the AdoMet level was observed in guinea pig liver. A comparison of phosphatidylethanolamine methyltransferase activity with the hepatic concentrations of AdoMet and AdoHcy in mouse, rat, rabbit and guinea pig is presented. Regulation of the methylation pathway is discussed.

S-Adenosylmethionine (AdoMet) serves as the methyl donor for phosphatidylcholine (PtdCho) biosynthesis from phosphatidylethanolamine (PtdEtn). This reaction, catalyzed by phosphatidylethanolamine methyltransferase (EC 2.1.1.17), occurs primarily in the endoplasmic reticulum of rat liver and was first described by Bremer and Greenberg and Bremer et al. (1,2). In the three-step methylation of PtdEtn to form PtdCho, 1 mol of PtdEtn and 3 mol of AdoMet are utilized to form 1 mol of PtdCho and 3 mol of the second product, S-adenosylhomocysteine (AdoHcy). In 1961, Gibson et al. (3) presented the first evidence that AdoHcy was inhibitory to rat liver phosphatidylethanolamine methyltransferase. Kaneshiro and Law (4) reported the Km value for AdoMet as 200 μM for this enzyme from *Agrobacterium tumefaciens* and that AdoHcy inhibited the reaction by 50% at 4 μM. A greater affinity for inhibitor, AdoHcy, than for substrate, AdoMet, has been reported for several other methyltransferases (5-11). Consequently, several investigators have proposed that AdoHcy acts as a bioregulatory compound in the metabolism of transmethylation (11-14).

Previously, the concentration of AdoMet in rat liver was reported to be 70-90 nmol/g tissue (15-19) and the AdoHcy concentration as 40-60 nmol/g (15-17). At these concentrations of substrate and inhibitor, the majority of transmethylation reactions would be markedly inhibited. We have reported that the estimated in vivo hepatic concentration of AdoHcy was 5 nmol/g in developing rat liver and increased to 14 nmol/g in the adult (20). Nevertheless, the ratio of AdoMet to AdoHcy in cellular organelles may have some function in the regulation of methylation. It appears that in certain pathological conditions where either AdoMet or AdoHcy concentrations are altered, transmethylation is likewise affected (21,22).

We report here a study of AdoHcy inhibition on the three phosphatidylethanolamine methylation reactions in rat and guinea pig liver, including inhibition constants and the hepatic AdoHcy and AdoMet concentrations in various species.

MATERIALS AND METHODS

Chemicals

S-Adenosyl-L-[methyl-^{14}C] methionine (45.9 mCi/mmol) was purchased from New England Nuclear, Boston, MA, and diluted to 0.1 mCi/mmol with unlabeled AdoMet purchased from Sigma Chemical Co., St. Louis, MO. S-Adenosyl-L-homocysteine and 3-*sn*-phosphatidylcholine were also obtained from Sigma. Egg phosphatidylethanolamine, monomethyl-phosphatidylethanolamine and dimethyl-phosphatidylethanolamine were from Avanti Polar Lipids, Birmingham, AL. Vydac cation exchange resin was obtained from the Separations Group, Hesperia, CA.

Animals and Tissue Preparation

Except where otherwise stated, animals used in the experiments were female albino rats of the Sprague-Dawley strain, 80-100 days old, and female Sprague-Dawley BALB/c mice, 50 days old, from ARS Sprague-Dawley, Madison, WI; female guinea pigs, 15 weeks old, and female rabbits, 11 weeks old, from Gopher State Caviary, Minneapolis, MN. All animals were fed Purina chow ad libitum and were

maintained on an 8:00 p.m. to 8:00 a.m. dark period. Tissue for enzyme assay was obtained and prepared the same as detailed previously (23).

Enzyme Assays

Measurement of the first reaction in the methylation of PtdEtn to PtdCho has been described previously (20,24). The reaction mixture contained 2 mg egg PtdCho, 0.9 mM sodium deoxycholate, 0.3 M Tris/HCl buffer (pH 8.6), 0.2 mM S-adenosyl-L-[methyl-^{14}C]-methionine (0.1 mCi/mmol), microsomes (1-3 mg protein) and the indicated amounts of AdoHcy in a final volume of 1.4 mL. The 15-min incubation at 37 C was initiated by addition of microsomes and terminated with 0.15 mL conc HCl. The reaction product was extracted by the method of Bligh and Dyer (25) and radioactivity determined in a Packard liquid scintillation spectrometer.

The second and third methylation reactions were assayed by previously described methods (23,24). The reactions were identical except for the use of either 1.0 mM monomethylphosphatidylethanolamine or 3.0 mM dimethylphosphatidylethanolamine as substrate. The reaction mixtures contained lipid substrate, 6.3 mM sodium deoxycholate, 0.3 M Tris/HCl buffer (pH 8.6), 0.35 mM S-adenosyo-L-[methyl-^{14}C]methionine (0.1 mCi/mmol), microsomes (1-3 mg protein) and varying amounts of AdoHcy in a total volume of 1.15 mL. The 8-min incubation at 37 C was initiated with microsomes and terminated with 0.1 mL conc HCl. The product was isolated and analyzed as already described.

Extraction of AdoMet and AdoHcy from Liver

The animals were killed by decapitation; segments of tissue were removed between 3 and 80 sec following the start of decapitation and frozen in liquid nitrogen. The remaining liver was prepared as just described and used for enzyme assay. Extraction of AdoMet and AdoHcy from tissue was according to the method of Hoffman et al. (20). The frozen tissue was weighed and homogenized, 20 strokes, in a Teflon plug homogenizer with 0.1 M mercaptoethanol/5% sulfosalicyclic acid (1-2 mL/g). The homogenate was centrifuged at 10,000 G for 10 min and the supernatant fluid filtered through a 1-μm glass fiber filter. The extraction procedure was repeated and the filtrate pooled.

Chromatography

AdoMet and AdoHcy were fractionated by high pressure liquid chromatography on Vydac cation exchange resin (20). The column (7 x 300 mm) was conditioned with 10 mL of 2 M ammonium formate (pH 4.0) and equilibrated with 40 mL of 0.01 M ammonium formate (pH 4.0). An aliquot of extract containing from 1 to 10 nmol of AdoHcy and AdoMet was applied to the column with a larger volume sample loop. Interfering compounds were removed with 0.01 M ammonium formate before a linear gradient to 1.2 M ammonium formate (pH 4.2) was used to elute AdoHcy and AdoMet. Detection of the compounds was by ultraviolet absorbance at 251 nm.

RESULTS

A series of experiments was designed to obtain the AdoHcy inhibition constants exhibited by the six reactions in rat and guinea pig livers (Fig. 1, A-F). In the rat liver microsomes, the Ki (AdoHcy) inhibition constants for the first, second and third methylation of phosphatidylethanolamine to phosphatidylcholine was 3.8 ± 0.7, 127.7 ± 4.9 and 61.8 ± 14.6. The values observed in the microsomes of the guinea pig livers were reversed in magnitude with 68 ± 28 for the first, 46 ± 8 for the second and low value of 2.1 ± 0.7 for the third (Table 1). Marked inhibition of the first reaction of microsomal phosphatidylethanolamine methyltransferase is evident in Figure 2. Regardless of the absolute concentration of AdoMet or AdoHcy, an equivalent amount of inhibition is manifest at a given AdoMet:AdoHcy ratio.

The AdoMet:AdoHcy ratio in rat liver drops markedly from 12:1 in early development to 5:1 at maturity (Fig. 3). The AdoMet concentration did not vary significantly from 69 nmol/g tissue throughout the lifespan of the rat; however, the AdoHcy concentration increased significantly from 5 to 14 nmol/g.

An increase in the concentration of AdoHcy in both rat and guinea pig liver was observed for up to 40 sec following decapitation (Fig. 4). We have previously reported a similar finding for 9-day and 450-day-old rats (20). A concomitant decrease in the hepatic AdoMet concentration occurred following the death of the animal (Fig. 4). The decrease in the concentration of AdoMet was found more consistently in guinea pig than in rat liver. An approximation of the in vivo levels of AdoHcy and AdoMet may be obtained by extrapolation of the curves to zero time; however, the determinations at 3-6 sec were usually sufficient.

The AdoMet and AdoHcy concentrations were determined in the livers of several species in an attempt to correlate them with the activ-

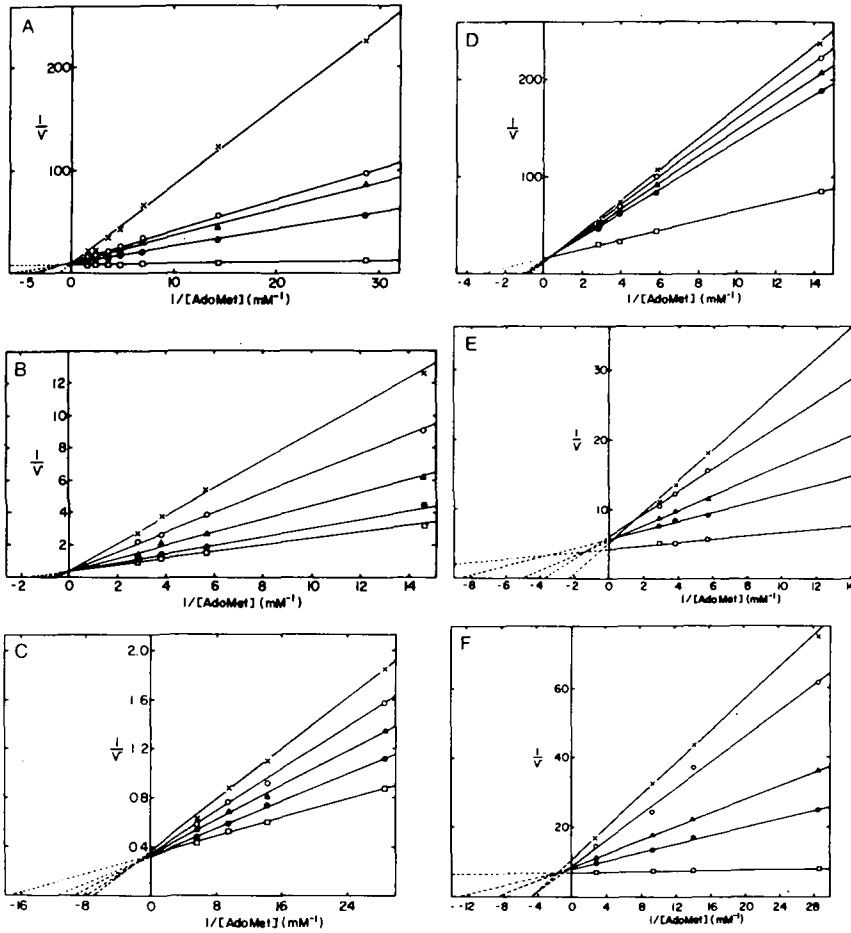

FIG. 1. (A) Inhibition of the first methylation reaction of rat liver phosphatidylethanolamine (PE) methyltransferase by S-adenosylhomocysteine. The initial velocity (expressed as nmol/min/mg) of the synthesis of PtdCho was determined at various concentrations of AdoMet in the presence of AdoHcy at the concentrations of 0.14 mM (X—X), 0.07 mM (○—○), 0.052 mM (▲—▲), 0.035 mM (●—●) or (□—□). Endogenous PdtEtn served as the lipid substrate. Details of the reaction are as described in Materials and Methods. Each point is the mean of dupliate determinations and liens were drawn according to least squares linear regression. (B) Inhibition of the second methylation reaction of rat liver PE methyltransferase by S-adenosylhomocysteine. The initial velocity (expressed as nmol/min/mg) of the synthesis of PtdCho was determined at various concentrations of AdoMet in the presence of AdoHcy at the concentrations of 0.036 mM (X—X), 0.24 mM (○—○), 0.12 mM ▲—▲), 0.048 mM (●—●) or 0 (□—□). 1.0 mM egg monomethylphosphatidylethanolamine served as substrate. (C) Inhibition of the third methylation reaction of rat liver PE methyltransferase by S-adenosylhomocysteine. The fixed concentrations of AdoHcy were 0.12 mM (X—X), 0.072 mM (○—○), 0.048 mM (▲—▲), 0.024 mM (●—●) or 0 (□—□). Except for the use of 3.0 mM egg dimethylphosphatidylethanolamine as substrate, details are as given for B. (D) Inhibition of the first methylation reaction of guinea pig liver PE methyltransferase by S-adenosylhomocysteine. The fixed concentrations of AdoHcy were 0.36 mM (X—X), 0.24 mM (○—○), 0.12 mM (▲—▲), 0.048 mM (●—●) or 0 (□—□). (E) Inhibition of the second methylation reaction of guinea pig liver PE methyltransferase by S-adenosylhomocysteine. The fixed concentrations of AdoHcy were 0.36 mM (X—X), 0.24 mM (○—○), 0.12 mM (▲—▲), 0.048 mM (●—●) or 0 (□—□). (F) Inhibition of the third methylation reaction of guinea pig liver PE methyltransferase by S-adenosylhomocysteine. The fixed concentrations of AdoHcy were 0.36 mM (X—X), 0.24 mM (○—○), 0.12 mM (▲—▲), 0.048 mM (●—●), or 0 (□—□). Except for the use of 3.0 mM egg dimethylphosphatidylethanolamine as substrate, details are the same as given for B.

TABLE 1

Competitive Inhibition of AdoHcy on Microsomal Phosphatidylethanolamine Methylation in Rat and Guinea Pig Livers

Reaction	Ki (AdoHcy) (μM)	n
First		
Rat	3.8 ± 0.7	4
Guinea pig	68.0 ± 28.0	4
Second		
Rat	127.7 ± 4.9	4
Guinea pig	45.0 ± 8.0	4
Third		
Rat	61.8 ± 14.6	4
Guinea pig	2.1 ± 0.7	4

Values (followed by standard deviations) are the means of duplicate determinations from n number of analysis. Inhibition data are the summation of results shown in Figs. 1 and 2. Lipid substrate for the first methylation reaction was endogenous microsomal PtdEtn; the second reaction used 1.0 mM egg monomethylphosphatidylethanolamine and the last reaction used 3.0 mM egg dimethylphosphatidylethanolamine. Details for each reaction are given in Figs. 1 and 2 and Materials and Methods.

ity of the first reaction of phosphatidylethanolamine methyltransferase (Table 2). It was anticipated that, in species exhibiting low methyltransferase activity, such as guinea pig, the concentration of the inhibitor, AdoHcy, would be high or the substrate concentration would be low; however, it is evident that neither is true (Table 2).

DISCUSSION

The substantially lower level of polyunsaturated fatty acid containing phosphatidylcholines in guinea pig livers as compared to rat liver (26-28) may indeed be attributable to species differences in the CDP-choline and methylation pathways of phosphatidylcholine biosynthesis. Perhaps these differences lead to alteration in the lipid environment which are responsible for interspecies variation in the phosphatidylethanolamine methyltransferases. It has been demonstrated by Rytter et al. (29) that the methylation pathway synthesizes the more polyunsaturated species of phosphatidylcholine, however, the CDP-choline pathway contributes the majorityof the phosphatidylcholine in rat liver (30-32). Skurdal and Cornatzer (33) have shown that the activity of choline phosphotransferase in guinea pig liver microsomes is about half that in the rat. The microsomal enzyme activities of phosphatidylethanolamine methyltransferase is also lower in the guinea pig than in the rat (24,33). The enzyme, choline

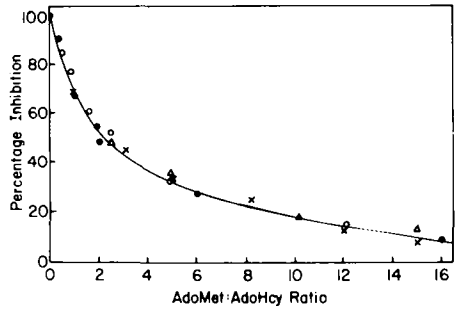

FIG. 2. Inhibition of the first methylation reaction of rat liver phosphatidylethanolamine methyltransferase by S-adenosylhomocysteine. Various concentrations of AdoMet were 214 μM (○), 88 μM (×), 30 μM (△) and 10 μM (●). Specific AdoHcy concentrations can be derived from AdoMet:AdoHcy ratios. 100% activity of the reaction represents 101 nmol/min/mg microsomal protein. Each point is the mean of duplicate determinations.

oxidase, that breaks down free choline in the liver had an activity 18 times lower in the guinea pig than the rat (34). Furthermore, the guinea pig does not produce a fatty liver like the rat when fed a choline-deficient diet (35, 36), which shows the marked differences in phospholipid metabolism in the guinea pig compared to the rat. Since production of the polyunsaturated phospholipids has recently been implicated in numerous cell membrane functions (37), it was desirable to further investigate the three methylation reactions of the conversion of phosphatidylethanolamine to phosphatidylcholine which form the highly unsaturated phosphatidylcholine species. Ki-

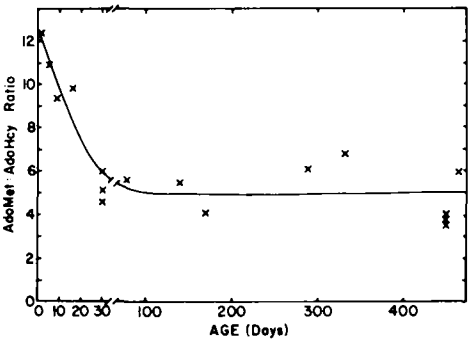

FIG. 3. Ratio of AdoMet:AdoHcy concentrations in the livers of various age rats. Livers were excised and frozen in 3 to 5 sec following decapitation. Specific AdoMet and AdoHcy concentrations are from ref. 20. Other details are as outlined in Materials and Methods." EAch point is the mean of dupliate determinations.

netic studies of the rat and guinea pig liver phosphatidylethanolamine methyltransferases have been completed in an attempt to provide some insight toward the presence of one, two or three enzymes in this tissue (24). The maximal velocities of the first methylation reaction were 114 ± 35 and 48 ± 6 pmol phosphatidylcholine formed/min/mg microsomal protein for rat and guinea pig liver, respectively (24). The second and third reactions were very fast in rat liver and had Vmax values of 3340 and 5720 pmol/min/mg, respectively. These two reactions occur at a considerably slower rate in the guinea pig, 549 pmol/min/mg for the conversion of monomethylphosphatidylethanolamine to phosphatidylcholine and 95 pmol/min/mg for phosphatidylcholine synthesis from dimethylphosphatidylethanolamine. The greater affinity of the rat liver enzyme for substrate, AdoMet, in the rate-limiting step (Km = 18 μM) vs guinea pig (Km 302 μM) entirely accounts for the greater reaction velocity in the rat (24). The significant difference between the Km (24) and Ki values for the first methylation in both species would apparently verify the rate-controlling action of this reaction (Table 1). The Km values (24) for the second and third reactions in rat liver are high compared to the tissue level of AdoMet, 69 nmol/g. Thus, a greater inhibitory action of AdoHcy may be encountered in these two reactions in rat liver. Furthermore, the nearly equivalent Km (24) and Ki (Table 1) values of the second and third reaction in guinea pig liver may significantly limit methylation in this tissue.

The presence of two phosphatidylethanolamine methylation enzymes in rat liver has been indicated previously (38) and a magnesium dependency of only the first reaction in various cell types (39) would seem to verify this. However, a single enzyme has recently been partially purified and is capable of catalyz-

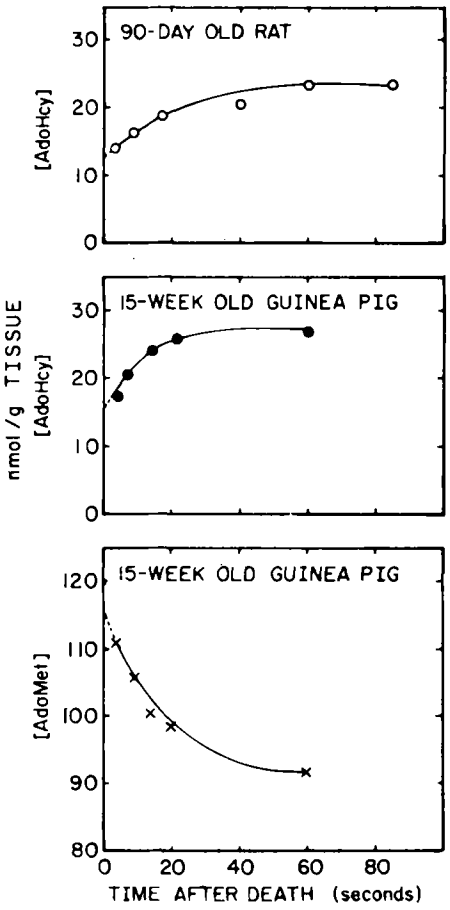

FIG. 4. Liver concentration of AdoHcy and AdoMet following death of the animal. Fractions (1-3 g) of the liver from a single rat (○—○) or guinea pig (●–●, X —X) were frozen in liquid nitrogen at specific times following decapitation. AdoHcy and AdoMet were extracted and fractionated chromatographically as described in Materials and Methods. Each data point is the mean from duplicate analysis of a single extract of a liver lobe.

TABLE 2

Relationship of Phosphatidylethanolamine Methyltransferase, S-Adenosylmethionine and S-Adenosylhomocysteine in Liver of Various Species

Species (age)	n	AdoMet nmol/g	AdoHcy nmol/g	AdoMet/ AdoHcy	Phosphatidylethanolamine methyltransferase pmol/min/mg
Mouse (50 days)	13	60.6 ± 10.0	14.1 ± 4.5	4.6 ± 0.9	206 ± 25
Rat (80-100 days)	17	68.9 ± 10.0	13.6 ± 2.9	5.2 ± 0.7	108 ± 23
Rabbit (11 weeks)	14	40.5 ± 4.9	12.4 ± 3.1	3.3 ± 0.5	50 ± 4
Guinea pig	10	114.4 ± 13.1	12.1 ± 2.8	9.9 ± 3.4	21 ± 11

Values (followed by standard deviations) are the means of n number of determinations. AdoMet and AdoHcy concentrations were obtained from tissue frozen 3-6 sec after death.

ing the three methyltransferase reactions (40).

Kinetic (24) and AdoHcy inhibition studies in rat and guinea pig liver give insight to the existence of one, two or three enzymes which catalyze the sequential methylation of phosphatidylethanolamine to phosphatidylcholine. However, the greater affinity for inhibitor than for substrate concurs with the findings of others for methyltransferases using tRNA, glycine, histones, histamine, acetylserotonin, phenylethanolamine, L-epinephrine and N-methyltrypamine as methyl acceptors (7-9,11, 40-42).

At an AdoMet:AdoHcy ratio of 1:1 or 2:1 as reported by other investigators (15-17), the activity of a majority of methyltransferases would be grossly reduced in vivo. Indeed, when the ratio of substrate to inhibitor is varied between 1:1 and 2:1, the in vitro activities of histone methyltransferase, DNA methyltransferase (20) and phosphatidylethanolamine-methyltransferase (Fig. 2) were reduced to 40-60% of normal. However, if AdoMet and AdoHcy are differentially compartmentalized in the cellular organelles, regulation of transmethylation might occur. It has been reported that AdoHcy will not pass through the cellular membrane in dog liver (43). Furthermore, it has been reported that AdoHcy will unidirectionally traverse the membrane to leak out of the rat liver cell and that AdoMet would neither enter nor exit the cell. However, at an AdoMet:AdoHcy ratio of 0.3:1, the in vivo methylation of histones, DNA and PtdEtn was inhibited 90-99%, regardless of compartmentalization (44).

Hoffman et al. (20) have recently reported that the hepatic concentration of AdoHcy is 14 nmol/g, thus an AdoMet:AdoHcy ratio of 5:1 is maintained in the adult rat (Fig. 3). An AdoMet:AdoHcy ratio of 12:1 in developing rat liver would provide a favorable environment for transmethylation to occur. The activity of phosphatidylethanolamine methyltransferase in rat liver increases from birth to reach a maximum at 20 days and, thereafter, declines rapidly to adult levels by 50 days (20). This partially corresponds with the AdoMet:AdoHcy ratio in rat liver (Fig. 3).

The increase in AdoHcy levels following death of the guinea pig is readily attributable to the continued enzymatic activity of the numerous methyltransferases in the liver converting AdoMet to AdoHcy and methylating the acceptor compound. Furthermore, there is a concomitant decrease in the hepatic AdoMet level (Fig. 4). Failure of both adenosylmethionine synthase and adenosylhomocysteine hydrolase to maintain constant levels of AdoMet and AdoHcy is indicated. It is conceivable that these responses may result from a rapid hormone imbalance or maybe linked to the rapid decrease in ATP concentration which is required for the synthesis of AdoMet. Phospholipid methylation is apparently influenced by norepinephrine (45), cAMP (46) and beta agonists (39). However, no effect on the hepatic levels of AdoMet and AdoHcy or on the in vitro activity of liver microsomal phosphatidylethanolamine methyltransferase were observed in our laboratory following L-epinephrine or L-norepinephrine administration or rats (unpublished experiments).

The data reported in this study show that three methylation reactions of rat liver phosphatidylethanolamine methyltransferase are significantly greater than in guinea pig liver. We assumed that, if either the level of AdoMet was elevated or the level of AdoHcy was depressed in rat liver, the higher methylating activity could be explained. However, no correlation between the hepatic concentrations of AdoMet or AdoHcy with the activity of the rate-limiting reaction of phosphatidylethanolamine methyltransferase among the four species was observed (Table 2). In contrast, preliminary results of experiments in our laboratory suggest that a direct correlation exists between the hepatic AdoMet:AdoHcy ratio and microsomal phosphatidylethanolamine methyltransferase in rats subjected to either 72-hr starvation or dietary methyl group depletion. Thus, in some species, the AdoMet:AdoHcy environment may regulate transmethylation to a greater or lesser degree than in other species.

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Hepatic Contribution to Newly Made Fatty Acids in Adipose Tissue in Rats and Inhibition of Hepatic and Extrahepatic Lipogenesis from Glucose by Dietary Corn Oil

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ABSTRACT

We have reexamined an earlier rat study in which the authors concluded that 60 min after [U-^{14}C]-glucose injection half of labeled fatty acids found in adipose tissue had been made in liver and then transported to the adipose tissue. We have shown that even under conditions in which the lipogenic role of the liver is optimized (fed-refed rats on a fat-free, high-carbohydrate diet), almost none of the labeled fatty acids found in adipose tissue of rats 60 min after they were fed a labeled glucose test meal was derived from the liver. This conclusion was based experimentally on (a) the use of the blocking agent Triton WR 1339 to measure the total labeled triglyceride fatty acids (TGFA) synthesized and secreted by the liver in 60 min and (b) comparison of plasma TGFA-^{14}C data with radioactivity found in liver and in adipose tissue in 60 min. Without using Triton WR 1339, mathematical analysis of plasma TGFA-^{14}C following the glucose test-meal leads one to the same conclusion: 97% of ^{14}C-labeled fatty acids found in adipose tissue at 60 min was made in situ. Additional studies in rats established that the source of error in the earlier studies was an incorrect assumption that dietary corn oil could inhibit hepatic lipogenesis from glucose C without inhibiting fatty acid synthesis in adipose tissue. In our studies, 10% corn oil inhibited equally both hepatic and adipose tissue fatty acid synthesis from glucose C under conditions that precluded any significant transport of labeled TGFA-^{14}C from liver to adipose tissue.

Despite years of study, the relative contribution of liver and adipose tissue to the synthesis of total body fatty acids from dietary carbohydrate is still controversial (1-6). A number of investigators have shown that, under conditions that promote rapid lipogenic rates, the liver may synthesize as much as 30-50% of the body's fatty acids in mice and rats (1,2,5). It is likely that most of these newly made fatty acids are subsequently transported to the extrahepatic tissues for storage and use. Indeed, evidence has been presented by Borensztajn and Getz that, following injection of [U-^{14}C]-glucose, half of the newly synthesized fatty acids found in adipose tissue had been made in the liver and then transported in a relatively short period of time (60 min) to the adipose tissue of fed rats (6). If that were the case, in vivo studies of lipogenesis in adipose tissue would be extremely misleading unless the contribution of radioactive FA made in the liver and transferred to adipose tissue were taken into account.

However, we think that the experiments carried out by Borensztajn and Getz (6) are inconclusive in this regard and that other experiments are needed in order to answer the question that they posed. In this study, we have attempted to clarify this problem using several different experimental approaches. First, we have tried to optimize the liver's possible role with respect to total body lipogenesis by using fed-refed animals on a fat-free diet (2). We then attempted to determine both theoretically and experimentally the maximal contribution that the liver might make under those "optimal" conditions to the radioactivity found in adipose tissue TGFA-^{14}C 1 hr following the feeding of a [U-^{14}C]glucose labeled test-meal. We also attempted in another series of experiments to test the basic assumption on which Borensztajn and Getz' analysis of their data was based; i.e., that dietary corn oil could block lipogenesis in the liver without inhibiting fatty acid synthesis from glucose carbon in adipose tissue.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were housed in plastic colony cages and were maintained on experimental diets ad libitum for 10-11 days before each experiment. Body weights for all groups of animals ranged from 200 to 300 g. No anesthesia was used in any of the studies.

Diets

In the first two experiments, the rats were fed a fat-free, 58% glucose diet commonly used

in our laboratory (2). In the third experiment, based on the fat content of the diets used by Borensztajn and Getz (6), half of the rats were fed a diet containing 38.5% glucose and 10% corn oil; the other half received the fat-free 58% glucose diet. Both diets also contained 22% casein, 6% Hawk-Oser salt mixture, 2% Vio-Bin, 0.2% vitamin mix and the remaining percentage was nonnutritive cellulose.

Triton WR 1339

Some of the animals in experiments I and II received Triton WR 1339 (Ruger Chemical Co., Inc., Irvington, NJ), which was diluted with 0.9% saline (final concentration, 200 mg/mL) and injected (0.5 mg/g body weight) into a tail vein.

Radioactive Tracer Doses

Isotopes were purchased from Dhom Products, Ltd., North Hollywood, CA. Purity of [U-^{14}C] glucose was at least 98% as determined by thin layer chromatography (7). In experiments I and II, [U-^{14}C] labeled glucose was administered through gastric intubation of a 50% glucose-water solution (20-25 μCi and 2.25 g glucose/animal). In experiment III, 25 μCi of tracer [U-^{14}C] glucose in 0.5 mL saline was administered by intravenous (iv) injection to each rat.

Tissues

In experiments I and II, animals were killed by decapitation and terminal blood samples collected in preheparinized vacutainer tubes. Plasma was separated and stored at -16 C. Livers and epididymal fat pads were quickly removed, weighed and immersed in 30% (50% ethanolic) KOH solution, then stored at -16 C for later analysis. In experiment II, guts were tied off, removed and frozen in liquid nitrogen. Carcasses were immediately digested in 30% aqueous KOH (heated to 100 C) and stored in 1-L bottles.

In experiment III, serial blood samples were obtained from an opthalmic venous capillary sinus following the iv injection of tracer [U-^{14}C] glucose (2). Animals were killed by decapitation and terminal blood samples collected. Plasma, livers and epididymal fat pads were removed and stored, along with carcasses, in the same manner as in experiments I and II.

Analyses

Livers and epididymal fat pads were saponified in 30% (50% ethanolic) KOH (30 mL/sample) for 2-3 hr under reflux at 80 C. Ten-mL aliquots were taken of the aqueous carcass digests to which 10 mL of 95% ethanol were added. These aliquots were then resaponified for 2-3 hr at 80 C. Extraction of total lipid fatty acids (TLFA) from liver, carcass and adipose tissue samples were performed according to Baker et al. (2).

Frozen guts from experiment II were homogenized in 70% ethanol and diluted to 100 mL. The samples were then heated to 80 C for 3 min. Aliquots were taken directly for counting. Glucose absorbed was then calculated by difference (7).

Plasma glucose specific activity (sp act) was determined according to the method of Baker et al. (8). Plasma glucose concentration was determined by enzymatic assay of deproteinized samples of blood plasma (8). Plasma triglyceride concentration and triglyceride radioactivity were determined using the procedure of Galletti (9).

^{14}C-labeled samples were dissolved in scintillator solution (Instagel/toluene, 1:1 [v/v]) and assayed with a Beckman Model LS 3133P liquid scintillation spectrometer. Instagel was purchased from Packard Instruments Corp., Downers Grove, IL. Appropriate ^{14}C standards were counted and selected samples were spiked with [1-^{14}C] palmitic acid as internal standard to evaluate quenching (appropriate corrections were applied).

Experimental Protocol

Experiments I and II were both designed to determine quantitatively the amount of radioactive TGFA formed by the liver from a labeled [U-^{14}C] glucose load and then secreted and transported into all tissues in fed-refed rats fed a fat-free diet similar to that used by Borensztajn and Getz (6). The model on which this experiment was based is shown in Figure 1 and described in the legend to that figure. We measured the formation of labeled TGFA in the liver, the total labeled and unlabeled TGFA secreted, the labeled TGFA transported to all tissues, the maximal labeled TGFA transported to adipose tissue (assuming a maximum of 30% of total transport out of plasma went to adipose tissue, which is a high estimate [10]), and the appearance in adipose tissue of labeled TGFA (synthesized from glucose C and transported from the liver). In addition, we measured plasma glucose concentrations and sp act in both experiments and all data were normalized to the control plasma glucose sp act at 60 min. The effect of Triton injection on the rate of glucose absorption was measured in experiment II (along with several other controls for handling and injection of the rats).

In experiment I, animals were randomly assigned into 8 groups. All experiments were carried out between 6:00 and 9:30 a.m. so that the animals would be in the "fed" state (2). Each rat was then given an oral load of ^{14}C-labeled glucose by gastric intubation. Triton was administered to half of the groups. Groups 1 and 3 received Triton injections 5 min prior to feeding whereas groups 5 and 7 received Triton 5 min after feeding. Groups 2, 4, 6 and 8 were non-Triton controls. Rats were killed 5, 15, 30 and 60 min after feedings.

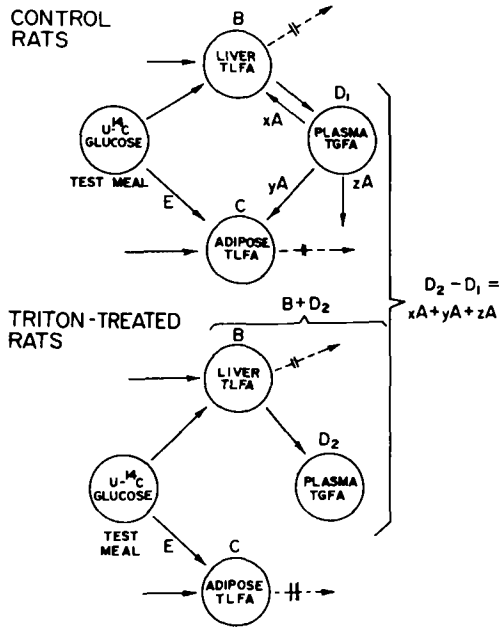

FIG. 1. Simplified model of TGFA-^{14}C synthesis, secretion (as VLDL-TG), and transport in control and Triton WR 1339-treated rats. Our experimental design is based on this model and the parameters shown. The quantity, A (% of the fed [U-^{14}C]glucose radioactivity) is the quantity of TGFA-^{14}C that leaves the circulation by all pathways during the 60-min experiment. The value, E, expressed in the same units, is the quantity of radioactive TLFA formed from [U-^{14}C]-glucose in 60 min in the adipose tissue. The values B, C, D_1, and D_2 represent the % of radioactivity fed in the labeled glucose test meal found in the respective compartments at 60 min. $x + y + z = 1$, where x, y, and z = the fraction of the total TGFA radioactivity leaving the circulation in 60 min in control rats that is transported back to the liver, to adipose tissue TGFA, and to all other tissues, respectively. Experiments I and II are designed primarily to evaluate the component E (de novo lipogenesis from glucose-C), relative to yA (maximal transport of TGFA-^{14}C from plasma to adipose tissue). We have assumed that y has a maximal value of 0.30 in glucose-fed, refed rats. Other assumptions: Triton WR 1339 does not influence the rate of TGFA-^{14}C secretion; loss of liver TGFA-^{14}C and of adipose tissue TGFA-^{14}C by arrows other than shown here are negligible (broken arrows) during the course of the experiment.

In experiment II, animals were randomly divided into 7 groups. Groups 1 and 2 both received Triton injections. Group 1 was fed an oral load of labeled glucose 5 min later and then sacrificed 60 min after feeding. Animals in group 2 were not given glucose and were killed 5 min after Triton administration. Groups 3 and 4 both received iv injections of 0.9% saline rather than Triton. Subsequent glucose feeding and killing times for groups 3 and 4 matched groups 1 and 2, respectively. Groups 5, 6 and 7 received no iv injections. Both groups 5 and 6 were given ^{14}C-labeled glucose loads and killed 5 min and 60 min after feeding, respectively. Group 7 was an untreated, zero-time control group.

Experiment III was designed to test the validity of the basic premise of Borensztajn and Getz (6), i.e., that dietary fat drastically inhibited lipogenesis in liver but not in adipose tissue under their experimental conditions. To test this hypothesis, which had been based on in vitro tracer studies (6), we measured the rate of body glucose carbon conversion to fatty acids in liver and adipose tissue of unanesthetized rats fed the two diets (fat-free and 10% corn oil) similar to those used by Borensztajn and Getz. No Triton was used and, although a glucose load was fed, the glucose was not labeled. Instead, tracer glucose was injected iv into the "fed-refed" (2) rats and the early rates of FA synthesis in the two organs was measured using the semicompartmental approach of Baker and Huebotter (11). The experimental design precluded any significant contribution of labeled TGFA derived from the liver into adipose tissue by restricting the lipogenic aspect of the study to a 20-min period. At that time, a negligible fraction of the labeled TGFA, newly synthesized from the injected [^{14}C]-glucose in the liver, will have been secreted into the circulation (12). Fifty animals were randomly divided into 4 groups of 12-13 rats each. Groups 1 and 2 had been fed a 58% glucose fat-free diet for 10 days prior to the experiment. Groups 3 and 4 had been fed a 38.5% glucose, 10% corn oil diet for 10 days. All animals were "refed" 4.5 mL (2.25 g) of 50% unlabeled glucose (in water) 15 min before iv injection of [U-^{14}C]glucose. For groups 1 and 3, blood samples were taken from the opthalmic venous capillary sinus 5 and 10 min after tracer injection. The animals were then sacrificed 20 min after tracer injection and blood, liver and epididymal fat were obtained. Groups 2 and 4 were sampled 30, 60 and 120 min after tracer

TABLE 1

Plasma Glucose Specific Activity and Glucose Absorption in Control and Triton-Treated Rats 60 Min after a Glucose Load[a]

	Experiment I	Experiment II	
	Plasma glucose sp act (t = 60 min) (cpm/mg) X 10^{-3}	Plasma glucose sp act (t = 60 min) (cpm/mg) X 10^{-3}	Fed glucose absorbed (%)[b]
Control	16.0 ± 0.62 (91)[c]	17.3 ± 0.6 (73)	26.9 ± 3.2
Triton	8.9 ± 1.04 (51)	16.0 ± 1.0 (67)	23.7 ± 2.4

[a]Mean ± SE from 6 rats/group.

[b]Based on analysis of the gut and its contents at t = 0 and t = 60 min (n = 6/group); the t_0 values were obtained immediately after intubation of the labeled glucose test meal.

[c]Mean % of glucose sp act in test-meal, in parentheses.

injection and killed 180 min after tracer injection (terminal blood sample).

RESULTS

Experiments I and II: Liver TLFA Synthesis, TGFA Secretion and TGFA Transport vs Adipose TLFA Synthesis

Plasma glucose specific activity. Following the feeding of the labeled glucose test meal, the sp act of plasma glucose rose steadily to a near plateau value which was reached at ca. 30 min (unpublished observations). For unknown reasons, the plateau value was significantly lower in Triton-treated than in controls in experiment I, but not in experiment II (Table 1). The plasma glucose concentrations between 30 and 60 min following the glucose test meals were constant at ca. 1.5 mg/mL and this level was unaffected by Triton treatment. Because of the low plasma glucose sp act observed in treated animals in experiment I, all subsequent data were normalized to the mean control plasma glucose sp act values at 60 min. This normalization in no way affects any conclusions with respect to relative rates of FA synthesis from plasma (body) glucose-C; it merely allows a clearer graphic comparison and consolidation of results from the two experiments. As shown in experiment II, there was no effect of Triton treatment on glucose absorption, the rates of which were nearly identical to values published earlier (13).

Incorporation of dietary glucose-carbon into hepatic total lipid fatty acids. As shown in Figure 2, the rate of glucose carbon incorporation into hepatic TLFA was approximately linear (after an initial lag phase) and was unaffected by Triton injection, both in experiments I and II. Approximately 0.4% of the fed labeled glucose carbon was found in the liver in 60 min. This corresponds to the quantity B in Figure 1.

Accumulation of labeled and unlabeled triglycerides in plasma following Triton WR 1339 injection. The concentration of plasma TG rose linearly (not shown) from an initial value of ca. 0.4 mg/mL to ca. 4 mg/mL in 60 min (Table 2). This corresponds to a mean rate (experiments I and II) of TG secretion (TGSR, Table 2) of 0.22 mg/min/100 g body weight, a value that agrees well with earlier estimates in glucose-fed rats (12). Therefore, the Triton effectively produced its expected blocking action (Fig. 1) in both experiments. The mean fractional rate constant of plasma TG turnover

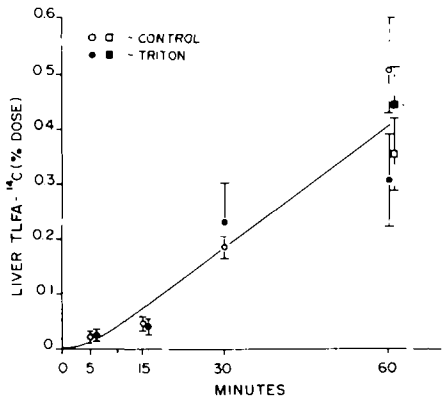

FIG. 2. Percentage of dose found in liver TLFA in controls (○,□) and rats administered Triton WR 1339 (●,■). Data are mean ± SE from 4 rats/group for each time point obtained in one experiment in which [U-^{14}C] glucose was given intragastrically and liver samples were collected 5, 15, 30 and 60 min after glucose administration. Data from another experiment in which only 60-min (terminal) samples were collected are included.

TABLE 2

Triglyceride Concentration and Radioactivity in Control and Triton-Treated Rats[a]

	TG (mg/mL)	TGSR (mg TG/min/100 g)[b]	^{14}C-TGFA (60 min) (% dose/plasma pool)[b]	^{14}C-TGFA (60 min) Uptake[c]	Maximal ^{14}C-TGFA transport to adipose tissue[d] (% dose/rat)
Experiment I					
Control	0.53 ± 0.09	0.24 ± 0.021	0.041 ± 0.013	0.065	0.022
Triton (60 min)	4.18 ± 0.30		0.106 ± 0.034	–	–
Experiment II					
Control	0.31 ± 0.05	0.21 ± 0.038	0.061 ± 0.013	0.265	0.088
Triton (60 min)	3.42 ± 0.57		0.326 ± 0.073	–	–

[a]Mean ± SE from 6 rats/group. Mean body weights of rats (control and Triton) in experiment I and experiment II were 250 g and 300 g, respectively.

[b]Total TG mass/plasma pool and ^{14}C-TGFA-% dose/plasma pool were calculated on the assumption that plasma volume in each group was 4.0% of body weight.

[c]Uptake (by all tissues) = difference (plasma ^{14}C-TGFA, Triton-treated minus controls).

[d]Maximal ^{14}C-TGFA transport from liver to adipose tissue assumed = 1/3 × "Uptake" shown in preceding column.

calculated from the values in Table 2 was 0.14 min^{-1}.

The appearance of labeled TGFA in the plasma of control rats was comparable in both experiments, 0.04-0.06% of the injected dose (Table 2). This value (0.05%) corresponds to the quantity D_1 in Figure 1. Following Triton treatment, the values of TGFA-^{14}C were higher than in controls, especially in experiment II in which an extra 0.27% of the fed glucose carbon accumulated as plasma TGFA. The mean value (experiments I and II) for uptake of TGFA-^{14}C by all tissues in the controls (i.e., D_2-D_1 in Fig. 1) was 0.17% of the fed test meal (Table 2, [0.065 + 0.265]/2).

Thus, on the average, the liver converted ca. 0.62% of the fed glucose carbon to hepatic TLFA in 60 min; 0.4% of the fed glucose carbon was still present in the liver and 0.22% had been secreted (Table 2 [0.11 + 0.33]/2). In non-Tritonized rats, 0.17% had been taken up by all tissues and 0.05% remained in the circulation. Of the total labeled TGFA uptake, assuming (10) that the uptake of labeled TGFA by adipose tissue was no more than 30% of the total uptake, we estimated that at most, 0.05% (i.e., 30% of 0.17%) of the fed glucose-^{14}C could have been converted to TGFA-C by liver and then transported to adipose tissue by way of plasma TGFA in 60 min (yA in Fig. 1).

Incorporation of labeled glucose carbon into adipose tissue. The conversion of ^{14}C-glucose carbon into adipose tissue TLFA (due to synthesis within both liver [followed by transport via TGFA] and adipose tissue TLFA-^{14}C) reached a value of ca. 2.0% of the fed dose at 60 min. The differences in TLFA-^{14}C between control and Triton-treated rats were not significant (Fig. 3). A significant lowering would not be expected since, as noted in the preceding paragraph, a maximum of only 0.05% of the dose could have been derived from plasma TGFA-^{14}C. Thus, with respect to Figure 1, yA = 0.05% and E = 1.95%. Clearly, almost all of the radioactive FA found in adipose tissue (97%) was synthesized in situ.

Experiment III: Does Dietary Corn Oil Cause Specific Inhibition of Lipogenesis in Liver and Not in Adipose Tissue?

Effect of dietary corn oil (10%) on the irreversible disposal rate of plasma glucose.

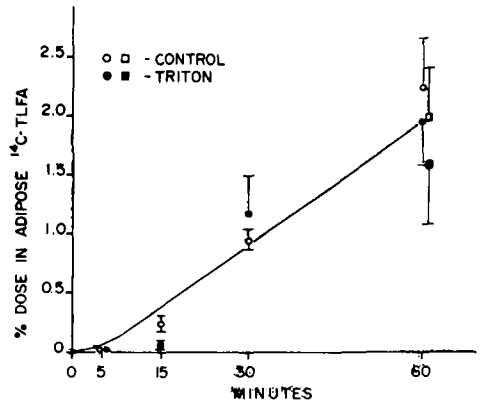

FIG. 3. Percentage of ^{14}C-radioactivity incorporated into TLFA of total adipose tissue in control rats (○,□) and Triton-treated (●,■) rats. Experimental conditions are the same as described for Fig. 2. Incorporation of radioactivity into TLFA at 60 min in control and Triton groups were not significantly different from each other. Data represent mean ± SE from 6 rats/time point/group.

Figure 4 shows the curves of plasma glucose sp act in rats fed fat-free and 10% corn oil diets. Both groups had the same mean plasma glucose concentration. Based on an assumed glucose space of 25% of body weight (14) and the measured grand mean plasma glucose concentrations (1.5 ± 0.10 mg/mL; mean ± SE) for the two groups of rats combined, the total body glucose pools could be calculated (98 ± 7 mg/rat). Since the body glucose pool sizes were identical, the calculated zero-time sp act of the two groups were also the same, 2.54×10^5 cpm/mg glucose in these rats (injected iv with 24.9×10^6 cpm). As can be seen in Figure 5, the mean plasma glucose sp act curve for the rats fed the 10% corn oil diet lies above that for those fed the fat-free diet indicating that there was a slower rate of irreversible disposal in the rats fed the polyunsaturated fat-containing diet. Indeed, a computer analysis of the data indicated that the respective IDR (mg glucose/min) were 9.3 (± 1.1%) and 7.5 (± 1.7%), mean ± SE, in the two groups—a statistically significant decrease of ca. 20% in the fat-fed rats.

The corn oil diet caused a significant decrease (74%) in the rate of glucose carbon conversion to TLFA in adipose tissue (from 0.23 ± 0.078 [n = 6] to 0.06 ± 0.011 [n = 6] mg glucose C/min/rat, mean ± SE, $p<0.01$), and a significant 44% fall in the corresponding rate in liver (from 0.039 ± 0.0086 [n = 6] to 0.022 ± 0.0033 [n = 6] mg glucose C/min/rat, $p<0.05$). The mean rate of glucose C conversion to total carcass FA was decreased by 50%, but the decrease was not statistically significant (from 0.32 ± 0.086 to 0.16 ± 0.026 mg glucose C/min/rat, $p<0.10$). However, the incorporation of glucose-^{14}C into carcass FA was significantly decreased, $p<0.05$ (from 3.20 ± 0.80% [n = 6] to 1.26 ± 0.23% [n = 6] of the injected dose at t = 20 min after iv tracer injection). Corresponding data for the incorporation of labeled glucose into liver and epididymal fat are presented in Figure 5. Thus, the composite data indicate that 10% dietary corn oil inhibited lipogenesis from glucose C in adipose tissue as well as in liver.

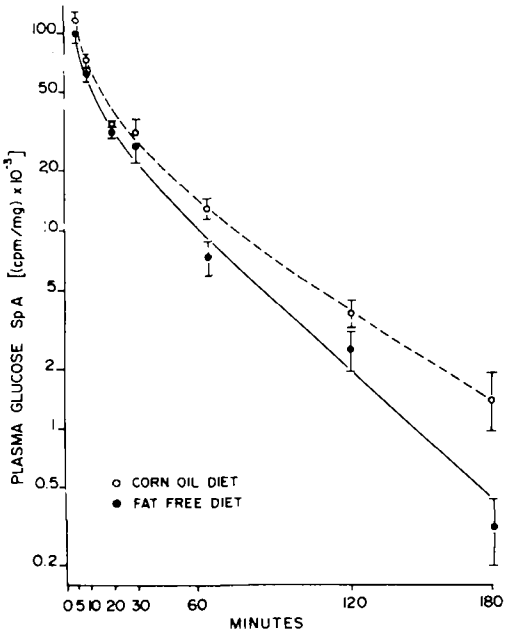

FIG. 4. Plasma glucose sp act in rats on a 58% glucose fat-free diet (•, controls) and 10% corn oil diet (○). The composition of the two diets is given in Materials and Methods. Data are mean ± SE from 6 rats/group.

DISCUSSION

We have attempted to quantify the order of magnitude of *labeled* FA movement (as VLDL-TGFA) from the liver by way of plasma TGFA to adipose tissue of rats under conditions in which the liver is rapidly synthesizing FA from dietary carbohydrate. We used two approaches, both of which are modifications and extensions of an earlier study of Borensztajn and Getz (6).

Our first approach is depicted in Figure 1. By blocking the exit of plasma TGFA with Triton WR 1339, we could obtain an estimate of the total labeled TGFA secreted by the liver. From this value, we could estimate the maximal amount of radioactivity that could have been transferred to adipose tissue TGFA (Fig. 1, yA). We could then relate yA to the TLFA-^{14}C synthesized by the liver (Fig. 1, B + D_2) and to the TGFA-^{14}C found in adipose tissue (Fig. 1, C).

Our second approach was based on the well known relationship among three quantities: (a) the area under a radioactivity vs time curve of a labeled substance, q(t), (b) the fractional catabolic rate of that substance, λ, and (c) the total labeled input into the compartment, q(t)*:

$$q(t)^* = \lambda \int_0^t q(t)dt$$

The value for q(60)* is equivalent to the quantity D_2 in Figure 1, i.e., the total radioactivity thatwould accumulate in plasma TGFA in 60 min if all exits were blocked. The rate constant λ can be estimated from the literature values for the fractional removal rate of TGFA in glucose-fed rats (12) or from rates of accum-

ulation of unlabeled TGFA in plasma after injection of Triton divided by the initial plasma TGFA pool size. In experiments I and II, we obtained a mean value of 0.14 min^{-1} which agrees with earlier values (12). The area of the curve for radioactivity in plasma TGFA was directly estimated from the control animal data (not shown) of experiment I; this gave a value of ca. 1.0% • min. Using the above equation, we obtained q(60)* = 0.14%. Thus, we could estimate values for A and D_2 (Fig. 1) without actually measuring the accumulation of labeled TGFA in plasma after blocking all exits with Triton WR 1339. The modeling approach also serves as a semi-independent check on the assumption that Triton WR 1339 blocks the exits of plasma TGFA without influencing the synthesis and secretion of labeled TGFA by the liver. The results of the two approaches are summarized in Table 3.

The results based on the second approach were almost identical to those based on the use of a blocking agent, Triton WR 1339. According to the first approach, 33% of the radioactive TLFA synthesized by the liver was secreted into plasma in 60 min, in close agreement with expectations based on the model of hepatic TGFA and TLFA turnover in fasted (15) and glucose-fed rats (12) presented in earlier studies by Baker and Schotz. The maximal quantity of radioactivity secreted by the liver and transported to adipose tissue in 60 min represented only a negligible fraction (0.05/2.0 × 100 = 2.5%) of the radioactivity found in the adipose tissue at that time. Thus, by both approaches, we conclude that virtually all of the radioactivity found in adipose tissue of rats under these conditions was actually synthesized by the adipose tissue itself. This contradicts the conclusion of Borensztajn and Getz (6) who estimated that about 50% of the ^{14}C-labeled TGFA in adipose tissue had been synthesized by the liver and then transported to the adipose tissue.

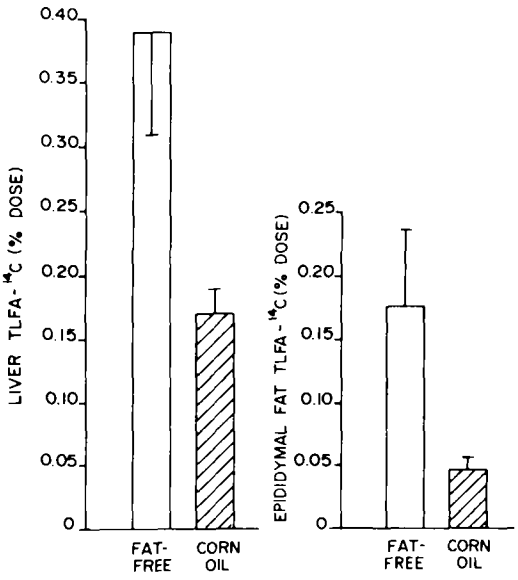

FIG. 5. Percentage of ^{14}C-radioactivity found in liver TLFA and epididymal fat TLFA in rats fed either a fat-free or 10% corn oil diet. Data were obtained 20 min after iv injection of [U-^{14}C] glucose to exclude contribution from hepatic transport to ^{14}C-TLFA found in epididymal fat pads. Incorporation data in liver and epididymal fat tissues of corn oil groups were significantly ($p<0.01$) less than the corresponding fat-free diet groups. Wet weights of adipose tissue and liver (mean ± SE, n = 6) did not differ significantly between the corn oil and fat-free groups: adipose tissue, 2.21 ± 0.16 g and 2.52 ± 0.17 g; liver, 10.2 ± 0.3 g and 9.9 ± 0.3 g. Data in figure are mean ± SE from 6 rats/group.

In order to reconcile our observations using Triton WR 1339 with those of Borensztajn and Getz using a different "blocking agent" (corn oil), we carried out a third study designed to test Borensztajn and Getz's major premise. On the basis of experiments done in vitro with both liver slices and adipose tissue of rats, they assumed that in vivo 10% dietary corn oil would block the synthesis of TLFA in fed rats injected with [U-^{14}C] glucose in liver but not in adipose tissue. Similar studies by others have been consistent with such a selective action of dietary fat on liver. For example, Clarke et al. (16) have reported that dietary linoleate inhibits lipogenesis in the liver but not in adipose tissue in meal-fed rats. Other reports of a selective action of corn oil on liver have been reviewed recently by Jeffcoat et al. (17), who have discussed some of the possible theoretical bases for such selectivity (short-term and long-term regulation in liver by essential FA; short-term regulation only in adipose tissue by saturated and not by essential FA). However, there is strong evidence in mice that dietary corn oil does inhibit lipogenesis from glucose C in adipose tissue in vivo (18,19), although less than in liver under the conditions studied (19). We thought that such an inhibition, if it occurs in rats under the experimental conditions of Borensztajn and Getz, could account for the 50% depression in adipose tissue TGFA-^{14}C that they observed. As we have shown in this study, regardless of the blocking agent, diminished transport to adipose tissue of TGFA-^{14}C derived from liver cannot produce a 50% lowering of adipose tissue TLFA radioactivity. A total blockage of hepatic TGFA synthesis and/

TABLE 3

Summary of ^{14}C-Labeled FA Synthesized from a Labeled Glucose Test Meal in Rats Fed a Fat-Free Diet

Labeled quantity (see Fig. 1)	Dose/Rat (%)	
	First approach (using Triton WR 1339)	Second approach (using modeling instead of Triton)
A	0.17	0.13
B	0.40	0.40
C	2.0	2.0
D_2	0.22	0.18
E	2.0	2.0
B + D_2	0.62	0.58
yA	0.05	0.04

A = Total plasma TGFA-^{14}C, outflow (60′) = xA + yA + zA; B = liver TLFA-^{14}C (60′); C = adipose TLFA-^{14}C (60′); D_2 = total TLFA-^{14}C secretion from the liver (60′); E = total TGFA-^{14}C synthesized by adipose tissue (60′); B + D_2 = total FA-^{14}C synthesized by liver (60′); yA = plasma TGFA-^{14}C transported to adipose tissue (60′).

or secretion or of plasma TGFA exit could only lower the adipose tissue by 0.5-2% in an hour under our conditions. Moreover, corn oil in the diet was shown in our experiment III to inhibit lipogenesis from glucose C in adipose tissue, and the degree of inhibition was sufficient to account for the lowering observed by Borensztajn and Getz.

Although additional work is needed to reconcile our work with some of the other studies in rats, there are many important variables that could account for the differences. As noted by Clarke et al. (16), the response of adipose tissue in meal-eaters may very well differ from that in nibblers. It is also possible that lipogenesis from 2C units in adipose tissue as measured with ^3H_2O by Clarke et al. (20) is not inhibited whereas lipogenesis from glucose C is impaired (2). In view of these findings, it is important to reexamine the effects of dietary saturated vs unsaturated FA in nibbling rats on lipogenesis from glucose C in adipose tissue. Earlier studies have been negative using both saturated and unsaturated FA (methylesters), but meal-eating rats were used and ^3H_2O was the tracer (16).

Our analysis confirms, in some respects, our earlier studies of lipogenesis in liver and adipose tissue of Zucker rats and their lean controls (5), as well as an earlier study of Haft (3), all of which indicate that labeled fatty acids found in adipose tissue were formed in situ; however, one must be careful not to extrapolate incorrectly from these tracer experiments to the tracee. The failure to find negligible transport of labeled TGFA from liver to adipose tissue does not imply that the contribution of the liver to the deposition of TGFA under conditions of rapid hepatic lipogenesis is also negligible. As yet, no one (to our knowledge) has carried out the appropriate experiments under any nutritional state in which the parameter has been appropriately explored.

ADDENDUM

Following completion of this work, our attention was brought to an abstract by J. Borensztajn, Fed. Proc. 31, 2414, 1972, describing an experiment similar in design to ours using glucose-^{14}C as tracer and Triton WR 1339 as a blocking agent in rats. According to this abstract, over 65% of the labeled fatty acids found in adipose tissue were made in the liver and transported by way of plasma TG to the adipose tissue in 24 hr. This conclusion is opposite to ours. One possible explanation of Borensztajn's finding is that Triton WR 1339 lowered the plasma glucose sp act, as it did in our experiment I, and/or it decreased food intake or the rate of glucose absorption from the gut. Any of these explanations could account for a lowered incorporation of tracer glucose (given ip in Borensztajn's experiment) into adipose tissue TGFA.

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Tissue Culture of Cocoa Bean (*Theobroma cacao* L.): Changes in Lipids during Maturation of Beans and Growth of Cells and Calli in Culture

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ABSTRACT

Callus cultures of *Theobroma cacao* L., initiated from explants of immature cocoa bean cotyledons, contained 5.3%-6.4% lipids (dry wt basis). The major fatty acids were palmitic, oleic and linoleic acids. Cell suspensions contained 5.7-7.7% total lipids which had a higher polyunsaturated fatty acid content than total lipids of the calli. Phospholipids and glycolipids were the predominant lipid classes of calli and cell suspensions. Immature cocoa beans at early stages of development contained much higher polyunsaturated fatty acids, higher polar lipids and lower triglycerides than did mature ripe beans. Ripe cocoa beans contained 54% total lipids of which 96.8% where triglycerides. The fatty acid composition of total lipids of calli and cell suspensions were similar to those of the immature cocoa beans.

INTRODUCTION

Cocoa butter, because of its special physical properties, its attractive taste and flavor, is the premium confectionary fat. The unique melting properties of chocolate are attributed to the unusual structure of the triglycerides of cocoa butter in which palmito-oleostearin is the preponderant species. Little is known of the control of biosynthesis of the triglycerides of cocoa bean. Because it is difficult to study synthesis of triglycerides in maturing cocoa beans, we explored the feasibility of using tissue and cell culture to study the synthesis of fatty acids and triglycerides by growing tissues of cocoa bean.

The lipid composition of some plant tissue cultures other than cocoa bean have been studied extensively (1-10). Generally, the lipids of plant tissue cultures are different both in content and composition from those of the mature seeds from the same plants (7). However, little is known about the relationship between the lipids of plant tissue cultures and those of immature seeds at early stages of growth and development.

Recently, the fatty acid composition of polar and nonpolar lipids of *Theobroma cacao* L. were reported (11). However, the fatty acid composition of individual lipid classes of immature cacao has not been reported. In this paper, we report the changes in composition of lipids during growth of cocoa bean calli and suspensions, and compare these to the lipids of immature cocoa beans harvested at progressive stages of growth and maturation.

EXPERIMENTAL PROCEDURES

Tissue Cultures of Cocoa Bean

Callus cultures of *Theobroma cacao* L. were initiated from immature cotyledons harvested 130 days after pollination as described by Tsai and Kinsella (12). The callus cultures were grown on a modified Gamborg's B5 agar medium (13) containing 1.0 ppm of 2,4-D and 0.2 ppm of kinetin in the dark at 30 ± 1 C in an incubator with moisture control (Hotpack, Philadelphia, PA). They were subcultured at 4-week intervals. Calli between the 8th and 10th passages were used for analyses. Suspension cultures of cocoa bean were established from the calli and grown in a modified Murashige and Skoog medium (13) containing 0.5 ppm of 2,4-D and 0.1 ppm of kinetin. The suspension cultures were maintained at 27 ± 1 C in the dark under continuous shaking at 120 rpm (Lab-Line Orbit Environ Shaker, Melrose Park, IL 60160). They were subcultured every 14 days. Cells from the third passage were used for experiments.

Immature Cocoa Beans

Immature cocoa pods of "Criollo" trees were obtained from Ecuador (courtesy of the Nestle Company, Fulton, NY). The pods ranged from 5 to 22 cm and were representative of different stages of maturity. The beans were separated from the pods, sliced, freeze-dried and stored at -30 C before lipid analyses.

Analytical Methods

Callus cultures (12) were harvested at

various stages of growth. The tissues were dried at 80 C for 24 hr and weighed. Cells in suspension cultures (12) were harvested 14 days after transfer. The cells were filtered onto Micracloth (Calbiochem) and blotted dry with Kimwipes. The cells were dried at 80 C for 24 hr to constant weight.

Lipids

Callus tissues and cell suspensions were harvested, freeze-dried and stored at -30 C. The total lipids were extracted by the method of Folch et al. (14). The lipid classes were separated by silicic acid (Mallinckrodt, 100 mesh) column chromatography. Aliquots of total extract containing 50 mg lipid were added to the column (1 x 10 cm) and the lipids were progressively eluted with 10 column volumes of chloroform (neutral lipids), 40 column volumes of acetone (glycolipids) and 10 column volumes of methanol (phospholipids) (8,15). Phospholipids were quantified by the colorimetric method of Raheja et al. (16). Glycolipids were quantified by the phenol-sulfuric acid method as described elsewhere (17). Triglycerides, diglycerides, free fatty acids, sterols and sterol esters were separated by TLC on Silica Gel 60H with petroleum ether (bp 35-60 C)/ethylether/acetic acid (70:30:1, v/v). Triglycerides and diglycerides were further quantified by the colorimetric method of Chin et al. (18). Free fatty acids were determined by the method of Lowry and Tinsley (19). Sterols were quantified by Liebermann-Burchard reaction with acetic anhydride/sulfuric acid as described by Snell and Snell (20).

Fatty acid methyl esters were prepared using boron trifluoride (21). The content and composition of fatty acids in total lipids and lipid classes were analyzed using a Hewlett Packard 5830A automated GC using stainless steel columns (6 ft x 1/8 in.) packed with 10% EGSS-X (Applied Science). The conditions for GC were the same as described previously (21).

RESULTS AND DISCUSSION

Growth of Cocoa Callus and Cell Cultures

The growth rates of the cocoa bean calli and cell suspensions, as indicated by increases in dry wt (Figs. 1 and 2), were similar to those observed for calli and cell cultures initiated from different parts, i.e., stem and leaves, of the cocoa tree (22-27). Cell cultures proliferated much faster and had shorter culture periods than calli.

Lipid Composition of Cocoa Cultures

The total lipid contents of cocoa calli and suspensions are presented in Table 1. These

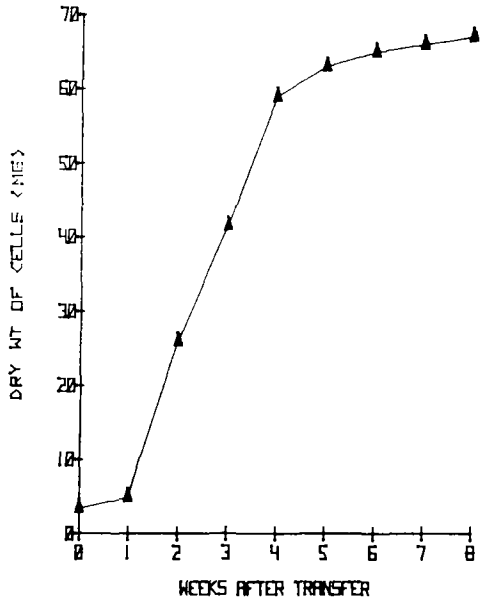

FIG. 1. Growth curve of cocoa calli after transfer to fresh medium and incubation at 30 ± 1 C. Dry wt was determined after 24 hr at 80 C.

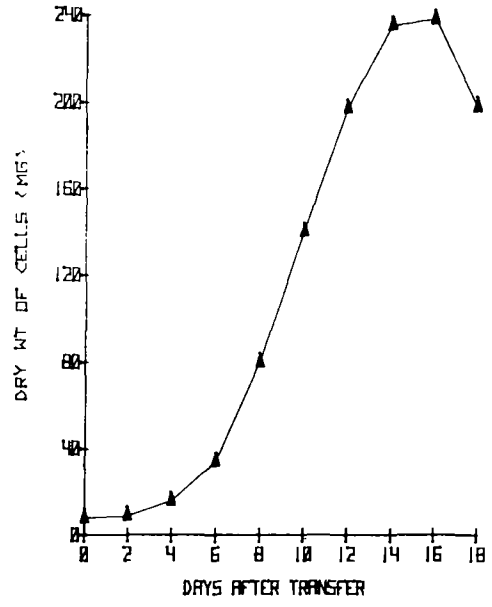

FIG. 2. Growth curve of cocoa suspensions after transfer to fresh medium and incubation at 27 ± 1 C. Cells from each culture flask were filtered onto Miracloth, washed, collected, and dried and weighed.

TABLE 1

Content and Fatty Acid Composition of Total Lipids Extracted from Cocoa Calli and Cell Suspensions at Different Stages of Growth

Cultures	Calli (weeks)				Suspensions (days)		
Time of growth	2	3.5	5	7	6	10	14
% Total lipids (w/w)[a]	6.4 ± 0.4	5.5 ± 0.5	5.5 ± 0.6	5.3 ± 0.2	7.7 ± 0.7	5.7 ± 0.5	6.5 ± 1.2
Fatty acid (% w/w)[b]							
16:0	24.9 ± 1.4	28.4 ± 0.1	29.0 ± 0.4	29.3 ± 1.4	24.6 ± 0.6	25.2 ± 1.0	28.3 ± 1.3
16:1	1.4 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.3
Unidentified acid	–	–	trace	3.1 ± 0.3	–	–	–
18:0	3.7 ± 0.4	2.9 ± 0.2	5.2 ± 0.6	5.2 ± 0.3	2.4 ± 0.3	2.6 ± 0.3	2.4 ± 0.2
18:1	22.4 ± 0.7	20.1 ± 1.0	20.6 ± 0.6	22.3 ± 1.2	15.8 ± 1.2	11.2 ± 0.8	11.5 ± 0.7
18:2	37.0 ± 2.0	40.7 ± 2.2	37.8 ± 0.8	34.4 ± 1.6	43.8 ± 1.0	47.3 ± 1.2	48.6 ± 1.2
18:3	10.0 ± 0.6	5.8 ± 0.5	5.6 ± 0.3	5.2 ± 0.4	10.5 ± 0.7	10.9 ± 0.8	7.7 ± 0.8
20:0	trace	–	–	–	1.7 ± 0.3	1.4 ± 0.2	–

[a]Dry weight basis.
[b]All samples were in duplicate and analyses in triplicate. Data were expressed as means ± standard deviations.

figures were comparable to those reported for soybean cultures (7,8). There were not significant changes in total lipid content of callus and suspension cultures over time of growth from 2 weeks up to 7 weeks and from 6 days to 14 days, respectively.

The distribution of major lipid classes of cocoa calli and suspensions is presented in Table 2. All cultures contained rather high proportions of polar lipids, especially glycolipids. Triglycerides were about 13% of total lipids in all cases. After 7 weeks of incubation, free fatty acids increased to 9.1% and diglycerides increased to 3.2%. The increase in these two lipid classes may reflect some lipolysis or a decrease in acylation as the cultures aged.

The phospholipid fraction of calli decreased after 7 weeks of incubation. By comparison, 5-day-old soybean suspensions were reported to contain 13.1% triglycerides, 0.1% free fatty acid, 7.1% steroids, 18% glycolipids and 59% phospholipids (8).

The fatty acid composition of total lipids of cocoa calli and suspensions is presented in Table 1. Both calli and suspensions contained high proportions of unsaturated fatty acids, especially linoleic acid. There was a slight increase in stearic acid and a concurrent decrease in linolenic acid after calli cultures aged. The fatty acid composition of total lipids of suspensions remained constant throughout a culture period. However, cells contained much higher linoleic acid and lower oleic acid than the calli. In general, the data on lipid composition were comparable to those of other plant tissue cultures (7,8).

Fatty acid composition of lipid classes is presented in Table 3. The major fatty acids associated with the triglycerides extracted from both cells and calli were palmitic, stearic, oleic and linoleic acid. Phospholipids and glycolipids of cells and calli contained little stearic acid. Phospholipids of both cultures contained relatively high linoleic acid levels whereas glycolipids contained relatively high levels of linolenic acid and arachidic acid (20:0).

Comparison of Lipids of Cocoa Cultures to Those of Maturing Cocoa Beans

In order to determine if lipids of cultured tissues resembled the lipids of cocoa bean, we compared them with lipids extracted from cocoa beans at progressive stages of maturation. The changes in total lipids and phospholipids in cocoa beans during maturation are shown in Figures 3 and 4, respectively. The total lipids were 11% on dry wt basis at 110 days post-pollination and increased to 54% upon ripening,

TABLE 2

Lipid Classes of Cocoa Bean Calli and Cell Suspensions

Lipids	Calli		Suspension cells
	5 wk[b]	7 wk[c]	14 days
	Percent by weight[a]		
Neutral lipids			
Triglyceride	13.2 ± 0.6	12.6 ± 0.6	12.6 ± 1.0
Diglyceride	0.6 ± 0.2	3.2 ± 0.2	0.2 ± 0.0
Free fatty acid	0.5 ± 0.2	9.1 ± 0.4	0.6 ± 0.2
Sterols	8.6 ± 0.7	9.2 ± 0.2	9.2 ± 1.0
Phospholipids	26.7 ± 0.4	13.9 ± 0.5	22.0 ± 2.6
Glycolipids	46.6 ± 2.2	49.4 ± 0.7	56.0 ± 2.2

[a]Five cultures were combined for analyses which were done in duplicate.

[b]Total recovery = 96.2%.

[c]Total recovery = 97.4%.

TABLE 3

Fatty Acid Composition of Lipid Classes from Cocoa Calli (5-7 wk) and Suspensions (10-14 days)

FA	Calli			Suspensions		
% by wt[a]	Triglycerides	Phospholipids	Glycolipids	Triglycerides	Phospholipids	Glycolipids
<16:0	trace	–	2.5 ± 0.3	trace	trace	–
16:0	25.7 ± 0.3	29.3 ± 0.4	28.6 ± 0.4	24.2 ± 0.4	30.7 ± 0.4	23.9 ± 0.5
16:1	1.4 ± 0.3	0.7 ± 0.2	1.6 ± 0.3	1.1 ± 0.1	1.2 ± 0.1	0.8 ± 0.2
18:0	17.2 ± 0.4	2.2 ± 0.2	6.6 ± 0.4	16.6 ± 0.2	1.9 ± 0.2	3.0 ± 0.3
18:1	24.4 ± 1.0	16.9 ± 0.5	15.9 ± 0.5	18.0 ± 0.8	6.2 ± 0.4	6.8 ± 0.4
18:2	23.4 ± 0.8	42.1 ± 1.2	20.7 ± 0.3	29.9 ± 0.4	53.0 ± 1.4	31.0 ± 0.3
18:3	7.0 ± 0.2	5.8 ± 0.2	15.8 ± 0.6	8.8 ± 0.3	5.2 ± 0.2	30.6 ± 1.4
20:0	0.7 ± 0.1	3.0 ± 0.3	7.1 ± 0.2	1.4 ± 0.3	0.8 ± 0.2	3.5 ± 0.2

[a]Mean of 3 determinations ± standard deviation.

whereas phospholipids decreased from 1.1% to 0.2% of dry matter upon ripening. The data generally agreed with those reported earlier (11).

The fatty acid composition of total lipids of cocoa beans at various stages of maturation is presented in Table 4. It is interesting that cocoa beans at early stages of maturation contained rather high proportions of linoleic acid and some linolenic acid. The high content of polyunsaturated fatty acids was related to the relatively high content of phospholipids and glycolipids in immature beans (Table 5). The nonpolar lipids of immature beans also contained a high amount of polyunsaturated fatty acids (11). It was reported that nonpolar lipids contained 19.1% linoleic acid in immature beans at 110 days postpollination (11).

Phospholipids and glycolipids contained much higher polyunsaturated fatty acids than triglycerides even in mature beans (Table 6). The glycolipids of immature beans had much lower stearic acid and higher linolenic acid than those of ripe beans.

The major lipid class of cocoa beans was triglycerides which were 85% of total lipids in immature beans and 96.8% in ripe beans. The fatty acids of the triglycerides of immature beans were similar to those of ripe beans whereas the phospholipids of immature beans had slightly higher levels of linoleic acid.

The data indicated that cocoa tissue cultures differ substantially from mature cocoa beans in the content and composition of lipids. Generally, plant tissue cultures contain lower amounts of lipids than seeds of the corresponding plant and the composition of lipids from tissue cultures are usually different from those of the corresponding seeds (7).

However, the similarity in content and composition of total lipids in tissue cultures of cocoa beans and those from immature cocoa beans harvested at very early stages of growth is notable. It has also been observed that the lipid composition of soybean cell suspensions was similar to that reported for immature soybean

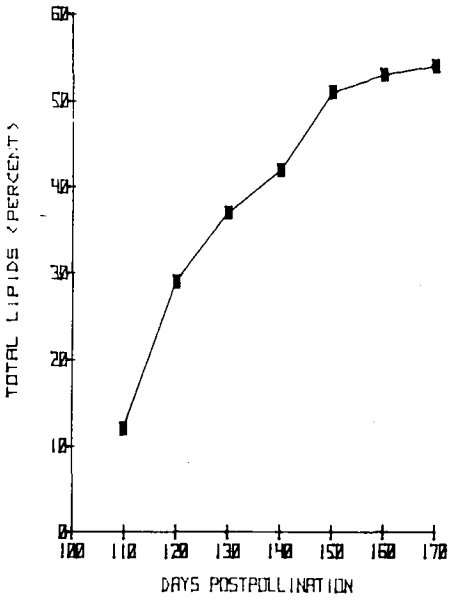

FIG. 3. The accumulation of total lipids in cocoa beans during growth and maturation. Each analysis was done in triplicate. The results are given as % of dry wt.

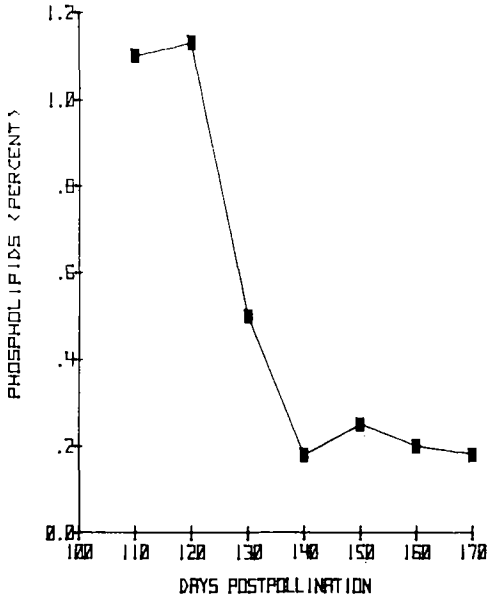

FIG. 4. Change in phospholipids of immature cocoa beans during growth and maturation. Phospholipids are expressed as % of dry matter. Analyses were done in triplicate.

TABLE 4

Fatty Acid Composition of Total Lipids Extracted from Cocoa Beans at Various Stages of Maturity (Criollo Group)

% by wt	Days postpollination					
FA	110	120	130	140	150	170
<16:0	1.2 ± 0.1	1.3 ± 0.1	trace	trace	1.6 ± 0.2	trace
16:0	31.1 ± 1.3	30.7 ± 1.6	26.6 ± 1.2	21.3 ± 1.0	17.7 ± 0.6	23.9 ± 1.3
16:1	–	–	trace	trace	–	–
Unidentified acid	5.4 ± 0.2	2.5 ± 0.3	–	–	–	–
18:0	5.8 ± 0.4	13.2 ± 0.6	30.8 ± 1.4	34.4 ± 1.2	33.7 ± 1.5	38.2 ± 1.2
18:1	7.6 ± 0.3	21.1 ± 1.0	36.1 ± 1.7	38.8 ± 1.9	41.8 ± 2.1	33.9 ± 1.3
18:2	45.4 ± 2.6	31.3 ± 1.5	5.8 ± 0.3	4.4 ± 0.3	5.2 ± 0.4	3.2 ± 0.2
18:3	3.6 ± 0.2	–	–	–	–	–

[a]All analyses were done in triplicate.

TABLE 5

Lipid Classes of Immature and Ripe Cocoa Beans (Criollo)

% by wt[a] Lipid classes	Immature beans (125 days postpollination)	Ripe beans (170 days postpollination)
Neutral lipids		
Triglycerides	85.0 ± 2.1	96.8 ± 0.4
Diglycerides	2.7 ± 0.4	0.6 ± 0.1
Free fatty acids	3.6 ± 0.4	0.5 ± 0.1
Sterols	1.8 ± 0.2	0.2 ± 0.0
Phospholipids	4.0 ± 0.4	0.5 ± 0.2
Glycolipids	1.2 ± 0.1	0.5 ± 0.1

[a]Mean of 3 determinations ± standard deviation.

TABLE 6

Fatty Acid Composition of Lipid Classes from Immature and Ripe Cocoa Beans (Criollo)

% by wt[a]	Immature beans (125 days)[b]			Ripe beans (170 days)		
FA	Triglycerides	Phospholipids	Glycolipids	Triglycerides	Phospholipids	Glycolipids
<16:0	–	–	trace	–	–	trace
16:0	23.7 ± 1.0	24.8 ± 0.5	17.8 ± 0.8	23.2 ± 0.2	25.0 ± 0.4	31.2 ± 0.8
16:1	trace	trace	trace	trace	trace	1.3 ± 0.8
18:0	37.4 ± 0.8	4.1 ± 0.1	4.0 ± 0.1	37.8 ± 1.8	10.0 ± 0.2	15.8 ± 0.6
18:1	32.1 ± 0.3	29.0 ± 0.4	21.6 ± 0.8	32.4 ± 0.6	30.9 ± 0.4	23.1 ± 1.1
18:2	4.6 ± 0.4	37.9 ± 0.6	23.8 ± 0.2	4.5 ± 0.3	30.0 ± 0.3	20.3 ± 1.0
18:3	trace	1.7 ± 0.2	31.6 ± 0.3	trace	1.2 ± 0.2	6.8 ± 0.6
20:0	1.0 ± 0.3	1.5 ± 0.3	trace	trace	1.8 ± 0.4	trace

[a]Mean of 3 determinations ± standard deviations.

[b]Days after pollination. Fatty acid composition of total lipid: 16:0 = 23.3; 16:1 = trace; 18:0 = 21.7; 18:1 = 42.0; 18:2 = 10.3; 18:3 = 1.0 and 20:0 = 1.0%, respectively.

seeds (8). The data suggest that plant tissue cultures fail to produce lipids similar to those from seeds, probably because of the lack of differentiation.

Most of the useful products obtained from plants are secondary metabolites produced in highly differentiated cells in plant body. Plant cells in culture are undifferentiated and do not produce these desirable products in useful quantities (28). Therefore, the induction of embryoids from cocoa cells in culture may have potential for the production of typical fats from tissues cultured in vitro.

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In Vitro and In Vivo Effects of Ethanol on the Formation of Endoperoxide Metabolites in Rat Platelets

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ABSTRACT

Preincubation of rat platelet-rich plasma (PRP) with ethanol resulted in dose-dependent inhibition of the formation of endoperoxide metabolites (EPM) when the PRP was aggregated by collagen suspension. The inhibition was manifested at concentrations normally attainable in blood of rats or humans by tolerable amounts of ethanol ingestion. Paradoxically, chronic ingestionof ethanol caused enhanced synthesis of EPM in platelets, indicating that the inhibitory effect of ethanol would be temporary, and that it can be reversed as soon as ethanol is eliminated. The level of arachidonic acid in platelet phospholipids of rats fed the ethanol diet was not different from that of the control, indicating that availability of immediate precursor acid would not be a factor for the enhanced synthesis of EPM in the ethanol group. This result suggested that platelets from rats subjected to chronic ethanol ingestion become hyperactive in synthesizing EPM through an unknown mechanism. When citrated whole blood was incubated in the presence of collagen suspension, amounts of EPM synthesized in the ethanol group were not different from those of the control group, but this was due to significant reduction of platelet counts in the ethanol group. Whether the effect of ethanol on other tissues would be similar to that on platelets is unknown. It is tempting to speculate that some of the pathological changes resulting from alcoholism might be mediated through the effect of ethanol on EPM formation.

INTRODUCTION

It has been shown that chronic or acute ethanol ingestion increases lipid peroxidation (1-3). This was based on increased values of thiobarbituric acid (TBA) reactive chromogen of liver microsomal fraction in rats receiving ethanol, and enhanced amounts of pentane in the expired air of rats which had ingested ethanol as compared to controls. It is not fully understood how ethanol ingestion increases lipid peroxidation. However, it has been generally suggested that increased activity of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase by ethanol ingestion may contribute to enhanced lipid peroxidation (4). Involvement of NADPH oxidase in lipid peroxidation in rat liver microsomes has been demonstrated (5). The oxidation of NADPH by microsomal NADPH oxidase can generate H_2O_2, which can oxidize ethanol to acetaldehyde by the action of catalase. It was shown that xanthine oxidase acting on acetaldehyde caused the peroxidation of linolenate (6) and arachidonic acid (7). The peroxidation of linolenate was due to generation of superoxide anion O_2^- by the system. This peroxidation was inhibited by superoxide dismutase or catalase, implying that both O_2^- and H_2O_2 are involved in the peroxidation. Therefore, it can be speculated that enhanced production of acetaldehyde and increased activity of NADPH oxidase are attributed to enhanced lipid peroxidation by ethanol ingestion.

Arachidonic acid can be metabolized in animal tissues via cyclooxygenase and/or lipoxygenase pathways in which formation of peroxides are involved. In addition, there is indication that alcoholic fatty liver might be associated with a disturbance in prostaglandin (PG) synthesis inasmuch as some PG or endoperoxide intermediates are considered to be endogenous feedback regulators of lipolysis in adipose tissue (8). Therefore, it is an intriguing question whether ethanol could affect arachidonic acid metabolism through the cyclooxygenase pathway. In this study, we determined in vitro and in vivo effects of ethanol on the formation of endoperoxide metabolites (EPM) from the endogenous precursor in rat platelets.

MATERIALS AND METHODS

Platelet Aggregation

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) fed commerical stock diet for 12 weeks were used. Blood was withdrawn from abdominal aortae of rats that were anesthetized with ether, using a plastic syringe containing 0.1 vol of 3.8% trisodium citrate. Platelet-rich plasma (PRP) was prepared by

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centrifuging the blood at 100 G for 10 min at room temperature. Platelet-poor plasma (PPP) was prepared by centrifuging the remaining blood at 1000 G for 10 min. Aggregation of platelets was studied photometrically using a dual-channel Chrono-log aggregometer (Havertown, PA) in siliconized test tubes at 37 C with a continuous stirring rate of 1,200 rpm as described in a previous report (9). Twenty μL of various concentrations of ethanol in Tyrode's buffer (NaCl, 137 mM; KCl, 2.7 mM; $NaHCO_3$, 11.9 mM; NaH_2PO_4, 0.04 mM, pH 7.35) were preincubated with PRP for 3 min prior to the addition of collagen suspension to initiate aggregation. The degree of aggregation in response to collagen suspension was determined from the maximal increase in light transmittance as the percentage of the transmittance of PPP.

Preparation of Samples for Radioimmunoassay

Five min after the addition of collagen suspension, the PRP was immediately centrifuged (1,000 G for 5 min) at 4 C. The platelet-free plasma was then stored at -15 C until it was assayed. The major EPM synthesized in rat platelets was thomboxane B_2 (TXB_2) as was shown for human platelets (10). We have previously reported that the concentration of TXB_2 in PRP aggregated by collagen suspension reached the maximum within 5 min (9). The concentration of TXB_2 remained the same even after 30 min of incubation. This might be due to rapid inactivation of cyclooxygenase by the substrate released from platelet phospholipids as a result of aggregation as shown by Lapetina and Cuatrecasas (11), and the apparent inability of platelets to further metabolize TXB_2. Diluted plasma samples were analyzed for TXB_2 and PGE_2 by radioimmunoassay as described in previous reports (12,13). The TXB_2 antiplasma we prepared showed neglible cross reactivities with major PG, their analogs or precursor acid; PGD_2 showed the highest cross reactivity (0.5%) among compounds tested (20:4ω6, 12-hydroxy-5,8,10,14-eicosatetraenoic acid [12-HETE], 12-hydroxy-5,8,10-heptadecatrienoic acid [HHT], 15-HETE, PGA_2, PGB_2, PGD_2, PGE_1, PGE_2, dihydro-PGE_2, 15-keto-PGE_2, eicosatetraynoic acid [ETYA], PGE_3, $PGF_{1\alpha}$, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, $PGF_{3\alpha}$, U51605). The antiplasma at 1-12,000 final dilution bound 52% of the total radioactivity of tritiated TXB_2 (New England Nuclear, Boston, MA, 120 Ci/mmol) added. Serial dilutions of a plasma sample showed a parallelism with the standard curve. It was shown in a standardization experiment that ethanol at 2,264 mg% in the plasma did not affect the radioimmunoassay; the plasma samples were diluted 20 times before radioimmunoassay.

Animal Care and Diet

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) weighing 150 to 200 g were housed individually in stainless steel cages. They were divided into 2 groups of 7 each. After its overnight starvation, the first group was fed DeCarli/Lieber (14) liquid diet (Bio-Mix #711A, Bio-Serv., Inc., Frenchtown, NJ) containing 10 caloric % ethanol for the first 2 days. The ethanol content in the diet was increased to 20 caloric % for the next 2 days and then subsequently increased to 36 caloric % during the rest of the feeding period. The control group was pair-fed isocaloric liquid diet in which ethanol was replaced with a dextrin-maltose mixture. Ritchter tubes were used to feed and measure liquid diets. The control diet was composed of casein supplemented with methionine (0.3 mg/kcal) and cystine (0.5 mg/kg) giving 18% of the total calories; and the dextrin-maltose mixture giving 47% of total calories. Vitamin and mineral contents of this diet were described elsewhere (15).

After 20-21 days of feeding liquid diets, rats were starved overnight with drinking water available. An aliquot of citrated whole blood withdrawn from the abdominal aorta as already described was incubated for 20 min in the presence of collagen suspension in phosphate buffered saline (PBS, pH 7.0) with constant sitrring at 37 C. Serum was prepared after brief centrifugation (5 min at 100 G) of the incubation mixture. Formation of TXB_2 in this condition reached the maximum within 10 min, and stayed at a plateau even after 30 min. Platelet counts in the citrated whole blood were determined under a light microscope using Unopette (Becton-Dickinson, Rutherford, NJ). Platelet-rich plasma and PPP were prepared as already described. An aliquot of PRP from each rat (control vs ethanol group) was transferred to a siliconized test tube, and the platelet concentration was equalized using PPP. Platelet aggregation, sampling and radioimmunoassay were done as described. The remaining PRP was centrifuged at 1,000 G for 10 min to isolate platelets. Platelets were washed once in PBS-EDTA (0.05 M), and stored at -15 C until they were assayed for fatty acid composition.

Platelet lipids were extracted by the Folch et al. method (16). The chloroform/methanol solution for the extraction contained BHT (0.02%) as an antioxidant. Phospholipids from the total lipids were separated by thin layer chromatography (Silica Gel G plate, Kontes Scientific, Vineland, NJ) using a solvent system

TABLE 1

Effects of Ethanol on the Formation of EPM and Platelet Aggregation in Citrated PRP[a]

Ethanol preincubated (mg %)	TXB_2	PGE_2	Platelet aggregation
	(ng/mL)		(%)
Vehicle	345.5 ± 14.8	45.0 ± 6.4	73.4 ± 1.6
36	308.0 ± 20.7	39.9 ± 5.4	68.2 ± 2.3
72	270.9 ± 58.0	19.3 ± 4.1[b]	73.5 ± 2.3
144	208.4 ± 35.7[b]	18.4 ± 2.7[b]	72.0 ± 1.8
288	150.5 ± 22.7[b]	13.2 ± 2.1[b]	69.8 ± 2.5
566	61.6 ± 14.3[b]	4.1 ± 0.3[b]	58.5 ± 4.1[b]
1132	6.4 ± 1.4[b]	0.2 ± 0.04[b]	23.3 ± 8.3[b]
2264	1.8 ± 0.5[b]	0.4 ± 0.01[b]	0

[a]Values are mean ± SEM (4 to 8 observations for TXB_2; 3 observations for PGE_2; 5 to 8 observations for platelet aggregation).

[b]Significantly different from the control ($p<0.01$, Duncan's multiple range test).

of diethyl ether/petroleum ether/acetic acid (30:70:1, v/v). The phospholipid fraction remaining at the origin was scraped off, and the lipid was eluted from the silica gel using 20 mL of chloroform/methanol/acetic acid mixture (1:1:0.1, v/v). Fatty acid composition of phospholipids was analyzed by gas liquid chromatography (GLC) as described in a previous report (12). Fatty acid methyl esters that were derived from platelet phospholipids from one rat were enough for 2 injections to GLC. Liver triglyceride was extracted by the Folch et al. (16) method and quantitated according to the method described by Fletcher (17) using *trans* trilinolein (99%, Nu-Chek-Prep, Elysian, MN) as a standard.

The blood ethanol level was determined by GLC using a stainless steel column (6-ft) packed with 0.2% Carbowax 1500 on Carbopak, 60/80 mesh (Supelco, PA). The ethanol level of the diluted serum sample was quantitated using isopropyl alcohol as an internal standard.

RESULTS AND DISCUSSION

In Vitro Effects of Ethanol

Preincubation of ethanol with PRP resulted in dose-dependent inhibition of the formation of endoperoxide metabolites in platelets aggregated by collagen suspension as shown in Table 1. The minimal concentrations of ethanol required to inhibit TXB_2 and PGE_2 were 144 and 72 mg %, respectively. These concentrations were reached in the blood (135 mg %) of a rat receiving the liquid diet containing 36 caloric % ethanol. When 10 mL of the liquid diet was administered orally to the rat by stomach tube, blood ethanol level reached 268 mg % in 2 hr. Such inhibitory concentrations of ethanol could also be attained in rat blood after oral administration of ethanol at 2 g/kg (18). In humans, ethanol ingestion to give 90-220 mg % in blood can cause emotional instability (19).

Aggregation of platelets was inhibited only when ethanol concentration exceeded 566 mg %. Blood levels of ethanol in rats receiving an oral, acute dose of ethanol (6 g/kg) did not exceed such concentration (18). In humans, blood levels above 450 mg % can cause death (19). Therefore, the inhibition of platelet aggregation at high concentrations of ethanol does not seem to have physiological significance.

Whether ethanol inhibits EPM formation in tissues other than platelets is unknown. It is known that accumulation of lipids in liver after an acute dose of ethanol to rats is primarily a result of increased mobilization of fatty acids from adipose tissue (20). The mechanism by which an acute dose of ethanol can increase lipolysis is not fully understood, although fatty liver induced by chronic ethanol ingestion is believed to be due to increased lipogenesis in liver as a result of an increased NADH/NAD ratio (20). It has been observed that basal and hormone-stimulated lipolysis in adipose tissue of rats are inhibited by PGE (21), which can be synthesized in adipose tissue (22). Thus, elevated plasma free fatty acids and increased lipolysis in essential fatty acid deficient animals (23,24) could be due to a lack of synthesis of PG having antilipolytic action (25). Accordingly, PG or their intermediates have been suggested as endogenous feedback regulators of lipolysis in adipose tissue (8). If ethanol also inhibits EPM formation in adipose tissue as it

did in platelets, this effect could provide at least part of the biochemical basis for lipid accumulation in liver after acute ethanol ingestion. It is not known how ethanol inhibits EPM formation in platelets. Collier et al. (26) showed that 2,500-5,000 mg % of ethanol increased the formation of PG-like material (based on rat stomach strip bioassay) by 50% from exogenous arachidonic acid in bovine seminal vesicle homogenate. Panganamala et al. (27) showed that ethanol at 2% (v/v, or 1,600 mg %) stimulated PGF biosynthesis from bovine vesicular gland microsomal fraction containing epinephrine and reduced glutathione. It is inconceivable that effects of ethanol at such pharmacological concentrations in cell-free, in vitro systems bear any physiological significance. Pennington and Smith (28) showed that ethanol at or above 0.2 M (920 mg %) resulted in dose-dependent inhibition of TXB_2 formation from exogenous arachidonic acid in unstimulated human platelets. Manku and Horrobin (29), however, showed that ethanol up to 330 mg % did not affect the conversion of exogenous arachidonic acid to TXB_2 by washed human platelets that were not stimulated. Inhibition of EPM formation from the endogenous substrate in rat platelets by ethanol in this study was manifested within concentrations normally attainable in blood of rats or humans by tolerable amounts of ingestion.

In Vivo Effects of Ethanol

Average weight gains of rats in the ethanol and control groups were 2.94 and 3.53 g/day, respectively. These values are comparable to those of the other studies that used a diet with similar composition (14). Liver weight and liver triglyceride of the ethanol group were significantly ($p<0.01$) greater than those of the control group (45.4±2.0 vs 28.1±0.6 g/kg for liver weight; 14.8±3.1 vs 5.8±1.6 mg/g for liver triglyceride), indicating that alcoholic fatty liver was manifested. Platelet counts in the blood of the ethanol group were significantly ($p<0.05$) less than those of the control group (6.43±0.43 vs 7.84±0.38 $\times$ $10^5/mm^3$). Thromocytopenia was also observed in severe alcoholism in humans (30). It is, therefore, necessary to equalize platelet concentrations of PRP of paired groups in order to compare EPM formation by platelets.

Paradoxically, prolonged ingestion of ethanol caused enhanced synthesis of EPM in platelets. Because rats had been starved overnight (15-18 hr) before they were sacrificed, the direct effect of ethanol on platelets was eliminated; no detectable amounts of ethanol were found in sera of rats after starvation. These results indicate that the inhibitory effect of ethanol on the biosynthesis of EPM in platelets is temporary, and it can be reversed as soon as ethanol is eliminated. The level of EPM precursor acid (arachidonic acid) in platelet phospholipids was not different between the 2 groups (Table 2) indicating that difference in availability of immediate precursor acid would not be a contributing factor for the enhanced synthesis of EPM in the ethanol group. Consequently, these results suggested that chronic ethanol ingestion induced platelets to be hyperactive in synthesizing EPM (Table 3). If the effects of ethanol on other tissues are similar to that on platelets, physiological and metabolic responses which are sensitive to endoperoxides or their metabolites would vary with time after alcohol ingestion. Thus, metabolic disturbances which could be caused by the inhibitory effect of ethanol on EPM formation can be reversed as ethanol concentration falls below a certain threshhold concentration. It is notable that the degree of lipid accumulation could be lessened by reducing the amount of ethanol intake (18), and fatty liver was not manifested by chronic alcohol ingestion if the ethanol level in the diet is low (31). It has been shown that lipid accumulation reached a maximum after 15-20 hr of a single dose of ethanol ingestion, and returned to normal after 30-50 hr in rats (32). Ethanol can be cleared from blood after 18 hr of ingestion (18). The dose-dependent fashion and reversibility of alcoholic fatty liver mimicked the

TABLE 2

Effects of Chronic Ethanol Feeding on the Fatty Acid Composition of Platelet Phospholipids[a,b]

Fatty acids	Control	Ethanol
16:0	30.5 ± 0.8	27.4 ± 0.8
16:DMA	8.0 ± 0.7	7.0 ± 0.6
16:1	2.4 ± 0.4	3.0 ± 0.2
18:0	12.9 ± 1.9	10.8 ± 0.8
18:DMA	5.0 ± 0.2	4.7 ± 0.5
18:1ω9	10.2 ± 1.1	12.4 ± 2.1
18:1DMA	6.6 ± 0.7	8.8 ± 0.5
18:2ω6	5.0 ± 0.4	4.9 ± 0.8
18:3ω6	0.3 ± 0.03	0.6 ± 0.07
18:3ω3	0.3 ± 0.1	0.2 ± 0.03
20:2ω6	0.3 ± 0.02	0.4 ± 0.05
20:3ω6	0.2 ± 0.02	0.1 ± 0.01
20:4ω6	19.0 ± 1.0	18.2 ± 0.7
22:4ω6	1.7 ± 0.04	1.4 ± 0.1

[a]Values are mean ± SEM (5 observations for control; 6 observations for ethanol group, wt %).

[b]Dimethyl acetals (DMA) were tentatively identified based on their retention times.

TABLE 3

Effects of Chronic Ethanol Feeding on the Formation of EPM During the Collagen-Induced Aggregation of Citrated PRP[a]

Rat number	TXB_2		PGE_2	
	Control	Ethanol	Control	Ethanol
		(ng/mL)		
1	130.0	163.3	9.4	21.2
2	116.6	176.2	10.4	21.6
3	460.8	444.5	56.1	61.3
4	208.4	413.9	22.4	67.4
5	292.6	504.2	33.6	76.6
6	192.1	322.2	14.9	36.1
7	256.4	452.0	17.9	59.3
Mean ± SEM	236.7 ± 44.3	353.8 ± 51.8	23.5 ± 6.2	49.1 ± 8.5[b]

[a]Platelet concentrations of paired PRP were equalized using PPP.

[b]Significantly different from the control ($p<0.01$).

TABLE 4

Effects of Chronic Ethanol Feeding on the Formation of EPM by Platelets in Citrated Whole Blood Incubated in Presence of Collagen Suspension

Rat number	TXB_2		PGE_2	
	Control	Ethanol	Control	Ethanol
		(ng/mL)		
1	94.0	110.6	0.5	0.6
2	106.8	128.7	0.4	0.6
3	145.4	129.6	1.9	1.4
4	83.6	132.0	1.0	1.5
5	122.6	107.9	0.6	0.7
6	90.8	68.8	0.6	0.3
7	83.8	105.7	1.5	0.8
Mean ± SEM	103.8 ± 8.7	110.6 ± 7.3	0.9 ± 0.2	0.8 ± 0.2

effect of ethanol on EPM formation in platelets, although this does not necessarily prove direct involvement in alcoholic fatty liver of EPM or their intermediate.

It is unknown why chronic ethanol ingestion induced platelets to be hyperactive in synthesizing EPM, whereas ethanol itself directly inhibited the formation of endogenous EPM. When citrated whole blood was incubated in the presence of collagen suspension, amounts of EPM synthesized in the ethanol group were not different from those in the control group, as shown in Table 4. Apparently, reduced platelet concentration in the ethanol group compensated hyperactivity of platelets.

In summary, the effect of ethanol on the biosynthesis of EPM in rat platelets was unique in that ethanol itself was inhibitory, whereas prolonged ingestion induced platelets to be hyperactive in synthesizing EPM. Excessive, lack of, or imbalanced synthesis of EPM in tissues could trigger development of certain pathological conditions which may not be manifested clinically at the initial state of development. Our results warrant further studies for the effect of ethanol on arachidonic acid metabolism in various tissues in order to understand the biochemical basis for pathological changes resulting from alcoholism.

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Structure of Biliary Phosphatidylcholine in Cholesterol Gallstone Patients

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ABSTRACT

The fatty acid composition of biliary phosphatidylcholine was analyzed in 13 patients with radiolucent gallstones undergoing elective cholecystectomy, and in 11 normolipemic patients without gallstones undergoing abdominal surgery. The only difference in the percentage fatty acid composition between the two groups was a significantly ($p<0.05$) higher percentage arachidonic acid in the first group. This acid was exclusively located in the *sn*-2 position of phosphatidylcholine (PC), accounting for 13.0 ± 4.9% in the first group and 8.2 ± 4.9% in the second ($p<0.05$). The percentage arachidonic acid of PC was negatively correlated ($p<0.001$) with the percentage biliary chenodeoxycholate in gallstone patients, but not in controls. Explanation of these findings is, at present, only speculative.

INTRODUCTION

The formation of cholesterol (Ch) gallstones is thought to result from changes in the relative composition of bile lipids. According to current concepts, gallstones form when the Ch concentration in bile is increased vs that of bile salt (BS) and phosphatidylcholine (PC). The influence of structural changes in biliary phospholipids is not completely understood. Few data are available on the composition and structure of biliary PC (1-3) and on their importance in determining the detergent effect of PC in bile (4,5). Moreover, there appears to be no comparative studies on the fatty acid (FA) composition and distribution of biliary PC between healthy subjects and patients with Ch gallstones.

This report deals with the positional distribution of the individual FA of biliary PC in patients with and without Ch gallstones undergoing, respectively, cholecystectomy or abdominal surgery.

METHODS

Patients

Studies were performed on 13 normolipemic patients with radioluscent gallstones undergoing elective cholecystectomy (group A) and 11 normolipemic patients without gallstones (controls) undergoing abdominal surgery (group B).

Group A consisted of 5 males and 8 females, ranging in age from 28 to 68 years. Upon chemical analysis, stones were 70 ± 10% (range 58-97%) Ch.

Group B was composed of 5 males and 6 females, ranging in age from 32 to 65 years, of whom 8 submitted to gastrectomy for gastric ulcer and 3 to partial cholectomy for colonic cancer.

None of the patients studied presented clinical or biochemical evidence of liver disease. All patients were hospitalized 1 week before surgery, during which period they all received the same regular hospital diet. Furthermore, none received antibiotics or drugs known to affect lipid metabolism. Surgery was performed in all cases between 8 and 11 a.m. after at least 12 hr of fasting.

Upon access to the biliary region, 2-5 mL of bile was collected directly from the gall bladder by needle aspiration using a sterile technique.

Two mL of bile was immediately poured into a graduated cylinder containing 40 mL of a chloroform/methanol (2:1, v/v) mixture and the cylinder was vigorously shaken.

Six mL of aqueous NaCl 0.15 M were added and the cylinder was again shaken. After separation of the two layers, the upper phase was dicarded and total phospholipids were measured in the lower phase as inorganic phosphorus according to Bartlett (6).

Analysis of the FA Composition of PC

An aliquot of 0.5-1.0 mL of the lower chloroform phase was loaded as a long band on a precoated silica gel plate for thin layer chromatography (TLC) (Merck, Darmstadt, W.G.).

The plate was developed in a chloroform/methanol/water/ammonium hydroxide (65:35:4:0.25, by vol) solvent system.

The bile PC was visualized by iodine vapors and identified by comparison with a standard of egg PC (Supelco Inc., Bellefonte, PA). The PC band was scraped off the plate, poured into a filter tube with fritted disc, medium porosity,

sealed in and eluted by 6 mL of a chloroform/methanol (2:1, v/v) mixture. The solvent was collected into a glass stoppered tube and dried under nitrogen. The purified extract was redissolved in 1 mL of benzene and used to determine the total FA composition after transesterification of PC with 1 mL of 2 N sodium methoxide in methanol (Supelco Inc., Bellefonte, PA). The mixture was heated for 10 min at 80 C, cooled at room temperature and, after addition of 5 mL of water, extracted twice with 5 x 2 mL of petroleum ether (bp 40-50 C). The extract was concentrated under nitrogen and used for gas liquid chromatography (GLC). Analysis of FA methyl esters was performed with a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector and a glass column (6 ft x 0.25 in. id) packed with 10% DEGS-PS on Supelcoport, 80-100 mesh (Supelco Inc., Bellefonte, PA). The following operation conditions were used: oven temperature 185 C, carrier gas (nitrogen) at flow rate of 28 mL/min.

The gas chromatograph was calibrated and checked daily with an American Oil Chemists' Society oil reference mixture RM-3 (Supelco Inc., Bellefonte, PA).

Positional Distribution of FA in PC

An aliquot of 5 mL of the lower chloroform phase was used to determine the positional distribution of FA in PC. The solvent was dried under reduced pressure and analyses were carried out according to the procedure described by Robertson and Lands (7) using phospholipase A_2 from Naya Naya snake venom (Calbiochem, La Jolla, CA).

Analysis of Bile Salts and Cholesterol

Conjugated BS were enzymatically hydrolyzed in fresh bile according to Nair and Garcia (8) and extracted in acidified diethyl ether. Bile acids and cholesterol were simultaneously fractionated by GLC after preparation of methyl ester trimethyl-silyl-ether derivatives (9), and quantitated against 5α-cholestane (Supelco Inc., Bellefonte, PA).

Bacteriology

Samples of undiluted gall bladder bile obtained under sterile conditions were cultured for anaerobic and aerobic bacteria and examined under light microscopy. Anaerobic bacteria were identified according to the manuals of the Virginia Polytechnic Institute. Aerobic bacteria were identified by conventional methods.

Statistical Analysis

Statistical studies of correlations and differences between means were carried out as described by Snedecor and Cochran (10).

RESULTS

The total FA composition and positional distribution of the biliary PC are shown in Table 1. Palmitic acid was the most prevalent FA of PC, accounting for 40-50% of total FA in each group. No significant differences were

TABLE 1

Mean Percent Fatty Acid Composition (± SD) of Biliary PC

FA	Total FA		*sn*-1 position		*sn*-2 position	
	Gallstone patients	Controls	Gallstone patients	Controls	Gallstone patients	Controls
16:0	45.8	48.9	74.0	75.5	17.5	22.3
	(6.1)	(9.2)	(6.8)	(6.6)	(11.3)	(16.6)
16:1	2.6	3.6	3.1	3.3	2.2	4.0
	(0.9)	(1.3)	(1.1)	(0.9)	(1.5)	(2.7)
18:0	5.4	5.2	10.0	9.9	0.9	0.6
	(1.3)	(0.8)	(2.1)	(1.9)	(2.0)	(0.9)
18:1	12.9	13.4	9.3	8.8	16.5	18.0
	(2.4)	(2.9)	(5.3)	(4.6)	(6.0)	(4.9)
18:2	24.5	23.1	4.2	2.3	45.7	43.9
	(3.8)	(7.4)	(3.1)	(1.5)	(7.6)	(14.2)
18:3	0.8	0.4	0.1	tr	1.5	0.8
	(0.6)	(0.4)	–	–	(1.3)	(0.8)
20:3	1.4	1.0	tr	tr	2.7	2.0
	(0.9)	(0.7)	–	–	(1.7)	(1.5)
20:4	6.5*	4.1	tr	tr	13.0*	8.2
	(2.3)	(2.5)	–	–	(4.9)	(4.9)

*$p < 0.05$.

TABLE 2

Mean Concentration of Bile Salts, Cholesterol and Phosphatidylcholine (left side) and Relative Bile Salt Composition (right side) in Bile of Cholesterol Gallstone Patients and Controls

	Gallstone patients	Controls		Gallstone patients	Controls
			C%	37.2 ± 10.1	40.4 ± 8.0
Bile salts mmol/L	109.9 ± 69.0	126.4 ± 54.9	CDC%	46.6 ± 7.2	43.1 ± 8.5
Cholesterol mmol/L	22.5 ± 12.9	23.2 ± 13.6	DC%	14.2 ± 10.5	14.4 ± 11.6
Phosphatidylcholine mmol/L	47.5 ± 17.9	63.5 ± 19.4	L%	1.2 ± 1.4	1.4 ± 1.7

Results are expressed as means ± SD.
C=cholate; CDC=chenodeoxycholate; DC=deoxycholate; L=lithocholate.

observed in the total FA composition between group A and B, except for the arachidonic acid, which was significantly higher ($p<0.05$) in group A. With respect to the positional distribution of FA, the only difference was found in position *sn*-2, in which the percentage arachidonic acid was significantly higher ($p<0.05$) in gallstone patients than in controls.

The mean concentrations of BS, Ch and PC in bile as well as the relative BS composition are shown in Table 2. No significant differences were observed between the two groups with respect to any of these parameters.

A negative correlation ($r=0.8660$, $p<0.001$) between the percentage arachidonic acid of PC and percentage chenodeoxycholate in bile was found in group A, but not in group B (Fig. 1). No correlation was found between the PC composition and the biliary lipid concentrations.

Three of the 13 gallstone biles and 1 of the 11 nongallstone biles had a significant bacterial growth (two aerobic, one anaerobic and one mixed infection). Bacterial infection of bile was not related to changes in the biliary composition of PC or BS.

DISCUSSION

The total FA composition of biliary PC in this investigation is very similar in gallstone and nongallstone patients, which is in keeping with previous findings (11,12). Analysis of the positional distribution of FA within biliary PC in a large series of patients confirms the prevalence of saturated FA in the *sn*-1 position (85%) and of unsaturated FA in the *sn*-2 position (80%). Thus, at least 15-20% of the PC

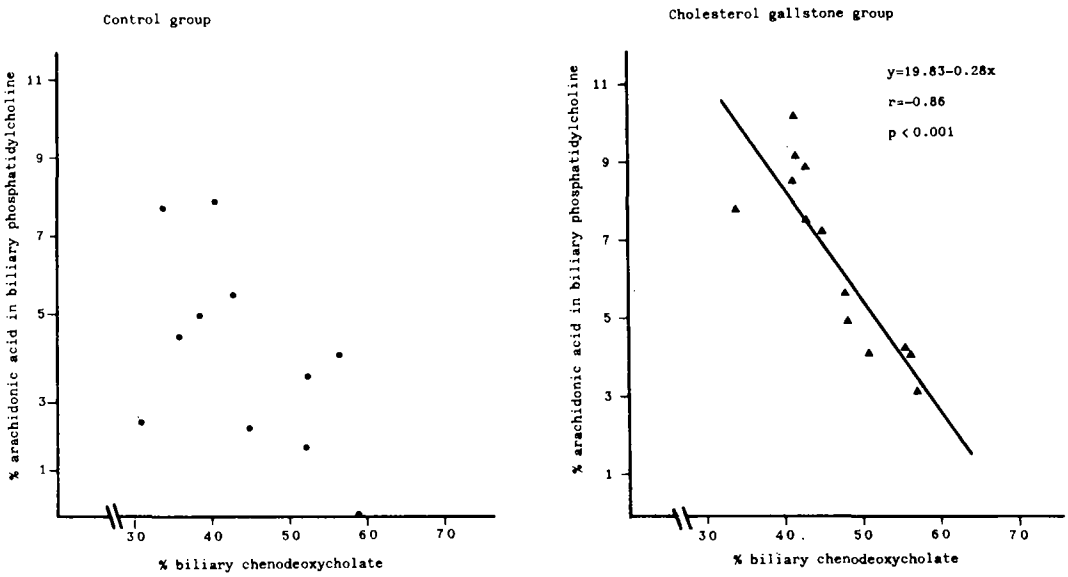

FIG. 1. Correlation between percent arachidonic acid in biliary phosphatidylcholine and percent biliary chenodeoxycholate in control group and in patients with cholesterol gallstones.

molecules in bile appear to have uncommon structures; it is not yet known, however, how FA are coupled.

Data from this study indicate that the arachidonic acid content in biliary PC of gallstone patients is higher than in patients without gallstones. The distributional analysis reveals that this acid is exclusively located in the *sn*-2 position of PC.

The differences in the bile arachidonoyl-PC content between gallstone and nongallstone patients may be due to contamination with plasma PC, which, as is well known (11,12), is richer in arachidonic acid than bile PC, or to the shedding of inflammatory of mucosal cells into the bile within a diseased gall bladder. However, no aerobic or anaerobic infection of bile was found in those patients with higher amounts of arachidonoyl-PC. Although the presence of a certain degree of aseptic cholecystitis cannot be excluded, this seems unlikely, as our patients presented no specific symptoms in the recent history and no inflammatory cells were detected in the bile specimens.

The type of diet might also influence the FA composition of biliary PC (13,14), in that the ratio between linoleic and arachidonic acid of PC rises when polyunsaturated FA are increased in the diet (13). During this study, however, patients were on the same hospital diet and, furthermore, no negative correlation was observed between the linoleic and arachidonic acid percentages in PC.

Thus, the reason for the higher arachidonoyl-PC content in bile of cholesterol gallstone patients should be sought elsewhere, presumably within the liver.

Although the origin of bile PC has not yet been fully elucidated (15,16,17), the secretion of PC into bile is thought to be bound to BS secretion, either as a result of their membrane-solubilizing properties (18,19) or as a result of their different micelle-forming properties (20, 21). It is tempting to hypothesize that the flow of BS within the liver cell might also influence the acyl composition of bile PC. Our results show that (a) no significant differences in biliary BS patterns exist between gallstone and nongallstone patients, and (b) a significant negative correlation exists between the percentage arachidonic acid of PC and the percentage chenodeoxycholate in bile within the gallstone group (Fig. 1). The correlation, however, in spite of a similar distribution of chenodeoxycholate percentages, does not exist in the control group. This finding could be either coincidental or could indicate a linkage between chenodeoxycholate and arachidonoyl-PC secretion in bile; however, since, in the present investigation, only the gall bladder bile concentration of chenodeoxycholate was measured, and not the actual secretion, it is not possible to offer a definite explanation.

ADDENDUM

Some of our data (predominance of palmitic acid in position *sn*-1 and of linoleic acid in position *sn*-2, and a higher presence of arachidonyl-PC in gallstone patients) seem to be, at least partially, confirmed by a recent paper (Ahlberg et al., J. Lipid Res. (1981) 22, 404-409). By converse, we failed to observe the higher proportion of oleoyl-PC found by these authors in gallstone patients.

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Essential Fatty Acids in Trout Serum Lipoproteins, Vitellogenin and Egg Lipids

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ABSTRACT

This paper describes evidence of (n-3) and particularly of 22:6 (n-3) fatty acid enrichment in trout lipoproteins as well as in vitellogenin, egg lipovitellin and oil globule. Among the lipoproteins, HDL and LDL were the main forms of blood lipid transport, whereas phospholipids and cholesteryl esters are the preferential chemical carriers for (n-3) fatty acid transport. However, cholesteryl esters were less important as esterified fatty acid carriers than in man. Taken together with the data obtained in mammals, our results suggest that there may be a relationship between EFA activity and the distribution of the EFA among the lipoprotein lipid fractions in vertebrates, irrespective of the EFA series. Administration of an (n-3) fatty acid deficient diet for three months prior to trout spawning produced a significant increase in egg lipid content, primarily as a result of the increase of the oil globule composed almost exclusively of triacylglycerols. This diet decreased the 22:6 (n-3), as well as the (n-3) fatty acid contents of lipoproteins, lipovitellin, vitellogenin and the oil globule. In contrast, the (n-3) fatty acid level was always higher in lipoproteins and lipovitellin than in the vitellogenin and the oil globule. Moreover, the relative levels of 22:6 (n-3) and total (n-3) fatty acids were quite similar in lipoproteins and lipovitellin on the one hand, and in vitellogenin and the oil globule on the other. These findings suggest a direct relationship between the two forms of plasma lipid transport and the two egg compartments. During ovogenesis, dietary lipids seemed to be diverted from the adipose tissue and essentially deposited in the egg.

ABBREVIATIONS

C = cholesterol, CE = cholesteryl esters, PL = phospholipids, OG = oil globule, LV = lipovitellin, TAG = triacylglycerols, VLDL = very low density lipoproteins, HDL = high density lipoproteins, LDL = low density lipoproteins, EFA = essential fatty acids.

INTRODUCTION

Knowledge of the fatty acids essential in fish has progressed considerably in the last few years (1,2). The efficiency of 18:3 (n-3) (linolenic acid) as an EFA appears to depend on the bioconversion capacity of the desaturase system of the animal (3-5). It is clear that fish possessing insufficient Δ6-, Δ5-, and Δ4-desaturase enzyme capacity must find the highly polyunsaturated (n-3) fatty acids ($C \geq 20$, called [n-3] HPUFA) which they are incapable of synthesizing from 18:3 (n-3) in their diet. The (n-3) HPUFA could also play an important role in fish reproduction and in their embryonic or larval development. The 22:6 (n-3) could be a crucial factor in the hatching of carp eggs (6) and might be preferentially released from the vitellus yolk into the sac-fry in brook trout (7). When 18:3 (n-3) is the only source of dietary lipid, the normal processes of reproduction and development are ensured in trout (8).

In recent years, many studies have been devoted to the understanding of lipid transport (9). Circulating lipoproteins, classically subdivided into VLDL, LDL and HDL, are lipid-carrying macromolecular components of the blood plasma of mammalian as well as non-mammalian vertebrates (10). Vitellogenin, however, is a specific form in which plasma lipids may be transported and is encountered in mature oviparous female and estrogen-injected male species of amphibia, birds and fish (11-16). It is synthesized by the liver and seems to be the precursor of two egg components: the lipovitellin, a proteolipid containing more protein than lipid constituents; and the phosvitin, a serine-rich phosphoprotein. Moreover, fat depots exist in eggs at the final step of vitellogenesis. They customarily form a unique droplet called the oil globule (17), which is composed almost exclusively of triacylglycerols. The oil globule is essentially the counterpart of the fat depots in the adipocyte.

In attempting to obtain further information on EFA in trout, we have compared the (n-3) fatty acid contents of the different plasma lipoproteins and of the different lipid classes of plasma lipoproteins in fish fed a commercial diet. We have also examined the respective effects of (n-3)-deficient and non-(n-3)-deficient diets on egg lipid distribution and on the fatty acid composition of total plasma lipoproteins, vitellogenin, adipose tissue, the oil globule and lipovitellin.

MATERIALS AND METHODS

Plasma lipoproteins were analyzed in sexually active males weighing 100-120 g at their first spermiation in January 1979. Blood was withdrawn 3 hr after the last feeding. Table 1 shows the fatty acid composition of the commercial diet A fed to the fish. The water temperature was 17 C.

We used 2 groups of 6 females (average wt 810 g), held at a water temperature of 10-12 C, for the study of the fatty acid composition of vitellogenin and egg lipids and for determination of the distribution of different classes of egg lipids. The first group (CLO) was fed an experimental diet containing 10% of cod liver oil; the second group (L) received the same diet, but the CLO was replaced by non-(n-3)-fatty acid supplemented lard. The gross and fatty acid compositions of the CLO and L diets are presented in Tables 1 and 2. The female trout were fed from September 15 (eggs were 1/5 developed as determined by the volume ratio) to the spawning period (November 17, 1979-January 5, 1980). They were starved for 2 days before sampling; blood was withdrawn and the perigastic adipose tissue (PAT) was removed as already described (18).

Ripe eggs were removed from trout ovaries, lyophilized and their dry weight determined. The total lipids were extracted according to the Folch et al. procedure (19). An adequate volume of a 0.75% NaCl solution was added to obtain a chloroform/methanol/water (8:4:3, v/v/v) solvent mixture. The methods for PAT lipid extraction and for the isolation and chemical analysis of plasma lipoproteins have been previously reported (18,20).

The following procedure was used to determine the respective amounts of oil and yolk globule lipids. The eggs (1-g samples) were ground in 3 mL of a solution containing 0.73% NaCl and 0.005% BHT. After removing the egg membranes, the solution was centrifuged at 15,000 G for 40 min. The top layer of the tube corresponding to the oil globule was then removed by aspiration, diluted in the same salt solution, and centrifuged again. The infranatant solutions (IS) of the first and second centrifugations, representing the yolk globule, were pooled (IS 1). The triacylglycerol contents of the yolk globule and of the whole egg were

TABLE 1

Fatty Acid Composition of the Diets

Diet	A[a]	CLO[b]	L[b]	B[a]
14:0	3.1	3.7	1.5	3.3
16:0	13.6	11.7	26.7	29.9
18:0	3.2	2.6	19.3	9.7
20:0	2.9	0.1	–	0.6
Sat.	23.6	19.4	48.0	45.3
16:1 (n-7)	3.2	10.5	1.7	3.7
18:1 (n-7)	–	4.1	2.0	2.2
20:1 (n-7)	–	0.6	t	–
Σ (n-7)	3.2	15.4	3.7	5.9
18:1 (n-9)	19.5	15.2	31.6	12.1
20:1 (n-9)	5.0	9.9	1.0	2.2
22:1 (n-9)	8.7	6.4	–	1.8
Σ (n-9)	33.2	31.5	32.6	16.1
18:2 (n-6)	28.4	6.6	12.0	18.8
18:3 (n-6)	t	0.2	t	–
20:2 (n-6)	0.1	0.3	0.3	0.2
20:3 (n-6)	0.1	t	t	–
20:4 (n-6)	0.1	0.5	0.2	0.6
Σ (n-6)	28.7	7.6	12.5	19.7
18:3 (n-3)	4.5	1.3	1.1	2.6
18:4 (n-3)	0.9	1.8	t	0.8
20:3 (n-3)	–	0.1	0.1	–
20:4 (n-3)	0.2	0.8	t	–
20:5 (n-3)	1.5	9.3	0.6	4.5
22:5 (n-3)	0.2	1.0	–	0.5
22:6 (n-3)	2.9	10.0	1.0	4.6
Σ (n-3)	10.2	24.3	2.8	13.0

[a]A and B are commercial diets (Aqualim and Trouvit containing 10% and 8.5% lipid, respectively).

[b]CLO (cod liver oil) and L (lard) diets contained 10% added lipid; replicate diet lots did not show more than minor differences in fatty acid composition.

TABLE 2

Composition of the Cod Liver Oil and Lard-Supplemented Diets

Components[a]	p. 100
Casein	15
Blood powder	5
CPSP[b]	10
Wheat	30
Soya meal	20
CLO or L lipid	10
Vitamin mixture[c]	2
Mineral mixture[d]	2
Cellulose	4
Lignosulfite	2

[a]The diets contained 38% protein.

[b]CPSP: soluble fish protein concentrate containing 83% protein as compared to wet weight (water solubility: 75 to 80%).

[c]The composition of the vitamin mixture was: DL α-tocopherol 200 mg, vitamin K 0.5 mg, B_1 1.5 mg, B_2 3 mg, B_6 1.5 mg, nicotinic acid 17.5 mg, vitamin C 50 mg, folic acid 0.5 mg, vitamin B_{12} 10 mg of concentrate at 500 mg/kg, biotin 25 mg, Ca pantothenate 5 mg, choline at 50% 400 mg, vitamins A and D_3 were added only to the L diet mixture in order to adjust the quantities to those supplied by the CLO in the CLO diet: A, 15 mg at 500,000 IU/g and D_3, 7.5 mg at 100,000 IU/g. Sufficient quantities of cellulose were added to adjust the mixture weight to 2 g.

[d]The composition of 1 g of the mineral mixture was: $CaCO_3$ 212 mg, $MgCO_3$ 124 mg, KCl 90 mg, Fe citrate 20 mg, $CaHPO_4$, $2H_2O$ 500 mg, NaCl 40 mg, $CuSO_4$ 3 mg, $ZnSO_4$ 8 mg, $CoSO_4$ 0.02 mg, $MnSO_4$ 3 mg, KI 0.04 mg.

TABLE 3

Plasma LP and Esterified Lipoprotein FA in Male Trout at First Spermiation (see text for details)

Total LP[a]		2420[b]		100[b]		100[b]	
VLDL (d<1,015 g/mL)		170		7.0		11[c]	
LDL (1,015<d<1,085 g/mL)		880		36.4		47[c]	
HDL (1,085<d<1,21 g/mL)		1370		56.6		42[c]	
Total esterified FA		993[d]		41.0		36.9	
in VLDL	TAG	112	91.2	4.6	3.8	7.1	5.3
	PL		17.2		0.7		1.2
	CE		3.8		0.1		0.6
in LDL	TAG	396	225	16.4	9.3	18.6	4.5
	PL		124		5.1		6.6
	CE		46.8		2.0		7.5
in HDL	TAG	485	133	20.0	5.5	11.2	2.4
	PL		312		12.9		6.3
	CE		39.8		1.6		2.5

[a]Traces of chylomicrons.

[b]The first column represents the values in mg/100 mL of plasma; the second column represents these values in percentages of the total LP; the third column presents indicative percentages in man calculated from the data of references 21 and 22.

[c]Mean values of men and women aged 16-29 years (see ref. 21).

[d]The values of the esterified FA were calculated from the percentages of TAG, PL and CE in the three classes of LP (i.e., 59.6, 15.8 and 5.3 in VLDL, 28.1, 22.1 and 12.1 in LDL, 10.8, 35.6 and 6.6 in HDL, respectively) and by using the values of the ratio FA/FA esters obtained from the average molecular weight of fatty acid in each lipid class (0.90, 0.91 and 0.90 for TAG, 0.64 for PL and 0.43, 0.44 and 0.44 for CE of VLDL, LDL and HDL, respectively).

determined by an enzymatic procedure (Boehringer, Mannheim GmBH), thus avoiding the less accurate direct assay of oil globule triacylglycerols.

Since only the lipovitellin provided the yolk globule lipids, and since the oil globule is composed almost exclusively of triacylglycerols, the following relationships are proposed:

TAG from OG = OG = *total egg TAG - TAG from LV*
LV lipids = *total egg lipids* - TAG from OG
LV non-TAG lipids = $(C + CE + PL)_{LV}$ = LV lipids - *TAG from LV*
Egg non-lipid components = *egg dry weight* - (OG + LV lipids)
The italics show the data obtained by direct assay.

Vitellogenin was obtained as follows. After adding BHT (0.005%) and the usual antibacterial agents (20), the density of the serum sample was raised to 1.21 g/mL by the addition of a suitable volume of an NaCl-KBr solution of d = 1.34 g/mL, and centrifuged at 45,000 rpm (145,000 G) for 48 hr. After removing the lipoproteins in the top layer (d<1.21 g/mL) of the tube, the infranatant solution (d>1.21 g/mL) was collected (IS 2). The usual precipitation in double-distilled water for further purification of vitellogenin was intentionally omitted in order to avoid any oxidation of the polyunsaturated fatty acids.

Lipid extracts from lipovitellin and vitellogenin were obtained from IS 1 and IS 2, respectively, according to the Folch et al. procedure (19) with a minimal solvent/sample ratio of 20:1 (v/v).

Preparation of fatty acid methyl esters from lipid extracts has been reported previously (18). Esters were submitted to gas chromatographic analysis using a glass wall-coated open-tubular column (0.35 mm id x 46 m). The general preparative procedure for coating the column has already been reported (18). The stationary phase used in the present report was FFAP from Supelco, Inc. (Bellefonte, PA).

RESULTS AND DISCUSSION

As shown in Table 3, the plasma lipoprotein content of male trout was very high, confirming previous data (10,20). There is a great difference in the total lipoprotein contents between trout and mammals such as man (21) and rat (23). HDL was the principal class of lipoproteins found in this study, confirming the works of Chapman et al. (10) and Léger et al. (24) on adult trout (female and male) and on immature trout, respectively, and the work of Nelson and Shore (25) on pre-spawning

salmon. This type of HDL distribution is not markedly different from that of some mammals (23). This result was, however, contrary to that reported in Barclay's review (21) for normal human subjects, and to that of Frémont et al. (20), who showed a predominance of LDL in adult male trout 3-4 months after the spawning period. In order to shed some light on fish lipoprotein metabolism, further work is in progress to determine to what extent the stage of sexual maturation affects the distribution of LDL and HDL in fish.

Table 3 shows that the percentages of VLDL, LDL and HDL are 7, 36 and 57%, respectively, and that the esterified fatty acid content of the total lipoproteins is 41%. The values reported for man are very different. For every 100 mg of trout lipoproteins, 4.6 mg of esterified fatty acids were transported by VLDL, 16 mg by LDL and 20 mg by HDL. It is interesting to note that about one-half of the esterified fatty acids are present in trout HDL. The greatest proportion of esterified fatty acids (about one-half) in man is encountered in LDL.

In trout, a major part of the esterified fatty acids were present as triacylglycerols in VLDL and LDL and phospholipids in HDL. About 45% of the total esterified fatty acids were transported as phospholipids and 45% as triacylglycerols in the total lipoproteins, and about one-third as HDL phospholipids. In man, these values are 33, 35 and 17%, respectively. Fatty acids as cholesteryl esters represented hardly 10% of the total esterified fatty acids in trout plasma, as against 29% in man.

It is evident from these comparative data that more blood lipids are present in trout than in man. Also, more fatty acids are transported by triacylglycerols and phospholipids in trout than in humans where the part of the fatty acids transported by cholesteryl esters is much more elevated.

The distribution of (n-6) and (n-3) fatty acids among the esterified lipids of the lipoprotein classes is shown in Table 4. There were 3.4 times more (n-3) fatty acids than (n-6) fatty acids in total lipoproteins, whereas the proportion in the diet was the opposite. The phospholipids quantitatively represented the most important form of transport of the (n-3) series and especially of the 22:6 (n-3). From a qualitative point of view, however, both phospholipids and cholesteryl esters were pathways for docosahexaenoic acid transport as shown by the percentages in the next column, higher than those cited in Chapman's review (23). As compared with the high levels of arachidonic acid in rat lipoprotein phospholipids and cholesteryl esters in particular, these results strongly suggest that there may be a relationship between EFA activity and distribution of the EFA among the lipoprotein lipid fractions, irrespective of the EFA series.

These results also suggest that dietary fatty acids may be entirely responsible for the discrepancy between mammalian and trout

TABLE 4

Contents of (n-3) and (n-6) Polyunsaturated FA in Serum Lipoproteins of Male Trout at First Spermiation (see text for details)

FA		(n-6)		(n-3)		22:6 (n-3)		$\frac{22:6\ (n\text{-}3)}{\text{total FA}} \times 100$
in total LP		111[a]		381[a]		306[a]		–
in VLDL[b]	TAG	14.2	12.2	32.5	24.2	23.0	15.8	17.4
	PL		1.6		6.7		5.8	33.9
	CE		0.4		1.6		1.4	36.2
in LDL[b]	TAG	45.8	30.4	156	76.0	124	54.3	24.2
	PL		10.5		56.8		50.3	40.6
	CE		4.9		23.6		19.8	42.1
in HDL[b]	TAG	51.3	18.1	193	38.2	159	25.4	19.1
	PL		29.0		136		118	37.8
	CE		4.2		18.6		15.2	38.1
$\frac{\text{FA} \times 100}{\text{(FA in the 3 esterified lipid classes}}$	TAG	54.6		36.3		31.2		–
	PL	36.9		52.3		56.9		
	EC	8.5		11.4		11.9		–

[a]In mg/100 mL of plasma. See footnote c of Table 3 for method of calculation.

[b]For characteristics of densities see Table 3.

TABLE 5

Ratio of (n-6)/(n-3) Compared to That of the Respective Diets in Serum of Male Trout and in Pig

Animals	Diet	Blood components		
		TAG	PL	CE
Trout	2.8	0.43	0.20	0.24
Pig[a]	0.62	0.75	0.54	2.6
	17	19	15	18

[a]Data from ref. 26.

fatty acid compositions. In this respect, two studies concerning pig and human plasma lipids are of considerable interest in order to demonstrate that this explanation is not the right one. Ruiter et al. (26) showed that a relatively low (n-6)/(n-3) ratio exists in the blood serum of young growing pigs on a diet containing mackerel oil (Table 5). This was not brought about either by an increased level of 22:6 (n-3) serum content, or by a decreased level of very high content of 18:2 (n-6), but it was the result of an increased level of 20:5 (n-3) in a particular manner. In humans, a particular case is shown by the Greenland Eskimos (27) who eat significantly higher quantities of (n-3) fatty acids than Eskimos living in Denmark (deduced from data in ref. 27. Although no details are given concerning the fatty acid composition of the diet, it is clear that it contains large proportions of 22:6 [n-3]). Only small quantities of 22:6 (n-3) were present in all lipid classes of plasma whereas the 20:5 (n-3) is the major (n-3) polyunsaturated fatty acid, and is particularly abundant in cholesteryl ester fractions. We conclude from these data that, *under similar dietary conditions*, the fatty acid composition of plasma lipoproteins are very different in mammals and trout. Furthermore, the 22:6 (n-3) acid in fish could be considered as the counterpart of the 20:5 (n-3) in mammals, even though the 22:6 (n-3) is the counterpart of the 20:4 (n-6) from a strictly biochemical viewpoint, since they represent major products of the bioconversion of 18:3 (n-3) and 18:2 (n-6), respectively.

Egg lipids were found in two morphologically distinct forms which were easily separated by centrifugation: (a) the oil globule is almost exclusively composed of triacylglycerols with traces of cholesterol and cholesteryl esters as shown by a TLC method (not given in the present paper), and (b) the yolk globule includes lipovitellin and phosvitin. The lipovitellin contained the same lipid forms as the serum lipoproteins, but they were associated with about 80% protein, thus resembling the

TABLE 6

Ripe Egg Composition from Trout Fed Either an (n-3) Fatty Acid Enriched (CLO) or an (n-3) Fatty Acid Deficient Diet (L) (see text)

Diet	Wet wt	Humidity	Dry weight	Total lipids	OG lipids[a]	LV lipids	TAG of LV	Nonlipid comp.	Total TAG
CLO	100 (64.9 ± 3.3)[b]	60.3 ± 1.3	39.7 ± 1.3	8.6 ± 0.2	2.3 ± 0.2	6.2 ± 0.3	2.2 ± 0.3	31.2 ± 1.2	4.5 ± 0.3
L	100 (61.1 ± 2.0)[b]	59.0 ± 1.8	41.0 ± 1.8	10.1 ± 0.4	3.7 ± 0.3	6.4 ± 0.3	1.8 ± 0.05	30.9 ± 1.5	5.5 ± 0.3
	(NS[b])	NS	NS	L>CLO***	L>CLO***	NS	NS	NS	L>CLO**
CLO	–	–	100	21.6 ± 0.8	6.0 ± 0.8	15.6 ± 0.4	5.4 ± 0.5	78.4 ± 0.8	11.4 ± 0.8
L	–	–	100	24.8 ± 0.8	9.1 ± 0.7	15.7 ± 0.3	4.5 ± 0.2	75.2 ± 0.8	13.6 ± 0.8
				L>CLO***	L>CLO***	NS	NS	CLO>L**	NS
CLO	–	–	–	100	27.3 ± 2.9	72.7 ± 2.9	25.1 ± 2.8	–	52.4 ± 2.8
L	–	–	–	100	36.3 ± 1.9	63.7 ± 1.9	18.3 ± 0.8	–	54.6 ± 1.6
	–	–	–	–	L>CLO*	CLO>L*	CLO>L*		NS

[a]For abbreviations see text.
[b]In mg for one egg.
*, **, ***Significantly different ($p<0.05$, <0.02, <0.01, respectively). NS: not significant.

TABLE 7

Simplified Fatty Acid Compositions of Adipose Tissue, Serum Lipoproteins and Ripe Egg Compartments in Mature Trout

	Adipose tissue*	Diet+	Total lipoproteins†	Lipovitellin*	Vitellogenin†	Oil globule*
Trout receiving a non-(n-3)-deficient diet						
Σ Sat.	20.02 ± 0.49	19.4 - 19.6	25.5 - 25.5	22.78 ± 0.47	34.2 - 34.2	19.68 ± 0.42
Σ (n-7)	11.63 ± 0.66[a]	15.4 - 15.1	7.8 - 7.8	10.73 ± 0.24[b]	8.5 - 9.0	13.05 ± 0.22[c]
Σ (n-9)	34.98 ± 0.87	31.5 - 31.8	14.4 - 15.6	17.58 ± 0.22[d]	14.1 - 14.3	20.95 ± 0.58[e]
Σ (n-6)	20.73 ± 0.76	7.6 - 7.6	9.2 - 9.3	10.50 ± 0.51[f]	13.9 - 10.5	14.00 ± 0.58[g]
Σ (n-3)	11.57 ± 0.87	24.3 - 24.2	41.3 - 41.7	37.45 ± 0.70[h]	27.5 - 30.8	31.15 ± 1.13[i]
(n-6)/(n-3)	1.86 ± 0.19	0.31 - 0.31	0.22 - 0.22	0.28 ± 0.02[k]	0.51 - 0.34	0.46 ± 0.03[l]
18:2 (n-6)	17.48 ± 0.63	6.6 - 6.6	4.6 - 4.6	5.37 ± 0.32[m]	5.5 - 4.8	9.37 ± 0.46[n]
20:4 (n-6)	0.60 ± 0.03	0.5 - 0.5	3.0 - 3.0	2.13 ± 0.19[o]	2.5 - 2.6	1.92 ± 0.08[p]
18:3 (n-3)	1.22 ± 0.08[q]	1.3 - 1.3	0.4 - 0.4	0.53 ± 0.02[r]	0.4 - 0.4	1.02 ± 0.03
20:5 (n-3)	1.82 ± 0.30	9.3 - 9.2	9.4 - 9.4	6.87 ± 0.36[s]	5.9 - 6.6	5.92 ± 0.33[t]
22:6 (n-3)	5.62 ± 0.46	10.0 - 9.9	28.3 - 28.6	27.23 ± 0.60[u]	18.1 - 20.5	20.78 ± 0.03[v]
Trout receiving a (n-3)-deficient diet						
Σ Sat.	22.38 ± 0.69	48.0 - 47.9	29.3 - 26.8	23.57 ± 0.28	30.2 - 35.2	22.38 ± 0.69
Σ (n-7)	7.48 ± 0.24[a]	3.7 - 3.8	5.9 - 7.1	9.43 ± 0.27[b]	6.9 - 7.3	10.45 ± 0.28[c]
Σ (n-9)	36.95 ± 2.68	32.6 - 32.6	17.0 - 17.2	19.47 ± 0.52[d]	19.6 - 16.9	27.10 ± 0.77[e]
Σ (n-6)	23.57 ± 1.04	12.5 - 12.5	20.2 - 20.7	17.22 ± 0.46[f]	24.2 - 22.1	18.73 ± 0.36[g]
Σ (n-3)	8.82 ± 1.58	2.8 - 2.7	27.1 - 27.8	29.47 ± 0.61[h]	19.1 - 17.5	22.12 ± 0.69[i]
(n-6)/(n-3)	2.99 ± 0.37	4.5 - 4.6	0.75 - 0.74	0.59 ± 0.03[k]	1.27 - 1.26	0.85 ± 0.03[l]
18:2 (n-6)	17.55 ± 0.83	12.0 - 12.0	6.7 - 7.4	7.27 ± 0.22[m]	10.4 - 8.9	11.90 ± 0.48[n]
20:4 (n-6)	1.77 ± 0.60	0.2 - 0.2	8.0 - 8.6	4.57 ± 0.38[o]	6.2 - 5.4	2.57 ± 0.16[p]
18:3 (n-3)	0.70 ± 0.05[q]	1.1 - 1.1	0.2 - 0.4	0.40 ± 0.03[r]	—	0.90 ± 0.07
20:5 (n-3)	0.97 ± 0.33	0.6 - 0.5	2.9 - 5.5	3.13 ± 0.30[s]	2.1 - 2.2	2.48 ± 0.24[t]
22:6 (n-3)	5.03 ± 1.25	1.0 - 1.0	22.8 - 20.5	23.45 ± 0.40[u]	16.5 - 14.8	16.18 ± 0.52[v]

[a-v]Significantly different values ($p<0.01$).

*Results expressed as the means of 6 determinations ± SEM.

+Results of 2 separate extractions and analysis of 2 samples.

†Results obtained after 2 separate treatments of a number of pooled samples. Number of samples is 6 and 4 for trout on the nondeficient diet, and 11 and 4 for trout on the deficient diet.

proportion found in vitellogenin (unpublished data).

As shown in Table 6, a dietary (n-3) fatty acid deficiency does not seem to modify egg growth or water content. On the other hand, this deficiency had a great influence on the dry weight components, leading to a 15% increase in egg lipids which was offset by a slight, non-significant decrease in nonlipid components (essentially protein; not given in the present paper). Considering that total egg triacylglycerols remained relatively constant (Table 6), it appears that dietary (n-3) deficiency brought about no modification in total egg triacylglycerols, but did enrich oil globule triacylglycerols, thus leading to an increase in the oil globule size and a decrease in lipovitellin triacylglycerol content. Lipovitellin lipid content, relative to dry weight, however, was unchanged as a result of a decrease of the lipovitellin triacylglycerols. Finally, the results emphasize that the increase in total egg lipid content in females fed a deficient diet was essentially due to more rapid growth of the oil globule, i.e., a 52% increase in dry weight as compared to the eggs of females on a nondeficient diet, whereas lipid components of lipovitellin exhibited an identical increase on both diets.

Table 7 shows the fatty acid composition of the blood lipid carriers (lipoproteins and vitollogenin), the PAT and the two lipid compartments of the egg (the oil and the yolk globules) in spawning female trout. The (n-9) and (n-6) fatty acid levels were higher in fish on the L diet than in those on the CLO diet. This cannot be explained by the slight increase in dietary levels of both fatty acid series. However, the case of the (n-3) fatty acids is very different. A low dietary level appears to result in a large decrease in the percentages of all (n-3) fatty acids. In addition, it is evident that the (n-6)/(n-3) ratios in the oil globule, lipovitellin, vitellogenin and lipoproteins are very low and quite similar in the CLO and L groups, although the L diet (n-6)/(n-3) ratio was high. Irrespective of the type of diet, the (n-3) fatty acid contents are always higher in lipoproteins and lipovitellin than in vitellogenin and the oil globule. Moreover, the most similar values of (n-3) and (n-6) fatty acid contents were obtained in lipoproteins and lipovitellin on the one hand, and in vitellogenin and oil globule on the other. This suggests a direct relationship between blood carriers and egg lipid compartments. A large part of the lipoprotein lipids could be directed into the yolk globule as lipovitellin, whereas the vitellogenin lipids might be preferentially enter the oil globule.

It is interesting that high levels of 22:6 (n-3) and of total (n-3) fatty acids were always obtained in lipovitellin. Thus, yolk globule lipids would have a special role, that of providing fatty acids for building the cellular structures of the embryo.

The polyunsaturated fatty acid content of adipose tissue is quite similar to that of the pre-experimental diet (diet B, Table 2). This means that the levels of these fatty acids, and particularly of 22:6 (n-3), were independent of the dietary levels in adipose tissue during ovogenesis. In contrast to data on the direct and established influence of dietary fatty acids on adipose tissue fatty acid composition (28), this emphasizes that, during ovogenesis, the dietary lipids are diverted from the adipose tissue and mainly deposited in the eggs after elongation-desaturation. Estrogen released during vitellogenesis might inhibit the lipoprotein lipase activity of adipose tissue, as already mentioned (29). In addition, there would be no compensation of (n-3) fatty acid deficiency of the diet by selective mobilization of EFA from the adipose tissue, thus confirming data obtained in starved fish (30).

The fact that some fatty acids of different series play specific EFA roles in certain animal species is helpful in determining the phenomena specific to the EFA. The trout seems to be a good model for obtaining a better understanding of processes involved in EFA transfer and for studying their mechanisms of action.

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Utilization of Polyunsaturated Long-Chain Alcohols by *Clostridium butyricum*

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ABSTRACT

When a trace amount of linolenyl alcohol (*cis,cis,cis*-9,12,15-octadecatriene-1-ol) was added to the culture media of *Clostridium butyricum*, the polyunsaturated alcohol was readily assimilated. After an initial 18-hr exposure interval, there was a large increase in the amount and a change in the composition of thelong-chain alcohols present. However, this alteration was temporary. As the duration of the exposure interval increased, the alcohol content and composition of the treated bacteria returned toward control values. This return of the long-chain alcohol composition toward control values was associated with an increase in the proportion of linolenic acid in the phospholipid fraction. The ether moiety of the alk-1-enyl phosphoglycerides was unchanged in amount or composition upon exposure to linolenyl alcohol.

INTRODUCTION

Long-chain alcohols occur as trace lipids in *Clostridium butyricum*. Stearyl, oleyl and palmityl alcohol are the major moieties present (1,2).

Enzymatic reduction of palmitic acid to the corresponding aldehyde or alcohol as well as the incorporation of these lipid moieties into the complex lipids of this microorganism has been demonstrated (1,3). Recently, it has been shown that inhibition of long-chain alcohol biosynthesis in this bacterium is associated with a decreased rate of cell growth. This decrease in growth was reversed, in part, by inclusion of a naturally occurring alcohol in the culture medium (2). Considering these observations, this study was devoted to an evaluation of the utilization of the nonphysiological alcohol, linolenyl alcohol, by *C. butyricum*.

EXPERIMENTAL PROCEDURES

Chemicals

Fluid thioglycollate was obtained from Bio Quest Laboratories, Cockeysville, MD. Linolenyl alcohol (*cis,cis,cis*-9,12,15-octadecatriene-1-ol) was purchased from Supelco, Inc., Bellefonte, PA. The source of the other chemicals has been described (2).

Growth Conditions

The conditions employed for the propogation of *C. butyricum* (ATCC 6015) have been described (2). At all times, ethanol, 0.6% v/v, was added to the control and long-chain alcohol containing cultures. In this study, cultures were treated with linolenyl alcohol in several ways. Control cultures were grown in media devoid of linolenyl alcohol. Exposure I cultures were grown in the presence of linolenyl alcohol, 1.4 μM, for 16 hr at 37 C. Exposure II cultures were aliquots of bacteria removed from the exposure I cultures and again incubated at 37 C for 16 hr in the presence of linolenyl alcohol, 1.4 μM.

Lipid Analysis

Total lipid extracts were resolved into neutral and phospholipid fractions (2,4). The neutral lipids were saponified and total long-chain alcohols, free and esterified, were isolated by thin layer chromatography (TLC) and converted to acetoxy alkanes prior to gas liquid chromatography (GLC) (4-6). Phospholipids were treated with anhydrous methanolic HCl (7) and the resulting dimethyl acetals and methyl esters analyzed by GLC (6,7). The methods for quantitating lipid phosphorus, alk-1-enyl ethers and long-chain alcohols have been described (2). In all instances, solvents were equilibrated with nitrogen prior to use and a nitrogen atmosphere was maintained over the lipid fractions.

Autoxidation

Polyunsaturated lipids undergo autoxidation in the presence of oxygen, yielding peroxides (8) which inhibit cell growth. To minimize the possibility of peroxide formation, fresh stock solutions of linolenyl alcohol in 95% ethanol were prepared weekly and stored under nitrogen at -40 C. The ultraviolet spectra of these solutions were recorded prior to use. Autoxidation, as indicated by a change in absorbance at 233 nm, was not noted in any of the stock solutions used (8).

Statistical Analysis

The values presented here are the mean ±

SD. The difference between the individual means was evaluated by the Student t-test.

RESULTS

C. butyricum grown in control media contain trace amounts of long-chain alcohols (Table 1). In contrast, when this bacterium was grown for 16 hr in media containing linolenyl alcohol, the long-chain alcohol content of the treated microorganisms increased about 6-fold (exposure I). However, this increase was transitory. When bacteria from exposure I were subcultured in linolenyl-alcohol-containing media for an additional 16 hr (exposure II), the long-chain alcohol content of the cells approached that of the control cultures (Table 1).

The alk-1-enyl phosphoglyceride content was also assessed in the control and linolenyl alcohol treated groups. While the concentration of this lipid type varied in individual cultures, all values were within a similar range and the averages for each group were not different from reported control values (2) (data not shown).

The qualitative composition of the long-chain alcohols, the phospholipid esterified fatty acids, and alk-1-enyl ethers was determined in

TABLE 1

Long-Chain Alcohol Content in 16-hr Cultures of Control, Linolenyl Alcohol Treated and Adapted *C. butyricum*[a]

Groups	Total lipid phosphorus[b]	Total long-chain alcohols[c]
Control		
A	23.9	13.4
B	59.9	16.5
C	48.6	28.6
Exposure I		
A	19.2	145.6
B	19.2	152.0
C	8.0	72.4
D	7.4	76.7
Exposure II		
A	7.0	14.1
B	11.4	27.0
C	14.2	24.7
D	17.4	16.2

[a]All cells were grown in thioglycolate media containing 0.6% v/v 95% ethanol at 37 C for 16 hr. The media for exposure groups I and II also contained linolenyl alcohol, 1.4 μM. The letters A, B, C and D refer to 4 separate experiments in which cultures of control and treated bacteria were harvested and their lipid content analyzed.

[b]The lipid phosphorus content is expressed as the total μmol present in the sample.

[c]The total long-chain alcohols (free plus esterified) are expressed as nmol/μmol lipid phosphorus.

TABLE 2

Effect of Exposure to Linolenyl Alcohol on the Qualitative Composition of the Total Long-Chain Alcohols of the Neutral Lipids and the Esterified Fatty Acids and Alkenyl Ethers of the Phospholipids[a]

Carbon chain[c]	Total long-chain alcohols[b]			Phospholipid fatty acids			Alkenyl ethers		
	Control	Exposure I	Exposure II	Control	Exposure I	Exposure II	Control	Exposure I	Exposure II
14:0	4.4 ± 1.4	1.4 ± 0.2	3.5 ± 2.9	5.2 ± 1.3	5.2 ± 1.0	1.1 ± 0.7	nd	1.4 ± 0.9	3.6 ± 0.6
16:0	30.2 ± 9.1	15.9 ± 3.4	27.3 ± 7.2	57.7 ± 9.6	59.0 ± 4.7	53.3 ± 12.8	38.6 ± 5.1	44.4 ± 8.5	41.1 ± 7.2
16:1	4.8 ± 2.5	5.7 ± 0.9	6.7 ± 1.3	13.2 ± 1.4	15.4 ± 1.0	10.6 ± 1.8	23.7 ± 2.7	15.3 ± 1.4	10.4 ± 3.4
17:cyc.	5.8 ± 3.7	5.9 ± 1.2	7.0 ± 0.3	9.6 ± 0.4	9.6 ± 2.1	9.9 ± 3.2	15.1 ± 1.1	19.8 ± 2.2	18.5 ± 0.9
18:0	45.6 ± 8.1	22.0 ± 7.6	54.4 ± 12.4	5.2 ± 1.0	1.6 ± 1.0	3.8 ± 1.6	2.8 ± 1.0	4.4 ± 3.7	7.4 ± 2.3
18:1	7.1 ± 3.4	nd	nd	4.2 ± 0.7	4.2 ± 2.0	9.6 ± 5.7	10.1 ± 2.0	3.2 ± 1.3	6.4 ± 2.1
18:3	nd	47.2 ± 4.8	1.6 ± 1.0	nd	nd	7.6 ± 2.0	nd	nd	nd
19:cyc.	nd	nd	nd	2.8 ± 1.4	4.6 ± 0.8	6.0 ± 1.5	9.3 ± 3.1	9.3 ± 4.0	11.9 ± 2.8

[a]The qualitative composition is expressed at wt %. The values are the averages ± SD. The samples were obtained from the individual studies presented in Table 1. Fatty moieties representing less than 1% of the total are not reported.

[b]Total long-chain alcohols refers to both the free and esterified alcohols since these moieties were isolated together.

[c]Carbon chain is the carbon number:number of double bonds; cyc. refers to the cyclopropane moiety. Identification was based on the chromatographic mobility of the acetoxyalkanes, methyl esters, and dimethyl acetals derived from the long-chain alcohols, esterified fatty acids and alkenyl ethers relative to standrads on GLC using a polar SP-2340 and a nonpolar column OV-101 and confirmed by mass spectrometry; *nd* means not detected.

the control and alcohol-exposed bacteria (Table 2). The long-chain alcohol composition of the control bacteria was similar to that reported previously (1,2); palmityl and stearyl alcohols were the major species present. Growth in the presence of linolenyl alcohol (exposure I) resulted in a decrease in the proportion of palmityl and stearyl alcohols to levels half those noted in the control group; oleyl alcohol was not detected, whereas linolenyl alcohol accounted for half of the total alcohols. In contrast, prolonged growth in the presence of this alcohol gave different results. Now, the linolenyl alcohol content of the bacteria (exposure II) represented less than 2% of the total long-chain alcohols present. Again, oleyl alcohol was not detected, but the proportion of other alcohols was similar to the control group.

The qualitative composition of the esterified fatty acids of the phospholipids (Table 2) was similar in both the control and exposure I groups and consistent with pubished values (3). In contrast, in the exposure II group, linoleic acid now accounted for 7.6% of the total fatty acids. The composition of the alk-1-enyl ethers was similar in all 3 groups and in agreement with published values (3). A moiety corresponding to the dimethyl acetal of linolenyl aldehyde was not found in any of the lipid extracts. The alkyl phosphoglycerides were not evaluated.

DISCUSSION

As noted before, long-chain alcohols occur in trace amounts in *C. butyricum*, and stearyl alcohols are the major species present (1,2). Polyunsaturated alcohols do not occur physiologically in this bacterium. However, when *C. butyricum* was grown for 16 hr in the presence of linolenyl alcohol, there was a large increase in the amount of long-chain alcohols present. This increase was not a superimposition of the unnatural alcohol, linolenyl, upon the existing physiological alcohols of the cell. This conclusion is based on the observation that the amount of the major physiological alcohols, palmityl and stearyl, increased about 3-fold in the linolenyl-alcohol-treated cells (exposure I) despite a ca. 2-fold decrease in their proportional composition.

The observation that linoleic acid increased from undetectable amounts to 7.6% of the total phospholipids fatty acids following a prolonged exposure to linolenyl alcohol (exposure II) permits several observations. First, long-chain alcohols can penetrate the plasma membrane of this bacterium. Second, one of the events occurring in the adapted species of this bacterium (exposure II) is an induction of the enzyme(s) involved in the oxidation of long-chain alcohols to fatty acids. Since untreated *C. butyricum* is capable of converting long-chain acids to alcohols and aldehydes to acids (1), the enzyme induced here may be an alcohol dehydrogenase.

The observation that the large change in the linolenyl alcohol content of the treated bacteria (exposure I) is not associated with a quantitative or qualitative change in the alk-1-enyl ethers of this microorganism, would indicate that under these conditions the polyunsaturated alcohol is not a substrate for the synthesis of the ether moiety.

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Chain Length Dependent Thermodynamics of Saturated Symmetric-Chain Phosphatidylcholine Bilayers

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ABSTRACT

A molecular interpretation for the chain length dependent thermotropic behavior of saturated symmetric-chain phosphatidylcholine bilayers is proposed. It is suggested that the bilayer interface region and conformationally inequivalent terminal ends of the fatty acyl chains perturb the packing associations of the rest of the hydrocarbon chains in the gel phase of the bilayer. These perturbing effects, which are seen to increase with decreasing acyl chain length, have been quantitatively defined by a perturbation parameter, P. The thermodynamic parameters of the thermal phase transition of these phosphatidylcholines are found to be linearly correlated to P and these linear relationships can be used to predict the minimum number of carbon atoms in the acyl chain necessary in order for a bilayer phase transition to occur.

Of the properties which characterize synthetic saturated phospholipids in model membranes, the thermally induced gel ⟷ liquid crystalline phase transition has been the one most rigorously investigated, experimentally as well as theoretically (1-7). These studies have lead to a better understanding of the molecular structure of phospholipid molecules in the bilayer, and moreover, the phase transition appears to be critical to the proper function of biological membranes (8).

It has long been established that, for saturated symmetric-chain diacylphosphoglycerides, the main transition temperature and associated thermodynamic parameters, such as transition enthalpy and entropy, are dependent on the length of the lipid's fatty acyl chains (1). For the homologous series of saturated symmetric-chain phosphatidylcholines (PC), e.g., the transition temperature and other thermodynamic parameters (ΔS, ΔH, ΔV) increase with increasing acyl chain length. However, recent studies (5) have shown that these thermodynamic parameters are a curvilinear, rather than a linear, function of the chain length of these PC. This behavior contrasts with that of bulk hydrocarbons, corresponding in length to the PCs' acyl chains, for which thermodynamic parameters have been shown to be a linear function of hydrocarbon chain length (6). In the literature, there are a number of empirical methods to linearly correlate the melting behavior of diacylglycerides with acyl chain length (7,9). These methods are useful but are based purely on curve-fitting techniques rather than on molecular interpretation.

In this communication, the PC fatty acyl chains will be viewed as consisting of three regions of different structural order (Fig. 1). The bilayer interface (region 1) and conformationally inequivalent terminal ends of the acyl chains (region 3) are postulated as perturbing the packing associations of the rest of the hydrocarbon chains (region 2) in the gel phase of the bilayer. These perturbing effects, which increase with decreasing acyl chain length, will be quantitatively defined by a perturbation parameter, P. The thermodynamic parameters of the thermal phase transition of saturated symmetric-chain PC will be shown to be a linear function of P. These linear relationships can, in turn, be used to predict the minimum number of carbon atoms in the acyl chain that is necessary for a bilayer phase transition to occur.

THERMODYNAMIC DATA OF SATURATED SYNTHETIC PHOSPHOTIDYLCHOLINES IN BILAYERS

Listed in Table 1 are the literature values for the main transition temperature (T_m) and the observed transition enthalpy (ΔH) for a series of even-numbered PC with saturated symmetric

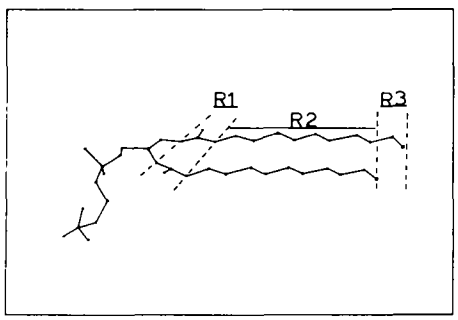

FIG. 1. Schematic diagram of 1,2-dilaurylphosphatidylcholine showing the partitioning of the fatty acyl chains into the structural regions R1, R2 and R3. Note the conformational inequivalence of the acyl chains which results in the *sn*-2 acyl chain being 1.5 carbon bond lengths shorter than the *sn*-1 acyl chain.

TABLE 1

Thermodynamic Parameters of Saturated Symmetric-Chain Phosphatidylcholines[a]

Parameter	1,2-Diacylphosphatidylcholine					LCC[b]	Linear equation
	C_{22}	C_{18}	C_{16}	C_{14}	C_{12}		
P	9.1	11.5	13.2	15.6	18.9	–	–
T_m (C)	75	55	41	24	-1.8	0.999	-7.77(P)+145
ΔH (kcal/mol)	14.8	10.6	8.6	5.4	1.7	0.994	-1.31(P)+26.4
ΔS_H(e.u./mol)	83.6	68.4	60.8	53.2	45.6	–	–
ΔS (e.u./mol)	42.5	32.3	27.4	18.2	6.27	0.998	-3.66(P)+75.3
ΔV (L/mmol)	–	3.56	2.72	1.83	–	0.993	-0.419(P)+8.33
$\Delta V/CH_2$ (mL/mol)	–	0.99	0.85	0.65	–	1.000	-0.083(P)+1.94

[a]C_n refers to the acyl chain length of the 1,2-diacylphosphatidylcholines. The values for T_m and ΔH are taken from ref. 6 for C_{22}, from refs. 5, 10 for C_{16}, and from ref. 5 for the rest of the phosphatidylcholines. Values for ΔV and $\Delta V/CH_2$ are taken from Nagel and Wilkinson (7). See the text for explanations of ΔS_H, ΔS and P.

[b]LCC is the linear correlation coefficient for a fit of the indicated parameter with the perturbation parameter, P.

acyl chains which undergo the gel → liquid crystalline phase transition. The values for dibehenoyl PC are from Phillips et al. (6), those for dipalmitoyl PC are the average of the values reported by Mabrey and Sturtevant (5) and Albon and Sturtevant (10), and the remaining values are from Mabrey and Sturtevant (5). The transition entropy is derived from the Clausius equality as: $\Delta S = \Delta H/T_m$, assuming an equilibrium first order transition (7). In Figure 2, this observed transition entropy (solid line) is plotted against fatty acyl chain length (N). The dashed line in Figure 2 is the fusion entropy of bulk hydrocarbons corresponding in length to the fatty acyl chains of the PC. This value is obtained as: ΔS_H = 1.9 e.u./mol × (2N), based on the value of 1.9 e.u./mol/carbon unit reported for the fusion entropy of bulk *n*-hydrocarbons which undergo the α → melt rotomeric chain disorder transition (6,11). Figure 2 reveals that the observed transition entropy in lipid bilayers is less than that for the corresponding bulk hydrocarbons by at least a factor of two. The smaller change in ΔS for the hydrocarbon chain in lipid bilayers is expected, since one end of the chain is in an ester linkage to the glycerol backbone of the phospholipid molecule, resulting in a decrease in motional freedom of the acyl chains relative to the free fatty acids (12). Interestingly, Figure 2 also shows that the observed change in ΔS for phospholipids in bilayers is not a linear function of the acyl chain length.

PHOSPHOLIPID STRUCTURE IN BILAYERS

The fatty acyl chains have been partitioned into three structural regions as diagrammed in Figure 1.

Region 1

Region 1 (R1) has been termed the bilayer interface (12) and consists of the carbonyl ester linkage and α carbons of both fatty acyl chains. The carbonyl-oxygen double bond and partial double bond character of the ester bond, which arises by resonance, imparts a coplanar structure to the interface elements (13). This structural planarity is similar to that observed for the peptide bond of proteins. Moreover, the C-H bond at the attachment site of the secondary ester to the glycerol backbone is arranged so as to be synplanar to the *sn*-1 acyl ester

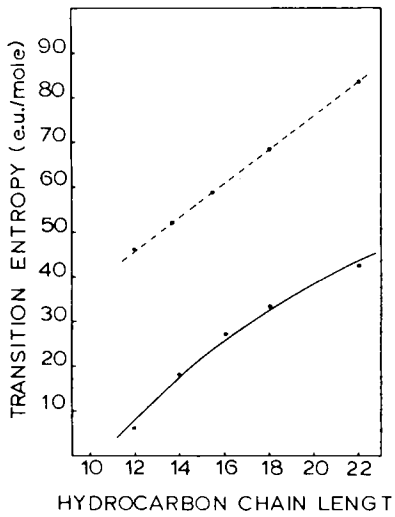

FIG. 2. Plot of the transition entropy of even saturated symmetric-chain phosphatidylcholines as a function of fatty acyl chain length. See text for an explanation of ΔS (—) and ΔS_H (-----).

linkage (see Fig. 1, ref. 12). The planarity of the bilayer interface would be expected to result in a highly restricted motion for the elements within this region. Indeed, both ^{13}C-NMR spin lattice relaxation times (14) and ^{2}H-NMR order parameters (15,16) indicate that this region possesses the smallest degree of segmental motion of all of the carbon bonds along the acyl chain. The relative structural rigidity of the bilayer interface, which persists in the liquid-crystalline phase, would lead us to predict that the acyl chain carbons within region 1 do not directly participate in the bilayer thermal phase transition.

Region 3

Although X-ray diffraction techniques have been employed to investigate the bilayer structure of membranes for some time, the crystalline structure of saturated symmetric PC at atomic resolution was reported only recently (17). X-ray data indicate that the initial segment of the *sn*-2 fatty acyl chain extends perpendicular to the *sn*-1 chain but the chain bends abruptly at the C(2) atom so that the rest of the *sn*-2 chain runs parallel to the linear *sn*-1 fatty acyl chain. Because of the abrupt bend, the terminal methyl groups of the two alkyl chains are not in register, but are separated by a distance of $\sim$3.7 Å. However, ^{2}H-NMR and neutron diffraction studies of saturated symmetric PC in the gel phase of the bilayer indicate that the two hydrocarbon chains are more nearly in register at the bilayer center (18,19). Instead of 3.7 Å as revealed by the X-ray crystal work, a separation of only 1.8 Å is observed for the two methyl groups in the gel phase of the bilayer. Experiments with dipalmitoyl PC carried out at temperatures higher than the phase transition temperature reveal that the first segment of the *sn*-2 chain, oriented parallel to the bilayer surface, has the largest statistical weight (20), indicating that the two acyl chains are, on the time average, inequivalent even in the liquid-crystalline state. In addition, ^{2}H-NMR studies by Seelig and Seelig (16) demonstrate that the conformational inequivalence of the *sn*-1 and *sn*-2 acyl chains is independent of the fatty acid composition of the PC. It would seem reasonable to assume, therefore, that the chain inequivalence in the saturated symmetric-chain PC persists independent of the chain length of the PC. Thus, in either the gel or the liquid-crystalline phase, there is a small segment of the terminal end of the *sn*-1 fatty acyl chain which must be distorted, possibly by *trans* $\leftrightarrow$ *gauche* rotational isomerization, to fill up the space under the methyl terminus of the *sn*-2 chain in order not to leave a region of vacuum. The *apparent* length of the *sn*-1 acyl chain of the saturated symmetric phospholipid that is actually in the *trans* configuration in the gel phase must, therefore, be 1.8 Å (or 1.5 carbon-carbon bond lengths) shorter than an all-*trans* chain configuration. Thus, the displaced terminal end of the *sn*-1 acyl chain would be expected to display a high degree of isotropic motion even in the gel phase of the bilayer. Region 3 may well be the source of the *gauche* rotomers, shown by Raman spectroscopy, to be present in the gel phase of saturated PC bilayers (21-23). Because of the large amount of disorder postulated to exist within region 3, it would be expected that this region will also not directly contribute, thermodynamically, to the thermal phase transition.

Region 2

Region 2 (R2) consists of the fatty acyl chain segments of the PC which lie between regions 1 and 3. In the gel phase of the bilayer, these segments can pack in association with one another and would be expected to show the largest van der Waals' attraction and the smallest volume/hydrocarbon unit of the three regions. The methylene carbon-carbon bonds within region 2 would also be expected to be predominantly in an all-*trans* configuration in the gel phase of the bilayer. It can be assumed, therefore, that region 2 will make the most significant direct contribution to the thermal phase transition of the bilayer.

Since the sizes of regions 1 and 3 remain constant, a decrease in acyl chain length will decrease the size of region 2. Thus, if region 2 were to undergo the gel $\leftrightarrow$ liquid crystalline phase transition independent of any interactions with region 1 or region 3, the thermodynamic magnitude (ΔH, ΔS, ΔV) of the transition would be expected to be a linear function of the acyl chain length. However, Figure 2 reveals that the transition entropy shows a progressively negative deviation from linearity with decreasing chain length. This observation can be interpreted in the following manner. The thermodynamic magnitude of the thermal phase transition is largely determined by the ability of the carbon units within region 2 to adopt an all-*trans* packing configuration in the bilayer gel phase which maximizes van der Waals contacts between chains. Any influence which acts to disrupt this optimal packing will decrease the thermodynamic magnitude of the phase transition. We propose here that the conformationally restricted motion of region 1 and rotomeric disorder within region 3 affect the phase transition indirectly by acting to

disrupt the regular packing of the acyl chains of region 2 within the gel phase of the bilayer. As the chain length of the PC is reduced, thus decreasing the relative size of region 2, the perturbing effects of regions 1 and 3 on the acyl chain packing would be expected to become more pronounced. This argument can account for the progressively negative deviation from linearity of the transition entropy as the PC acyl chain length is reduced.

Thus, to adequately explain the chain length dependent thermotropic behavior of these PC, it is necessary to consider not only the absolute chin length but also the relative distribution of the acyl chain carbons among the three structural regions just discussed. For this purpose we define the perturbation parameter, P, as: $P = (R_1+R_3)/R_2$. Region 1 consists of the carbonyl and α carbon of each acyl chain or 2 carbon-carbon bond lengths. Region 3, as discussed, is 1.5 C-C bond lengths. Thus, $R_1 + R_3 = 3.5$ C-C bond lengths. Region 2 contains the remaining carbon-carbon bonds or (2N-2) - 3.5 C-C bonds. The perturbation parameter, expressed as a percentage, is then given as: $P = [3.5/(2N-5.5)] \times 10^2$.

PLOT OF THERMODYNAMIC PARAMETERS VS P

The thermodynamic parameters given in Table 1 can be plotted against P; Figure 3 is a typical example of such a plot. In contrast to the curvilinear function of ΔS vs fatty acyl chain length (Fig. 2), the transition entropy is observed in Figure 3 to be a linear function of P (linear correlation coefficient = 0.998). In fact, changes in all thermodynamic parameters (Table 1) show excellent linear correlation with P.

DISCUSSION

In this communication, we propose a molecular interpretation for the chain length dependent thermotropic behavior of saturated symmetric-chain PC. It is suggested that the relatively rigid bilayer interface and rotomerically disordered terminal ends of the acyl chains perturb the conformational statistics of the rest of the hydrocarbon chain, as well as the interaction between chains, thereby preventing the acyl chains from maximizing van der Waals contacts in the bilayer gel phase. This treatment views the bilayer gel phase as becoming relatively less ordered for PC of progressively shorter acyl chain length.

It is not possible to distinguish by this analysis which of the two regions, 1 or 3, is more important to the perturbation of the

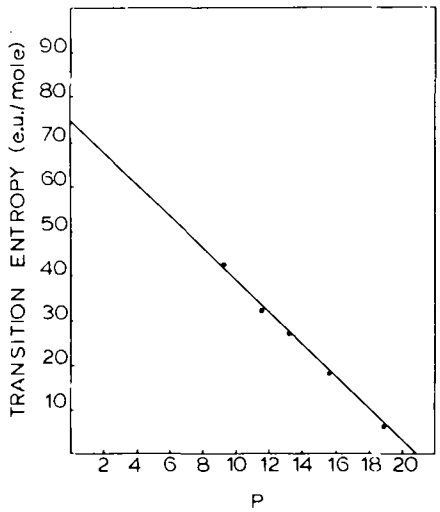

FIG. 3. Graphic demonstration of the linear correlation between the phosphatidylcholine transition entropy and the perturbation parameter, P. See text for explanation of P.

bilayer chain packing postulated here. A recent study of saturated mixed-chain PC (Mason, J.T., Huang, C., and Biltonen, R.L., submitted for publication) revealed that the thermotropic behavior of these PC is predominantly determined by the inequivalence in length of the two fatty acyl chains. The results of this study would lead us to predict that it is region 3 which is primarily responsible for the distortion of the gel phase chain packing in the saturated symmetric-chain PC, as well. However, the relative rigidity of the interface region would be expected to restrict the conformations available to the hydrocarbon methylene segments near the carbonyl end of the chain. In this way, region 1 might also make a contribution to the postulated perturbation of the hydrocarbon chain packing.

An inspection of Figure 3 and Table 1 will reveal that $\Delta H = \Delta S = \Delta V = 0$ when the perturbation parameter has a value of 20.5 ± 1.7 (SD). This observation suggests that any 1,2-diacylphosphatidylcholine with less than 11 carbons/acyl chain will not give rise to a bilayer phase transition. It is notable that Mabrey and Sturtevant (5) arrived at this same conclusion by considering the trends in transition temperature and enthalpy for saturated PC. It is obvious to state that any PC which does not form bilayers will not give rise to a bilayer phase transition. The converse statement, that any PC which does not display a bilayer phase transition will also not form bilayers, is less straightforward. It is known, however, that

diheptanoylphosphatidylcholine and dihexanoylphosphatidylcholine form micelles, not stable lamellar structures, in aqueous solution (24). These observations would suggest that the molecular interactions which are responsible for determining the magnitude of the thermal phase transition may also be involved in determining the stability of the lamellar structure.

As discussed by Phillips et al. (6), by analogy to long chain hydrocarbons, only the configurational term of the total transition entropy should be proportional to the acyl chain length in PC. The slope of Figure 3 (-3.7 e.u./mol/P) can therefore be interpreted as the decrease in configuration entropy of transition/unit increase in P. The configurational entropy of transition can reasonably be taken as a measure of the degree of rotomeric disorder along the hydrocarbon chains of the PC in the liquid-crystalline phase relative to the gel phase of the bilayer. A quantitative measure of this rotomeric disorder is the ratio of the average number of *gauche* to *trans* rotomers present along the PC fatty acyl chains (21-23). This would lead us to predict that the change in the ratio of *gauche* to *trans* rotomers which results from the thermal phase transition will decrease in strong linear correlation to an increase in P. We are currently in the process of attempting to verify this prediction experimentally.

In summary, we have stressed the importance of the bilayer interface and inequivalent conformations of the two acyl chains of the saturated PC molecule in determining the magnitude of various thermodynamic parameters associated with the gel $\longleftrightarrow$ liquid crystalline phase transition in bilayers of these PC. It would seem likely that these conformational differences will also affect other bilayer properties and should be taken into account when attempting to investigate the dynamic behavior of lipid membranes.

ACKNOWLEDGMENTS

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METHODS

High Performance Reversed Phase Chromatography of Cholesterol and Cholesteryl Esters of Human Plasma Lipoproteins

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ABSTRACT

Cholesterol and cholesteryl esters were separated according to their carbon number and number of double bonds by high performance reversed-phase chromatography (HPRC) using acetonitrile/chloroform/methanol (1:1:1, v/v) as a mobile phase. It was found that within the same equivalent carbon number (ECN) category, cholesteryl esters with the highest number of double bonds eluted ahead of those with a lower number of double bonds, and with the *cis* isomers eluting ahead of their *trans* partners. Thus, cholesteryl oleate (C27-18:1*c*) elutes ahead of cholesteryl palmitate (C27-16:0) and ahead of cholesteryl elaidate (C27-18:1*t*). Human lipoprotein, as well as rat liver cholesteryl esters, were separated using this technique.

The determination of cholesterol and total cholesteryl esters is frequently performed in clinical analysis (1). Classical methods of analysis such as enzymatic assays (2) and/or colorimetric reactions (3) are tedious and time-consuming. Interference of other serum components (4) are but one of the drawbacks of these methods. Cholesteryl esters can not be directly measured by the above methods, but must be assayed for cholesterol and the fatty acids analyzed as methyl esters by gas liquid chromatography (GLC).

Chromatographic methods are applicable to the separation of cholesterol and cholesteryl esters. Argentation chromatography has been used to separate cholesteryl esters into groups according to their degree of unsaturation (5). The separation of cholesteryl esters according to the carbon number of the fatty acids esterified to the cholesterol may be achieved using GLC (6-8). However, the unsaturated moieties are not separable by the stationary phases now available for gas chromatography.

The use of reversed phase chromatography resulted in improved separation of cholesteryl esters on the basis of their equivalent carbon number (ECN) with an indication of partial resolution of some critical pairs (9). A combination of two of these methods is required for a complete separation of the cholesteryl esters into individual components. However, these methods are not rapid enough nor reproducible enough for routine analysis.

Recently, high performance reversed-phase chromatography (HPRC) has been used to separate free and esterified cholesterol (10). The main disadvantage of this method was the inefficient resolution of critical pair esters. Even more recently (11), a method for the separation and quantification of cholesterol and its esters has appeared which uses high performance liquid chromatography (HPLC) for the separation of a series of saturated and unsaturated cholesteryl esters and free cholesterol.

In the study carried out in our laboratory and reported herein, a simpler isocratic nonaqueous reverse phase system was developed to separate cholesteryl esters, their critical pairs, two geometric isomers and free cholesterol from each other. This is illustrated by the separation of standard mixtures and the cholesteryl esters of human plasma lipoproteins and rat liver lipid.

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EXPERIMENTAL

Materials

A Tracor Model 950 dual piston chromatographic pump (Tracor Inc., Austin, TX)

equipped with a Rheodyne 1720 injector containing a 20-μL loop (Rheodyne, Berkeley, CA) and a Waters Model R401 differential refractometer detector (Waters, Inc., Milton, MA) was used as the basic instrument system. The minimal detected amount of cholesteryl palmitate was 5 μg with a signal-to-noise ratio of 10. The average component mass employed was from 20-40 μg in the standard mixture shown in Figure 1. The columns used in this study were Supelcosil LC-18, 5-μ octadecyl bonded phase (Supelco Inc., Bellefonte, PA). Data were recorded with a Hewlett-Packard Model 3380 electronic integrator (Hewlett-Packard Co., Fullerton, CA).

Reagents and Standards

A mixture of HPLC grade methanol/chloroform/acetonitrile (1:1:1, v/v) was used as the mobile phase. Cholesterol and cholesteryl ester standards were purchased from Supelco (Bellefonte, PA) and Nu-Chek-Prep. (Elysian, MN). Cholesterol and cholesteryl esters were solubilized in chloroform.

Sample Preparation

Lipids were extracted from human plasma lipoprotein fractions by suspending the individual fractions in 25 vol of chloroform/methanol (2:1) for 5 min (12) and filtering. The extract was then concentrated to ca. 5 mL with a rotary vacuum evaporator. The concentrated extract was then re-extracted two times with hexane and then concentrated to ca. 1 mL. This extract was then placed onto a silicic acid column to separate the lipid classes according to the method of Hirsch and Ahrens (13). The separation was verified with thin layer chromatography (TLC) on silica gel and standards of triglycerides and cholesteryl esters.

Rat liver lipids were extracted from fresh rat liver by homogenization of the liver in chloroform with a Waring blender. Cholesteryl esters were separated from other lipids with preparative TLC on silica gel with hexane/ethyl ether/glac. acetic acid (90:10:1) as the developing solvent (14).

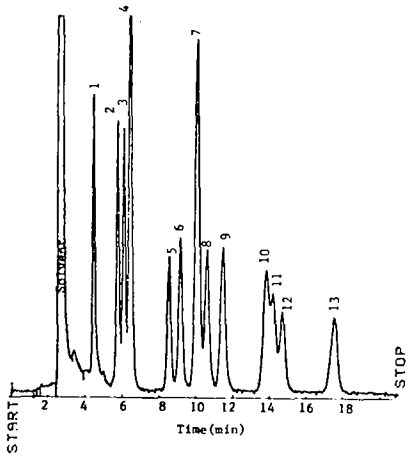

FIG. 1. Separation of cholesterol and cholesteryl ester standards. Column: 4.6 × 250 mm Supelcosil LC-18 reverse phase. Mobile phase: acetonitrile/methanol/chloroform (1:1:1, v/v). Flow rate: 1.0 mL/min. 1-Cholesterol, 2-C. Acetate, 3-C. Propionate, 4-C. Butyrate, 5-C. Nonanoate, 6-C. Decanoate, 7-C. Arachidonate, 8-C. Laurate, 9-C. Linoleate, 10-C. Oleate, 11-C. Elaidate, 12-C. Palmitate, 13-C. Stearate.

Isolation of Human Plasma Lipoproteins

Whole blood or plasma was obtained from the Shands Teaching Hospital of the University of Florida, College of Medicine. The plasma was separated from the whole blood and EDTA was added to the plasma to a final concentration of 0.025%. Sodium azide was then added to a concentration of 2.0 mM to prevent bacterial growth. Chylomicron ($d < 1.006$ g/mL), very low density lipoprotein (VLDL, $d < 1.006$ g/mL), low density lipoprotein (LDL, $1.006 < d < 1.063$ g/mL), and high density lipoprotein (HDL, $1.063 < d < 1.21$ g/mL) fractions were isolated sequentially by centrifugation in solutions adjusted with NaCl or KBr to the appropriate densities as described by Lindgren et al. (15) and Muesing and Nishida (16). All lipoprotein fractions were then dialyzed against phosphate buffer, pH 7.4, containing 0.25% EDTA.

Lipoprotein protein was determined by the method of Lowry et al. (17). Lipoprotein total cholesteryl was measured using the Libermann-Burchard reaction (18). The protein-to-cholesterol ratio for LDL and HDL were 0.44 and 2.26, respectively, similar to values reported by others (19). In addition, the lipoproteins were shown to be free of contamination with other plasma proteins and lipoprotein by electrophoresis on cellulose acetate membranes (Sephraphore III, Gelman Instrument Co., Ann Arbor, MI) separately stained for protein and lipid with Ponceau S or Fat Red 7B, respectively.

Component Identification

Components which eluted from the columns were identified by collecting them into individual scintillation vials, evaporating to dryness with dry nitrogen gas and converting the residue to the corresponding methyl esters followed by gas chromatographic analysis and component identification. Methyl esters were

prepared by addition of 5 mL of 1% sulfuric acid in anh. methanol to the vial and allowing the reaction to proceeed overnight or 12 hr. Hexane (5 mL) was then added to the vial and, after shaking, 5 mL of water was added. The hexane layer was removed, dried over anh. sodium sulfate, concentrated to ca. 1 mL, and an aliquot injected into the gas chromatograph equipped with a 3 mm x 6′ all glass column packed with a 10% SP 2330 stationary phase coated on 100-120 Supelcoport (20). Known standards were used to identify the methyl esters.

RESULTS AND DISCUSSION

The separation of cholesterol and a standard mixture of cholesteryl esters was carried out in this study using one or two LC-18 columns in series (Fig. 1). A difference of only one methylene group was required for a complete resolution of components. This is evident in the separation of cholesteryl acetate, cholesteryl propionate and cholesteryl butyrate from each other as well as the separation of cholesteryl nonanoate from cholesteryl decanoate. Baseline resolution of critical pairs was also achieved. These compounds are as follows: cholesteryl palmitate-cholesteryl oleate, cholesteryl myristate-cholesteryl linoleate and cholesteryl laurate-cholesteryl linolenate. The geometrical isomers cholesteryl oleate and elaidate were partially resolved from each other. The efficiency of separation was dependent on sample size and whether one or two columns were used.

The separation of the cholesteryl esters of the LDL fraction of human plasma lipoprotein is shown in Figure 2. The fatty acids esterified to cholesterol consist of arachidonic, linoleic, oleic and palmitic acids. The identical pattern was found for the other common lipoprotein fractions—VLDL and HDL. The similarity of patterns observed may have occurred as a result of using a pooled blood sample. It should be possible to observe significant differences in examination of each of the lipoprotein fractions from individually drawn samples.

The separation of rat liver lipid is shown in Figure 3. It is clear that free cholesterol is easily resolved but the presence of triglycerides interferes with the separation of cholesteryl esters. For instance, peak number 5 appears to be a mixture of cholesteryl arachidonate and the triglyceride composed of palmitic, linoleic and oleic acids (PLO); peak 7 is a mixture of cholesteryl linoleate and the triglyceride composed of palmitic and oleic acid (POO). Other identifications are listed in the figure

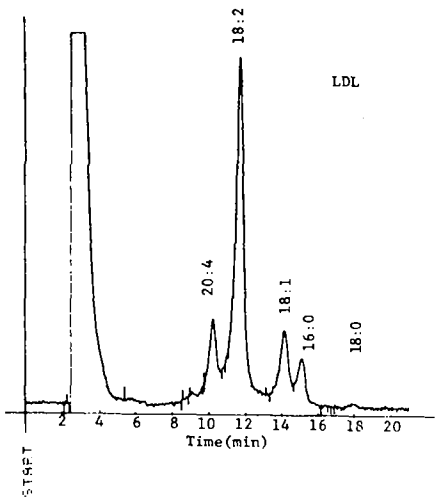

FIG. 2. Separation of the cholesteryl esters of low density lipoproteins of human plasma. Column: 4.6 x 250 mm Supelcosil LC-18 reverse phase. Mobile phase: acetonitrile/methanol/chloroform (1:1:1, v/v). Flow rate: 1.0 mL/min.

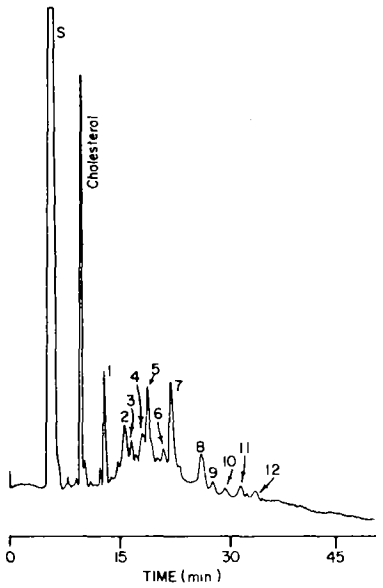

FIG. 3. Separation of rat liver lipid. Column: Two 4.6 x 250 mm Supelcosil LC-18 reverse phase in series. Mobile phase: acetonitrile/methanol/chloroform (1:1:1, v/v). Flow rate: 1.0 mL/min. 1-Unknown (diglyceride), 2-LeOL, 3-LePL, 4-LOL, 5-PLO + C. Arachidonate, 6-SOL+OOO, 7-POO + C. Linoleate, 8-SOO + C. Oleate, 9-SPO + C. Palmitate, 10-SPP, 11-SOS, 12-C. Stearate. (Triglyceride combinations composed of oleic (O), linoleic (L), linolenic (Le), Stearic (S) and Palmitic (P) acids).

legend. When rat liver cholesteryl esters were isolated via TLC and analyzed via HPRC, the chromatogram shown in Figure 4 was obtained. As indicated in the chromatogram, cholesteryl esters of arachidonic, linoleic, oleic, palmitic and stearic acids are present. Furthermore, it is evident that the sample was somewhat oxidized from the cluster of components eluting with the solvent front at ca. 10 min. In our experience, autoxidative products typically elute in this region when there are substantial amounts of polar material present, suggesting a technique to both isolate and evaluate these materials.

A linear relationship is obtained when the carbon number of the saturated acyl substituents of the cholesteryl ester is plotted vs their capacity factor k′ (Fig. 5). However, this relationship for unsaturated acyl substituents is nonlinear. Cholesterol did not elute with a k′ of a cholesteryl ester with a zero carbon number but rather at a place where a cholesteryl ester with -2.5 carbons esterified to cholesterol. This large shift is apparently the result of the presence of the hydroxyl group on the cholesterol moiety and the methanol in the mobile phase mixture resulting in a shorter residence time of cholesterol on the column. This indicates that the presence of a hydroxyl group in any cholesteryl ester such as that of a hydroxy acid would disrupt the elution pattern of a homologous series of esters. This is true for methyl esters and triglycerides, as well. Thus, a cholesteryl ester with a hydroxy-stearic acid will elute not only before cholesteryl stearate but also before cholesteryl palmitate.

A predictable pattern for the separation of cholesteryl esters (Fig. 6) was found to follow that observed for triglycerides and methyl esters (21) where the elution sequence starts with the highest carbon number components with greatest degree of unsaturation and terminates with the lowest carbon number of the series. Thus, cholesteryl arachidonate will elute before cholesteryl linolenate. The last cholesteryl ester to be eluted would be cholesteryl laurate (Fig. 6) which has the same carbon number as the equivalent carbon number of the group. The cholesteryl ester containing a *trans* fatty acid, i.e., elaidate, will have a longer residence time than its *cis* counterpart (21). However, the resolution of *cis-trans* esters would also be affected by the composition of the mobile phase and the efficiency of the stationary phases. A more uniform elution pattern has been observed using acetonitrile/chloroform (2:1) as the mobile phase but the resolution of the cholesteryl esters with low equivalent carbon numbers was drastically impaired. The addition of methanol to the mobile phase improved the resolution but disrupted the relative residence time sequence.

While the manuscript for this publication was in preparation, a similar method for the separation of cholesterol and its esters appeared (11). This method also reported the separation

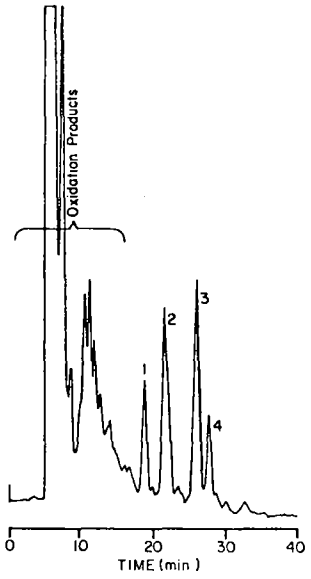

FIG. 4. Separation of rat liver cholesteryl esters. Column: Two 4.6 X 250 mm Supelcosil LC-18 reverse phase in series. Mobile phase: acetonitrile/methanol/chloroform (1:1:1, v/v). 1-C. Arachidonate, 2-C. Linoleate, 3-C. Oleate, 4-C. Palmitate, 5-C. Stearate.

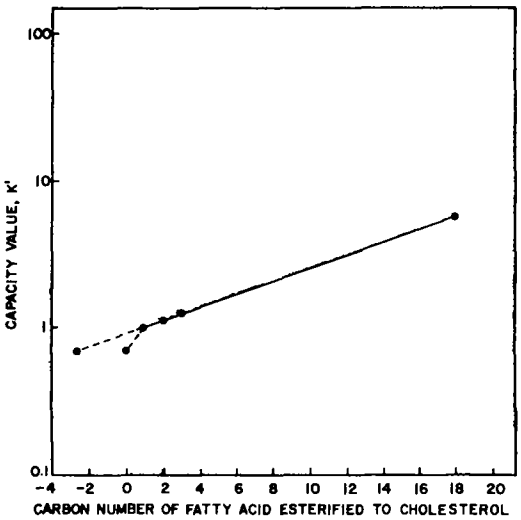

FIG. 5. Effect of fatty acid carbon number on the capacity value k′.

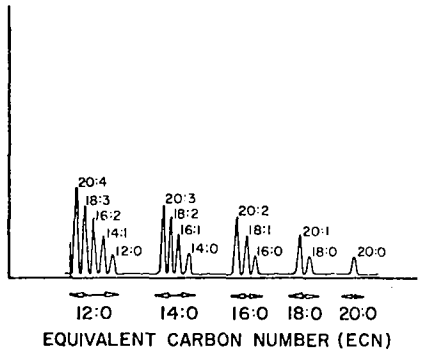

FIG. 6. Influence of unsaturation and equivalent carbon number on the elution profile of cholesteryl ester critical pairs.

of critical pairs. These authors employed a mobile phase composed of acetonitrile/tetrahydrofuran (65:35, v/v) and water. The water content was changed in a linear gradient from 3% to 0% over a 20-min period, at a column temperature of 37 C. A total column length of 40 cm of Zorbax ODS octadecyl bonded phase was used. Successful separation of compounds through cholesterol stearate was accomplished within ca. 25 min.

Results presented in this paper were obtained on a much simpler system requiring only a single mobile phase mixture and an isocratic system at room temperature. No gradient elution and hence no column regeneration was required. The separation achieved was comparable to that reported (11). A complete comparable separation of the test compounds was achieved within 17 min with one column and a significantly improved separation with two columns in series. However, as shown in Figures 1 and 2, adequate resolution of components is obtained with one column. The increased resolution of two columns is advantageous when the cholesteryl esters mixture is contaminated with triglycerides.

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Determination of Fatty Acid Composition via Chemical Ionization-Mass Spectrometry

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ABSTRACT

The analysis of fatty acid methyl esters by chemical ionization-mass spectrometry via a new interface system is described. The sample is applied to the interface system in dichloromethane containing trideuteromethyl (CD_3) fatty acid esters as internal standards and dodecanol as a carrier. The esters are evaporated into a carrier gas of nitrogen in the interface and then drawn through a transfer system where residual traces of solvent are removed and the esters are concentrated by a porous silver membrane separator. The sample, now enriched in the carrier gas, is passed into the mass spectrometer where it is analyzed by selective multiple ion monitoring using isobutane as the reagent gas. The method, which takes less than a minute, is computerized for the quantitative analysis of the 19 most common fatty acids occurring in major portions in animal and plant tissues. The accuracy and precision of the method was demonstrated and compared to fatty acid analysis by gas liquid chromatography on several fats.

INTRODUCTION

The coupling of gas chromatography with mass spectrometry and its application to fatty acid analysis, first reported by Ryhage and Stenhagen (1), greatly extended the analysis of these compounds. With the advent of CIMS (2,3), which gives a relatively simple fragmentation pattern, several investigations (4-9) have been directed to the use of this technique as a rapid method for the direct analysis of fatty acids. However, probe injection systems are not ideally suited to quantitative analysis inasmuch as they are not designed for multiple analyses; also, the mechanics of their operation predisposes against quantitative analysis of mixtures containing compounds with widely varying boiling points.

MATERIALS AND METHODS

Highly purified (> 99%) methyl esters, prepared by the Hormel Institute Lipids Preparation Laboratory or purchased from Nu-Chek-Prep, Inc., Elysian, MN, were used in this investigation. The eicosapentaenoate (20:5) and docosapentaenoate (22:5) methyl esters, which were not available commercially, were prepared using a combination of reverse phase HPLC and $AgNO_3$ TLC (O.S. Privett and W.L. Erdahl, unpublished data). The purity of each ester was monitored by GLC; the purity of the final preparations of these methyl esters was > 99%.

Dodecanol was purchased from Rohm and Haas, Philadelphia, PA, and used without further purification inasmuch as it gave no ions that interfered with the analysis of the methyl esters.

The CD_3 esters of hexadecanoate (16:0), octadecanoate (18:0), eicosanoate (20:0) and docosanoate (22:0) were prepared by esterifying the highly purified free fatty acids with tetradeuteromethanol using 2% dideuterosulfuric acid as the catalyst; the CD_3 esters had an isotopic purity of ca. 97%.

The methyl esters of soybean lecithin (Azolectin, Associated Concentrates, Woodside, Long Island, NY) and rat tissue lipids used for comparative analysis by GLC and our CI-MS method were prepared by interesterification with methanol using 5% HCl as catalyst (10).

Gas Liquid Chromatography

GLC analysis of methyl esters was performed with a Hewlett Packard Model 5840A gas chromatograph equipped with a 12′ × 0.125″ id column packed with 10% SILAR 10C on 100/120 mesh Gas Chrom Q (Laboratory Data Control/ASD, Parkridge, IL). A temperature program was used in the GLC analysis, starting at 170 C for 10 min, then increasing the temperature by 0.35 C/min to 205 C with an He flow rate of 15 mL/min.

Interface-Mass Spectrometry Apparatus

A block diagram of the complete system is shown in Figure 1 in order to indicate the

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CD_3, trideuteromethyl; HPLC, high pressure liquid chromatography; GLC, gas liquid chromatography; TLC, thin layer chromatography; CI-MS, chemical ionization-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; EI, electron impact; CI, chemical ionization; MID, multiple ion detection; D/A, digital-to-analog; A/D, analog-to-digital; FID, flame ionization detector; IF-MS, interface-mass spectrometry.

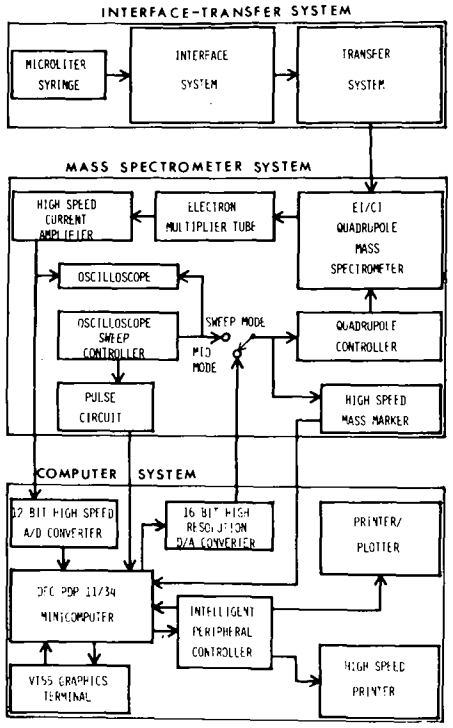

FIG. 1. Block diagram of the interface-transfer-mass spectrometer-computer system showing both the MID and sweep modes of collecting data and outputting results. A/D, analog to digital; D/A, digital to analog; DEC, Digital Equipment Corporation.

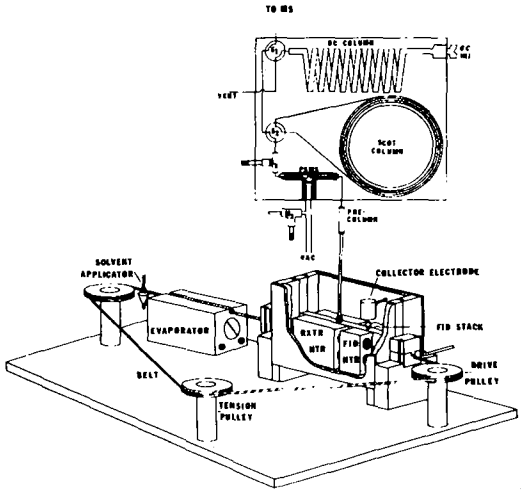

FIG. 2. Schematic of interface-transfer system showing method of application of sample on belt to its introduction into the mass spectrometer. RXTR HTR, reactor heater; FID HTR, flame ionization detector heater; VAC, vacuum; S_1 and S_2, switching valves; M_1 and M_2, micrometering valves; PSMS, porous silver membrane separator; MS, mass spectrometer; GC, gas chromatograph.

relationship of the interface-transfer system, which is crucial to the method, to the mass spectrometer system and the computer system. Details of the construction of the interface-transfer system, a schematic of which is shown in Figure 2, have been previously described (11-13).

The transfer system (Fig. 2) also contains a packed column that can be used for independent analyses by GC-MS. The SCOT column (Fig. 2) is used for secondary analysis when the system is used in conjunction with HPLC. It is bypassed in our method which passes the methyl esters directly into the source of the mass spectrometer through switching valves S_1 and S_2 where they are ready for analysis.

The mass spectrometer is a Biospect Model 7501 (Scientific Research Instruments Inc., Baltimore, MD). It is a quadrupole instrument with both EI and CI modes of operation and is equipped with a solids probe injector. Only the CI mode of operation is used for our method.

An MID data acquisition program and the hardware necessary to control the mass spectrometer and to collect data from the analysis were developed with the hardware and software necessary to operate the various peripheral devices. The program is run on a PDP-11/34 minicomputer by setting hardware and mass spectrometer parameters and selecting the methyl esters for analysis, using a VT55 graphics terminal. By program control, a 16-bit high resolution D/A converter generatres an analog signal which is sent to the quadrupole controller which, in turn, controls the quadrupole rods in the mass spectrometer. The analog signal from the D/A converter also operates a high-speed mass marker which gives digital information concerning the pseudomolecular (M+1) ion being monitored via a built-in A/D converter. The mass spectrometer is now monitoring only one (M+1) ion. The intensity of the sample ions present at the (M+1) ion is amplified by an electron multiplier tube. The ion current is further increased by a high-speed current amplifier which sends the signal to a high-speed 12-bit A/D converter in the computer. The digitized intensity data, along with the digitized (M+1) ion data, are stored in the computer. The program sorts and processes the data, outputting a table showing the analysis of each (M+1) ion through an IMSIA 8080 microprocessor peripheral controller to a Texas Instruments, Model Omni 810, high-speed printer. The data may then be stored on a hard

disk where, later, via other programs, it can be plotted on a Hewlett Packard Model 7221 printer/plotter.

A second data acquisition program was developed to collect intensity and ion mass information by linearly sweeping a set range of ion masses. The analog signal is supplied by the oscilloscope sweep controller. The analog signal operates the quadrupole controller and mass marker. To synchronize the data acquisition to the start of the sweep, a trigger pulse from a pulse circuit is used. The change in ion intensity as the ion mass is varied is amplified by the electron multiplier tube and current amplifier. The signal is digitized by the 12-bit A/D converter and stored in the computer. An ion mass reading from the mass marker is stored with each intensity reading. The sweep program generates an ion mass relative intensity table which is outputted to the high speed printer. This table can be stored on a hard disk and, later, via other programs, it can be displayed on the VT55 graphics terminal or a hard copy can be drawn by the printer/plotter.

Procedure

The sample of methyl esters containing the internal CD_3 ester standards and carrier, 40 μg of dodecanol, is applied in ca. 1 μL of dichloromethane to the belt of the interface system (Figs. 1 and 2). The belt is operated at 0.33 cm/sec; the reactor compartment of the interface and the transfer system are maintained at 280 C. Nitrogen is introduced into the reactor compartment at 50 cc/min at the front port and 20 cc/min at the central port (Fig. 2). The flow rate of nitrogen is adjusted to direct the methyl esters, which are in the vapor state, entirely into the transfer system. Hydrogen is introduced at 40 mL/min into the FID compartment which is maintained at 450 C. This compartment contains an FID which is not used in this procedure. The hydrogen is blocked

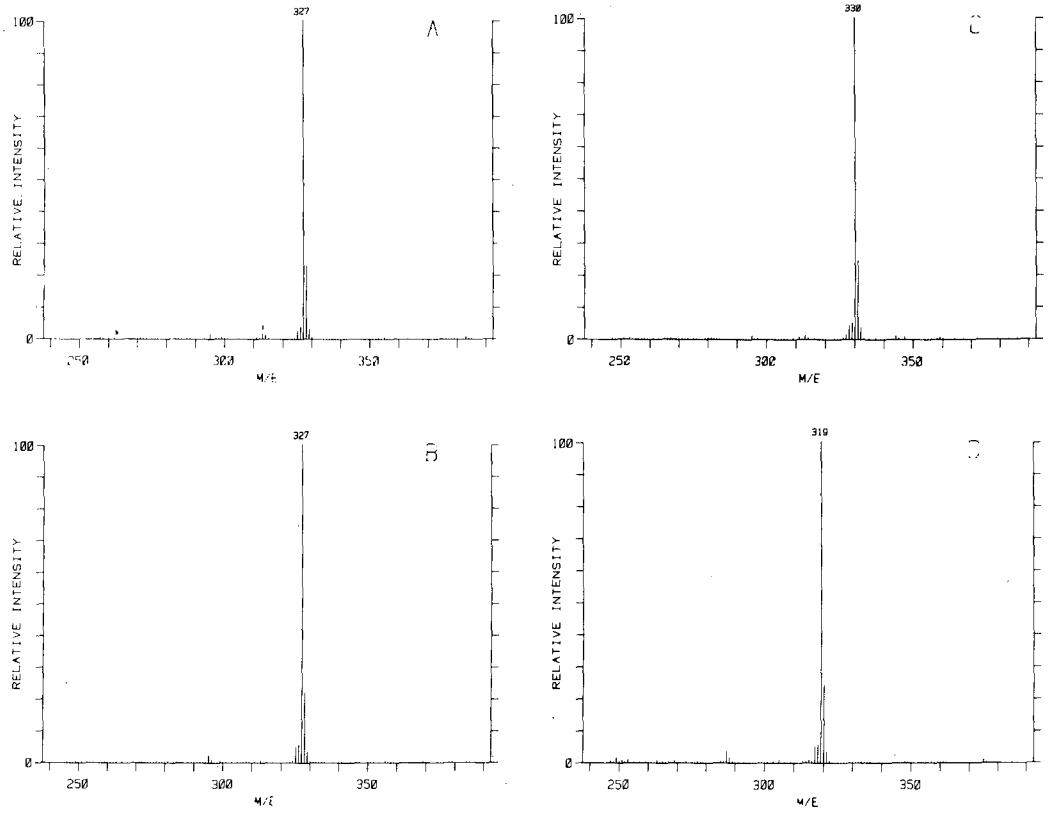

FIG. 3. Mass spectra of methyl esters using isobutane reagent gas under various conditions. A: methyl eicosanoate solids probe injection, source 225 C at 1.00 torr pressure; B: methyl eicosanoate, interface-transfer injection, 285 C, source 225 C at 2.00 torr pressure; C: trideuteromethyl eicosanoate, interface-transfer injection, 285 C, source 225 C at 2.00 torr pressure; D: methyl eicosatetraenoate, interface-transfer injection, 285 C, source 225 C at 2.00 torr pressure.

from the first compartment by nitrogen entering at the central port and is forced to exit through the flame detector by the nitrogen block at the end port. Nitrogen is passed into this port at 15 mL/min for this purpose. The main function of the FID compartment is to reduce oxides on the belt and, in effect, provide a chemical cleaning of the belt on each cycle through the interface. As the belt enters the atmosphere, a fresh layer of oxides is formed on the surface increasing its adsorptive properties.

The sample in the carrier gas of nitrogen is passed into the transfer system where it is concentrated by means of a porous silver membrane separator. The degree of concentration is controlled by the micrometering valve, M_1, which is set to allow a maximum of 8 mL/min gas to enter the source of the mass spectrometer and the external vacuum applied

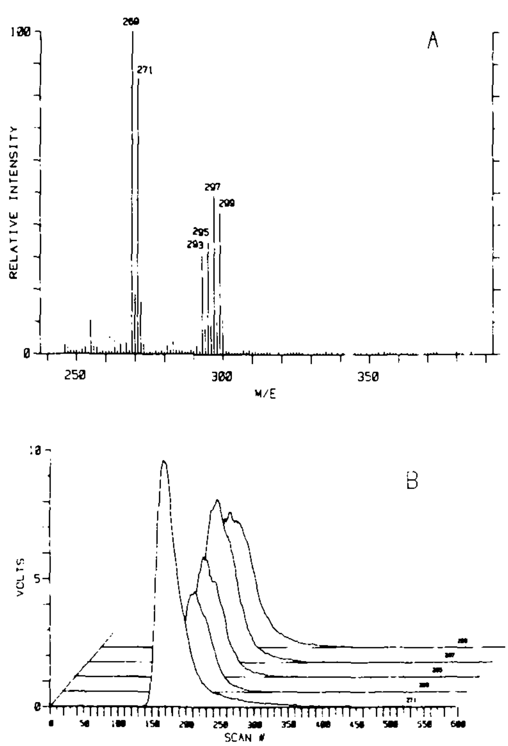

FIG. 4. A: mass spectrum of a methyl ester mixture using isobutane reagent gas at 2.00 torr pressure, interface-transfer injections at 285 C, source 225 C (269 [16:1]; 271 [16:0]; 293 [18:3]; 295 [18:2]; 297 [18:1] and 299 [18:0]); B: 3-dimensional (M+1) ion intensity (volts) vs time (scan number) plot of several methyl esters applied in a mixture to the belt of the interface-transfer system. The scan rate was ca. 15 scans/sec on all esters (271 [16:0]; 293 [18:3]; 295 [18:2]; 297 [18:1] and 299 [18:0]).

TABLE 1

Methyl Ester and Trideuteromethyl Ester Percentage of Relative Intensity

Methyl and CD_3 Ester	(M+1) ion 269	271	274	293	295	297	299	302	317	319	321
16:1	100.0	2.9	.1	.1	0	.2	.2	0	0	0	.1
16:0	4.5	100.0	.3	0	0	.1	0	0	0	0	.1
16:0(CD_3)	.1	.5	100.0	.1	0	.1	0	0	0	.1	.1
18:3	.2	.2	.2	100.0	4.7	.3	.2	.2	.1	.1	.2
18:2	.6	0	.2	15.9	100.0	6.6	.9	.4	.1	.2	.2
18:1	.8	.2	.1	1.4	7.1	100.0	3.8	.2	.1	.1	.2
18:0	.4	.6	.2	.2	.6	4.3	100.0	.7	.2	.3	.2
18:0(CD_3)	.4	.7	.1	.2	.1	0	.7	100.0	.2	.4	0
20:5	1.7	2.2	1.1	.8	.7	.6	1.5	.7	100.0	6.8	.8
20:4	4.8	1.2	.5	.6	.5	.3	.4	.2	9.5	100.0	5.3
20:3	.7	1.2	.2	3.2	.6	.1	.1	0	1.3	7.9	100.0
20:2	1.3	1.0	.6	1.5	1.9	.6	.3	0	1.0	2.2	12.5
20:1	1.8	.8	.3	7.2	.7	.7	.3	0	.3	.5	1.1
20:0	.8	.7	.5	1.1	2.9	.4	.1	.3	.2	0	.3
20:0(CD_3)	.8	.6	.4	1.2	3.3	.5	.4	.5	.3	0	.4
22:6	11.2	8.5	4.9	4.4	1.5	1.5	1.9	1.0	1.3	.5	.6
22:5	5.6	10.8	2.9	2.4	5.1	1.7	1.6	.7	1.9	1.1	.9
22:4	2.4	2.5	1.1	2.0	1.3	2.4	1.1	.8	1.3	1.8	.9
22:3	4.9	1.3	.7	6.2	2.1	1.7	1.4	.4	5.9	1.7	2.8
22:2	16.0	1.9	.5	3.9	6.5	1.5	.4	.6	1.2	23.7	1.7
22:1	6.4	1.1	.4	.5	.9	1.7	.7	.2	.4	2.0	7.4
22:0	1.9	1.0	.1	.8	1.2	1.5	1.5	.1	.1	2.1	1.4
22:0(CD_3)	1.9	.9	1.0	1.2	1.6	1.1	.9	.3	.3	1.1	1.6

Methyl and CD_3 Ester	(M+1) ion 323	325	327	330	343	345	347	349	351	353	355	358
16:1	0	.3	.1	.1	0	0	0	0	.1	.4	0	.1
16:0	.1	0	0	0	.1	.1	0	.2	.1	0	.1	0
16:0(CD_3)	.1	0	0	.1	0	.1	.1	.1	0	0	.1	.1
18:3	.2	0	.1	.1	0	.1	.3	.6	.2	.1	.1	.1
18:2	.6	.1	.2	.1	.2	.3	.5	.7	.7	.5	.3	.3
18:1	.2	.3	.2	0	.1	.2	0	.4	.1	.3	.2	.2
18:0	.2	.2	.3	.1	.1	.1	.1	.1	0	.1	.2	0
18:0(CD_3)	0	.1	0	.1	.1	0	0	.1	.1	.2	0	0
20:5	.5	.4	.9	.4	.2	.3	.6	.1	.3	.1	.9	.4
20:4	.3	.2	.5	.1	.2	0	.2	.4	.3	0	.1	.2
20:3	4.9	.4	.2	.1	0	.1	.1	.3	.2	0	0	.1
20:2	100.0	4.5	.6	.2	.1	0	.3	.1	.1	.4	.1	.1
20:1	6.5	100.0	4.0	.4	.1	.1	.1	.1	.1	.1	.1	.3
20:0	.7	5.0	100.0	.6	.1	0	.1	0	.1	.1	.2	0
20:0(CD_3)	.5	.1	.7	100.0	.1	.1	.5	.2	.1	.1	.1	.1
22:6	.8	.9	2.6	.9	100.0	9.5	.6	.2	.2	.2	.3	.7
22:5	.6	.6	1.8	1.1	15.7	100.0	8.8	5.5	2.3	1.6	.2	0
22:4	.6	.7	.4	.6	3.4	10.9	100.0	6.4	1.5	1.2	.3	.2
22:3	.9	.5	.7	.4	1.4	2.0	10.6	100.0	7.1	1.3	.4	.3
22:2	1.5	.4	.4	0	.7	.5	2.6	14.7	100.0	5.9	2.5	.1
22:1	.5	1.1	.6	.1	0	.1	.4	1.1	6.7	100.0	4.6	.1
22:0	3.4	.9	5.3	.5	0	.2	.1	0	1.1	6.1	100.0	.8
22:0(CD_3)	3.1	.3	.8	.4	.6	.4	.2	.2	.1	.2	1.3	100.0

to the separator which is also controlled by a metering valve, M_2. The mass spectrometer is operated at a pressure of 4×10^{-6} torr in the analyzer section and 1-2 torr in the source where the reagent gas, isobutane, and the sample, concentrated in carrier gas, are introduced.

The MID data acquisition program was set to monitor 23 different ester (M+1) ions. To allow for nonlinearity between molecular masses monitored and voltage applied to the quadrupole rods, 9 intensity readings were taken over the center, 50% of each (M+1) ion peak. Only the largest intensity reading was kept. Two intensity readings were averaged and a molecular ion reading was taken before the computer increased the voltage on the quadrupole rods, advancing the mass spectrometer to the next (M+1) ion to be monitored. This process was repeated until all 23 ester (M+1) ions had been measured. Then the entire process was repeated, starting at the lowest (M+1) ion. Ten averaged intensity measurements can be made for each ester (M+1) ion in 1 sec. A typical run of 30 sec generates 300 data points for each ester (M+1) ion.

An integration routine determined the peak area for each (M+1) ion, correcting, if necessary, for any shifts in baseline. A table of parameters, peak areas and relative percentage composition for each ester (M+1) ion was printed on the high-speed printer a few sec after the data acquisition was completed. The raw data can then be stored on a hard disk and programs, such as the plotting routine shown in Figure 4B, can be used to present the data graphically.

RESULTS

Typical mass spectra of the methyl esters of fatty acids under the conditions just described using isobutane as the reagent gas are shown in Figure 3. The solids probe was used to compare mass spectra of the methyl esters by probe injection with interface-transfer injection. Both spectra were essentially the same, as is shown in Figures 3A and 3B. The predominant ion (Table 1) is that of the M+1 ion. In most cases, the only other ions of significance are those of the naturally occurring isotopes. However, with the higher the degree of unsaturation in a methyl ester, the greater will be the intensity of the (M-1) ions which are produced by a secondary ionization mode. Thus, the peak area being measured at the (M+1) ion will be reduced giving different response ratios for each ester in the series. Also, with the 20 and 22 series esters, (M-31) ions, which are formed by the loss of an OCH_3 group, were observed at masses corresponding to the pseudomolecular ion of 16 and 18 series methyl esters.

Due to mass discrimination by the quadrupole, the response of the individual methyl esters varied considerably as shown in Figure 4. In order to correct for the different response resulting from mass discrimination and ionization mode, the CD_3 esters of the saturated

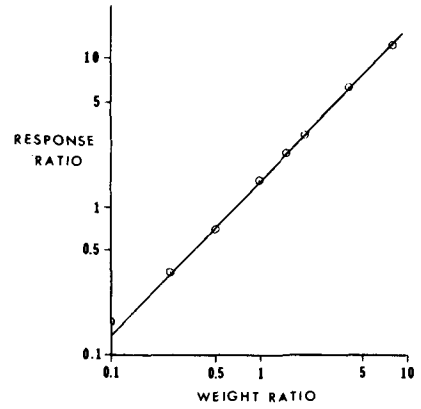

FIG. 5. Log-log plot of the mass spectral response ratio of the 18:1 methyl ester divided by 18:0 CD_3 ester vs the weight ratio of the 18:1 methyl ester divided by the 18:0 CD_3E. Applying a least squares fit to the equation, $y=Ax^B$, A=1.515, B=0.9995, thus demonstrating the linearity of the response vs weight ratios.

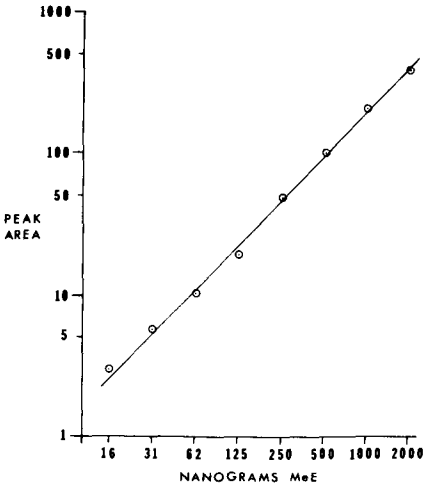

FIG. 6. Log-log plot of the peak area vs nanograms of 18:1 methyl ester applied to the belt in 1 μL of a dichloromethane solution containing 40 μg/mL of dodecanol. Applying a least squares fit to the equation, $y=Ax^B$, A=191,440; B=1.0269, thus demonstrating the linearity of the mass spectral response to the mass of methyl esters applied to the belt.

TABLE 2

Methyl Ester Analysis of 16-Carbon Fatty Acids

Step 1. Make MID run of pure 16:1 and 16:0 MeE and 16:0 CD_3E measuring intensities at the 3 pseudomolecular (M+1) ions, integrating the (M+1) ions for each ester.

Step 2. Set up a square matrix using relative peak areas from step 1: (Illustrated by using coefficients [X 10^{-2}] from Table 1).

$$\text{Mixture peak MeE 16:1} = M_{16:1} = 1.000\,X_{16:1} + 0.029\,X_{16:0} + 0.001\,X_{16:0\,d};$$
$$\text{Mixture peak MeE 16:0} = M_{16:0} = 0.045\,X_{16:1} + 1.000\,X_{16:0} + 0.003\,X_{16:0\,d};$$
$$\text{Mixture peak } CD_3E\ 16:0 = M_{16:0\,d} = 0.001\,X_{16:1} + 0.005\,X_{16:0} + 1.000\,X_{16:0\,d},$$

where $X_{16:1}$ = corrected peak area of 16:1 MeE (M+1) ion; $X_{16:0}$ = corrected peak area of 16:0 MeE (M+1) ion; and $X_{16:0\,d}$ = corrected peak area of 16:0 CD_3E (M+1) ion.

Step 3. Invert matrix using modified Gauss-Jordan elimination method:

$$X_{16:1} = 1.00131\,M_{16:1} - 0.0450565\,M_{16:0} - 0.000776025\,M_{16:0\,d};$$
$$X_{16:0} = 0.029033\,M_{16:1} + 1.00132\,M_{16:0} - 0.00497757\,M_{16:0\,d};$$
$$X_{16:0\,d} = 0.000914207\,M_{16:1} - 0.00295891\,M_{16:0} + 1.00002\,M_{16:0\,d}.$$

Step 4. Make MID run on a standard ester mixture of known MeE and CD_3E concentration as in step 1.

Step 5. Calculate corrected peak areas using the equations in step 3.

Step 6. Calculate the response factors for MeE relating known weights of the esters in the standard mixture to the measured responses by the following equation:

$$\frac{\text{corrected peak area MeE}}{\text{corrected peak area } CD_3E} = K \times \frac{\text{weight MeE}}{\text{weight } CD_3E},$$

where K = response factor, for the methyl esters, e.g., solving this equation for K (response factor) gives the following:

$$K_{16:1} = \frac{\text{weight 16:0 d}}{\text{weight 16:1}} \times \frac{X_{16:1}}{X_{16:0\,d}}$$

$$K_{16:0} = \frac{\text{weight 16:0 d}}{\text{weight 16:0}} \times \frac{X_{16:0}}{X_{16:0\,d}}$$

Step 7. Make MID run of sample to which a known amount of CD_3E 16:0 has been added as in step 1.

Step 8. Calculate correct peak areas using equations in step 3.

Step 9. Rearranging the equation in step 6 yields

$$\text{weight 16:1} = \frac{\text{weight 16:0 d}}{K_{16:1}} \times \frac{X_{16:1}}{X_{16:0\,d}}$$

$$\text{weight 16:0} = \frac{\text{weight 16:0}}{K_{16:0}} \times \frac{X_{16:0}}{X_{16:0\,d}},$$

where the weight of each MeE can be calculated from the known amount of CD_3E added to the sample and from the response factor calculated in step 6.

Step 10. Calculations of % composition is made from the calculated weights of the MeE:

$$\%\ 16:1 = \frac{\text{weight 16:1} \times 100}{\text{weight 16:1} + \text{weight 16:0}}$$

$$\%\ 16:0 = \frac{\text{weight 16:0} \times 100}{\text{weight 16:1} + \text{weight 16:0}}$$

Step 11. If more than one determination is made on each sample, the average, standard deviation and relative % error can be calculated.

Because of the complexity of inverting the matrix, anything beyond a three-component mixture would require the use of a computer. The computer also leads to automation of the entire process. This method can be expanded to include any number of compounds. It is necessary to have a spectrum of the pure compounds being measured as well as any internal standard. The square matrix can be set up and inverted. Any response factors can be calculated from a standard mixture of the components. Then, by adding the internal standard to the sample, all components can be determined.

TABLE 3

Methyl Ester Standard Mixture, Percentage Composition (wt)

	Standard A		Standard B		Standard C	
	IF-MS[a]	GLC[b]	IF-MS[a]	GLC[c]	IF-MS[a]	GLC[b]
16:0	18.7 ± 0.5	17.8 ± 0.2	5.3 ± 0.4	6.1 ± 0.4	29.8 ± 0.2	28.2 ± 0.1
16:1	17.6 ± 0.1	17.6 ± 0.1	10.6 ± 0.3	10.5 ± 0.6	21.7 ± 0.7	21.6 ± 0.1
18:0	19.5 ± 0.3	16.6 ± 0.1	14.4 ± 2.1	13.7 ± 0.3	17.4 ± 0.6	19.8 ± 0.1
18:1	16.5 ± 0.5	16.4 ± 0.1	18.3 ± 0.6	18.0 ± 0.1	15.6 ± 0.3	16.0 ± 0.1
18:2	15.1 ± 0.7	15.8 ± 0.1	21.7 ± 0.5	21.3 ± 0.4	11.4 ± 0.1	10.9 ± 0.1
18:3	12.5 ± 0.7	15.7 ± 0.2	29.8 ± 1.2	30.4 ± 0.7	4.3 ± 0.3	3.5 ± 0.2

[a]Average ± standard deviation, n = 5.
[b]Average ± standard deviation, n = 3.
[c]Average ± standard deviation, n = 4.

acids of each even chain length fatty acid from 16 to 22 carbon atoms were used as internal standards. The (M+1) ion of these esters, in relation to those of the corresponding methyl esters is illustrated in Figures 3B and 3C. These show that there are few differences in the spectra of the two analogs. The fact that highly unsaturated esters, such as eicosatetraenoate, are also not extensively fragmented, as shown in Figure 3D, aids greatly in the quantitation of the esters.

The technique of Gifford et al. (14) was used to take into account ions from esters that overlap each other. In order to determine the correct response of a particular methyl ester in a mixture and to develop a method for quantitative analysis, the mass spectral responses of the 19 most common fatty acids (as methyl esters) found in plant and animal tissue were determined along with the 4 CD_3 ester internal standards. The intensities at each of the 23 pseudomolecular ions for all 23 esters are presented in Table 1 in a 23 x 23 matrix. Inversion of this matrix by a modified Gauss-Jordan elimination method (15) produced a set of 23 simultaneous equations with 23 unknowns which allowed the corrected peak areas for each (M+1) ion in the mixture to be calculated. The relative concentration of each methyl ester was calculated from its response ratio with the corresponding chain length CD_3 ester standard.

Quantitative analysis is based on a linear relationship between the ratio of the response of the methyl esters and their corresponding even-chained saturated CD_3 esters to the ratio of their masses. The linearity of this relationship was demonstrated with a series of mixtures of 18:1 methyl ester and 18:0 CD_3 ester as shown in Figure 5. These data showed that the relationship was linear over a range of MeE to CD_3E from 0.1/1 to 8/1. By applying the method of least squares fit to this data in accordance with the equation $y=Ax^B$; linearity was demonstrated (B=0.9995).

The method also is based on the assumption of a linear relationship between peak area of each component and its mass. This relationship was demonstrated with 18:1 methyl ester as shown in Figure 6; the least squares fit to $y=Ax^B$ was B=1.0269. These results also showed that the useful range of the method extends into the low nanogram level. As an example of the procedure, each step is illustrated in Table 2 for a 3-component mixture.

To test the validity of the method, it was applied to standard mixtures of which the components were varied in concentration from low to high and high to low. These tests indicated that the method had a relative error of ca. 5% for the higher concentration of esters and 10% for the lower concentrations, which compares favorably to the GLC analysis also shown in Table 3.

As a practical test of the IF-MS method, the analyses of several fats were compared to that of GLC. These results, which are summarized in Table 4, showed that while the analyses by the IF-MS had a slightly higher standard deviation than those by GLC, the agreement between the methods was good. Some differences may arise due to the inability of a packed GLC column to separate all positional isomers and chain length series esters completely (8).

DISCUSSION

The method described here is designed to provide analyses of only predetermined fatty acids. It cannot be used to detect and quantify unknown components nor to provide a profile of more than the 23 components. However, the 19 fatty acids selected cover the range that one is likely to encounter in significant amounts in

TABLE 4

Fatty Acid Percentage Composition (wt) of Plant and Animal Tissues

Methyl ester	Soy lecithin		Rat liver[a]		Rat kidney[b]		Rat kidney[c]	
	IF-MS[d]	GLC[d]	IF-MS[d]	GLC[e]	IF-MS[d]	GLC[e]	IF-MS[d]	GLC[e]
16:0	18.7 ± 0.6	19.9 ± 0.3	29.5 ± 1.4	27.7 ± 1.2	18.9 ± 0.5	16.9 ± 0.4	23.1 ± 0.5	21.8 ± 0.3
16:1		0.2 ± 0.2	1.4 ± 0.1	3.9 ± 0.3	0.7 ± 0.2	0.7 ± 0.3	7.0 ± 0.2	7.8 ± 0.2
18:0	3.6 ± 0.3	3.6 ± 0.5	18.4 ± 0.3	16.9 ± 0.2	14.4 ± 0.3	12.8 ± <0.1	13.9 ± 0.4	12.5 ± <0.1
18:1	7.5 ± 0.2	8.7 ± 0.2	14.2 ± 0.5	14.2 ± 0.1	9.7 ± 0.2	11.0 ± <0.1	30.8 ± 1.3	32.6 ± 0.4
18:2	62.2 ± 0.7	59.2 ± 1.0	15.3 ± 1.0	15.1 ± 0.2	33.6 ± 0.6	35.2 ± 0.3	2.2 ± 0.4	2.1 ± 0.3
18:3	8.1 ± 0.3	8.4 ± 0.2	0.6 ± 0.1	0.5 ± <0.1			0.4 ± 0.2	0.3 ± 0.2
20:0			0.8 ± 0.3		1.1 ± 0.2		0.9 ± 0.5	0.5 ± 0.1
20:1			1.4 ± 0.2	0.9 ± <0.1	0.4 ± 0.3		0.6 ± 0.2	0.1 ± 0.2
20:2			0.3 ± <0.1	0.7 ± 0.3	0.4 ± 0.3	0.2 ± 0.2	0.3 ± 0.5	0.4 ± 0.2
20:3			1.3 ± <0.1	1.2 ± 0.4	1.0 ± 0.4	0.5 ± 0.3	9.9 ± 0.7	10.3 ± 0.3
20:4			16.5 ± 1.9	16.9 ± 0.3	17.8 ± 0.8	17.0 ± 0.3	8.2 ± 0.6	6.8 ± 0.3
20:5			1.1 ± 0.1	1.6 ± 0.6	0.8 ± 0.2	2.2 ± 0.5	0.9 ± 0.8	2.9 ± 0.4
22:0					0.7 ± 0.7	0.3 ± 0.2	0.7 ± 0.5	
22:1						0.5 ± 0.3	0.4 ± 0.4	
22:2							0.3 ± 0.4	
22:3						0.4 ± 0.3		1.5 ± 0.4
22:4					0.5 ± 0.7	0.8 ± 0.1	0.7 ± 1.2	
22:5						1.3 ± 0.6		0.3 ± 0.2
22:6					0.3 ± 0.3	0.2 ± 0.2		0.2 ± 0.2

[a]10% Safflower oil diet, analysis on 16-20 carbon series only.

[b]10% Safflower oil diet.

[c]10% Hydrogenated coconut oil diet.

[d]Average ± standard deviation, n = 5.

[e]Average ± standard deviation, n = 4.

most vegetable and animal fats and oils. Should there be a specific fatty acid that one might be required to measure other than the 19 acids selected, e.g., ricinoleic acid in castor oil, a method could be readily developed based on the same principles described here to provide an analysis of it, as well as any other components that may be desired.

Theoretically, our method can provide an analysis in less than 1 min and 60 analyses in 1 hr compared to 2 hr by GLC for complete separation of all components of the same mixture, i.e., one containing fatty acids with carbon chains of 16:0 to 22:0 and unsaturation to 22:6. Accordingly, the method described here or one developed on the same principle for a particular fat, not covered by the 19 acids designated in this method, should be very valuable where large numbers of samples must be analyzed, as in plant breeding or nutritional experiments.

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COMMUNICATIONS

Biosynthesis of Fatty Acids by *Trypanosoma cruzi*

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ABSTRACT

The incorporation of [1-^{14}C] acetate into fatty acids by cultured epimastigotes of *Trypanosoma cruzi* was studied. After 8, 24, and 48 hr incubation with labeled precursor, up to 2.8% of the initial radioactivity added to the medium was found in the *T. cruzi* long chain fatty acids. Saturated (16:0 and 18:0), monounsaturated (18:1ω9), and diunsaturated (18:2ω6) fatty acids were synthesized. Both the pattern of incorporation of labeled acetate into the fatty acids and the decarboxylation ratios found suggest that de novo synthesis of fatty acids has taken place.

Although total lipids represent about 20% of the dry weight of *Trypanosoma cruzi* (1,2), very little is known of the lipid biosynthetic capabilities in this protozoon (3). Since many microorganisms are able to incorporate lipids and fatty acids from the culture medium, their lipid and fatty acid compositions are not a reliable index of the mechanisms of lipid synthesis present, unless they are cultured in a lipid-free medium (4). *T. cruzi* is usually grown in crude, lipid-containing media, and only recently has the successful growth of *T. cruzi* in chemically defined media been reported (5-8). However, most of these media contain some lipid components, since exogenous fatty acids could be essential for growth (9), and the ability of trypanosomes to synthesize fatty acids had not yet been demonstrated (10).

T. lewisi (11,12) and *T. rhodesiense* (11) have been shown to carry out chain elongation and desaturation of long chain fatty acids, but de novo biosynthesis of these molecules was in doubt (12) or could not be proven (11).

This study describes the incorporation of [1-^{14}C] acetate into long chain fatty acids by cultured epimastigotes of *T. cruzi* in an attempt to elucidate the fatty acid biosynthetic mechanisms occuring in this organism.

EXPERIMENTAL PROCEDURES

Organisms

Epimastigotes of *T. cruzi* (Tulahuen strain) were cultured as previously described (3).

Incorporation Studies for Lipid Synthesis

[1-^{14}C] Sodium acetate (1.97 mCi/mmol, Comisión Nacional de Energía Atómica, Argentina) was sterilized by Millipore filtration before being added to the media (1 μCi/mL). Additions were made in duplicates after 72 hr of incubation during the logarithmic phase of growth. Inoculum densities were about 8.6 $\times$ 10^6 organisms/mL. All incubations were done at 28 C under a continuous stream of sterile air. Cultures were harvested after 8, 24 and 48 hr of incubation with the radioactive precursor. The parasites were collected by centrifugation at 1,000 G for 15 min, and then washed three times with a solution of 0.15 M NaCl.

Extraction of Cellular Lipids

Extraction of the total lipids and washing of the lipid extracts were done by the method of Folch et al. (13). Portions of the purified lipids were saponified with 90% methanolic sodium hydroxide (0.3 N) under reflux for 2 hr, and the nonsaponifiable lipids and the free fatty acids extracted as described by Kates (14). Duplicate aliquots of the purified total lipids and free fatty acids were taken for radioactivity determinations in a Beckman LS-100C liquid scintillation spectrometer, as in ref. 15. A toluene-based scintillation mixture was used. Correction for quenching was made by the external standard method. Efficiency for ^{14}C was ca. 85%.

All solvents were ACS reagent grade and distilled before use. A nitrogen atmosphere was

maintained throughout the whole procedure to prevent oxidation of unsaturated fatty acids.

Decarboxylation of Fatty Acids

Fatty acids were decarboxylated according to the procedure for the Schmidt reaction described by Brady et al. (16) and modified by Goldfine and Bloch (17). This method yielded 87% of the radioactivity as $^{14}CO_2$ from [1-^{14}C] palmitic acid, and 10.3% from a sample of uniformly ^{14}C-labeled palmitic acid (Amersham Corp., England).

Gas Liquid and Thin Layer Chromatography of Methyl Esters of Fatty Acids

Fatty acid methyl esters were prepared from the lipid extracts with 14% boron-trifluoride in methanol (18). Separation and radioactivity determinations of the methyl esters of fatty acids on the basis of their degree of unsaturation was carried out by $AgNO_3$-TLC as described by Cook (19). GLC of the methyl esters was carried out in a Varian 2100 gas chromatograph equipped with a flame ionization detector. A column of 16% diethylene glycol succinate on 100-120 Gas-Chrom P (Applied Science Laboratories, State College, PA) was operated at 180-190 C. Peaks were identified by chromatography with mixtures of standard fatty acid methyl esters. When standards were unavailable, peaks were given a tentative identity by a semilogarithmic plot of their relative retention times. Radioactive methyl esters were collected from the gas chromatograph as previously described (20) and counted.

Positions of the double bonds in the oleic and linoleic acids synthesized by *T. cruzi* and isolated by argentation chromatography were determined by ozonolysis (21).

RESULTS AND DISCUSSION

[1-^{14}C] Acetate was actively incorporated into the fatty acids of *T. cruzi* (Table 1). Incorporation into total lipids and fatty acids increased with longer incubation times. After 48 hr, up to 3.7% (for total lipids) and 2.8% (for fatty acids) of the total radioactivity initially added to the medium was recovered in the lipid fractions analyzed. The synthesized fatty acids were almost completely incorporated into complex lipids, mostly phospholipids, as has been reported elsewhere (3).

The ratios of radioactivity in the carboxyl carbon to that in the total fatty acid indicate the mechanism of synthesis. The ratios of 1:8.9 for the 24-hr sample and of 1:10.3 for the 48-hr sample (Table 1) are compatible with de novo biosynthesis of fatty acids. The values obtained are far from the 1:1 ratio that should be expected for chain elongation of endogenous fatty acids.

Although Korn et al. (12) for *T. lewisi* and Dixon et al. (11) for *T. lewisi* and *T. rhodesiense* also demonstrated incorporation of [^{14}C] acetate into long chain fatty acids, they were unable to prove the existence of de novo biosynthesis of these fatty acids.

The pattern of fatty acids synthesized by *T. cruzi*, as shown in Table 2, also suggests that a

TABLE 1

Incorporation of [1-^{14}C] Acetate into the Lipids of *T. cruzi*[a]

Incubation time (hr)	Total lipids A	Total lipids B	Fatty acids A	Fatty acids B	Decarboxylation ratio[b]
8	31	0.6	24	0.5	–
24	96	1.9	68	1.4	1:8.9
48	186	3.7	141	2.8	1:10.3

[a] 10 μCi of [1-^{14}C] sodium acetate (1.97 mCi/mmol) were added to 10 mL of the incubation medium. Results are expressed as (A), nmol of labeled acetate, and (B), as percentage of total radioactivity initially added to the medium, incorporated into the lipids of *T. cruzi*, and represent the mean of two experiments.

[b] Ratio of radioactivity in the carboxyl carbon to total radioactivity in the mixture of synthesized fatty acids.

TABLE 2

Distribution of Radioactivity in Methyl Esters of Fatty Acids of *T. cruzi* after Incorporation of [1-^{14}C] Acetate[a]

	Fatty acids	Incubation time 24 hr	Incubation time 48 hr
$AgNO_3$-TLC	Saturated	41.3	34.4
	Monounsaturated	32.4	19.1
	Diunsaturated	23.4	43.9
	Polyunsaturated	2.8	2.4
GLC[b]	16:0	–	15.7
	16:1	–	2.0
	18:0	–	14.1
	18:1ω9	–	16.1
	18:2ω6	–	50.4
	18:3	–	1.3
	Others	–	0.0

[a] Results are expressed as percentage of total radioactivity in methyl esters of fatty acids and represent the mean of two experiments.

[b] A standard mixture of equal parts (dpm) of [1-^{14}C] 16:0 and [1-^{14}C] 18:2ω6 yielded 48.4% of the radioactivity as palmitic acid and 47.5% as linoleic acid.

de novo mechanism occurs in these organisms. Elongation of medium chain fatty acids is unlikely because these acids are present in very small amounts in *T. cruzi* (22), and because of the decarboxylation studies already discussed. However, elongation of de novo synthesized palmitic acid to stearic acid by labeled acetate cannot be discarded (the theoretical decarboxylation ratio would be 1:9). Further experiments with labeled stearic acid should serve to clarify this matter.

Biosynthesis of oleic and linoleic acids was also found (Table 2) and suggests the presence of Δ9- and Δ12-desaturases in *T. cruzi*, rather than elongation of short or medium chain unsaturated fatty acids. These results are in agreement with those of Korn et al. (12) and Dixon et al. (11) for *T. lewisi* and *T. rhodesiense*, and of Meyer and Holz (23) in Kinetoplastid flagellates, regarding desaturation of long chain fatty acids. In contrast to their results, however, we found only very little synthesis of 18:3 or other polyunsaturated fatty acids. While oleic and linoleic acids are major components of the lipids in *T. cruzi* culture forms grown under identical conditions as those in our biosynthetic studies, 18:3 and other polyunsaturated fatty acids have not been detected (22).

Our studies clearly demonstrate that epimastigotes of *T. cruzi* can synthesize fatty acids by a de novo mechanism. They support the previous finding by Boné and Parent (9) that CO_2 had a stimulating action for growth in *T. cruzi* cultures in a medium lacking stearic acid but had no influence when this acid was supplied to the medium. Since this acid was found to be an essential growth factor for *T. cruzi* by these authors, their results in the CO_2 experiments suggested the existence of de novo biosynthesis of the stearic acid.

The uptake and metabolism of labeled fatty acids by culture forms of *T. cruzi*, and the in vitro incorporation of acetyl and malonyl-CoA into fatty acids by subcellular fractions of *T. cruzi*, now under investigation in our laboratory, would further substantiate our present findings.

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The Lipids of Kangaroo Meat

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ABSTRACT

Lipids were extracted from lean meat of kangaroos, sheep and beef cattle. Kangaroo meat lipids contained more phospholipid and less triacylglycerol than sheep meat or beef, and had a higher proportion of polyunsaturated linoleic and arachidonic acids. These results were obtained with kangaroos from the more arid inland region of Australia, and were similar to results obtained previously with kangaroos from a milder climatic region with a higher rainfall. Hence, the composition of the meat lipids is unaffected by the increased aridity of inland zones. The findings are of nutritional significance because kangaroo meat is now commercially available for human consumption.

Kangaroo meat is commercially available for human consumption in some parts of Australia. To provide meat for local and export markets, the controlled annual harvesting of at least 100,000 kangaroos is permitted under license from the Australian National Parks and Wild Life authorities.

In a previous study (1), the lipids of kangaroo meats were shown to be rich in polyunsaturated fatty acids. For that study, specimens of the Eastern gray kangaroo (*Macropus giganteus*) were taken from a relatively high rainfall region (26 in. annual average) of Eastern Australia. We now report the composition of meat lipids from the same species taken from a drier region of the inland plains with an average annual rainfall of only 16 in. For comparison, the simultaneous analysis of sheep meat and beef is also reported.

MATERIALS AND METHODS

Kangaroos were shot on a farm near Deniliquin, New South Wales. Meat was immediately taken from the hindquarter and chilled in ice before being frozen and transported to Melbourne for analysis. Samples of beef (rump steak) and sheep (loin chops) were obtained from butcher shops in Melbourne. For analysis, about 2.5 g of lean meat, without visible fat, was finely chopped with scissors, and then extracted overnight in 20 vol of chloroform/methanol (2:1, v/v). The lipid extract was purified (2), evaporated to dryness under a stream of nitrogen and made up to 10 mL vol with light petroleum, bP 60-80 C. Portions were then taken for saponification and cholesterol assay (3), for evaporation of solvent at 80 C for several hours then gravimetric assay of total lipid, or for gas chromatographic analysis after conversion to methyl esters with a mixture containing benzene/14% boron trifluoride in methanol/methanol (35:35:30, v/v/v). Conditions for gas chromatography were as previously described (1) and satisfactory separations of 18:3 from 20:0 and 20:1 were achieved. The distribution of the lipid classes was determined after their separation on sintered-glass rods (4) in the solvent system light petroleum (bP 40-60 C) diethylether/acetic acid (60:1.2:0.7, v/v/v) in the Iatroscan TH-10 (Iatron Laboratories, Tokyo, Japan), using calibration factors calculated from standard mixtures 18-5A, B and C, obtained from Nu-Chek-Prep, Inc., Elysian, MN.

RESULTS AND DISCUSSION

Table 1 shows that kangaroo meat contained less lipid overall than sheep meat and beef, and

TABLE 1

Lipid Composition of Meat Lipids

Meat	Total lipid (mg/g wet wt)	Distribution of lipid classes (%)			
		Phospholipid	Triacylglycerol	Cholesterol	Cholesteryl esters
Kangaroo	13.4 ± 0.8	60 ± 6.5	30 ± 7.1	4.5 ± 0.4	3.1 ± 0.8
Sheep	20.9, 20.9	17 ± 1.0	76 ± 2.0	4.0 ± 0.5	2.3 ± 0.8
Beef	19.8, 20.6	25 ± 3.5	66 ± 5.4	3.3 ± 0.3	4.0 ± 1.3

Results are mean ± SD for 5 samples, or each of duplicate assays on 2 samples of sheep meat and beef.

TABLE 2

Fatty Acid Composition of Meat Lipids

Meat	16:0	16:1	18:0	18:1	18:2	18:3	20:4	PS ratio
	(g/100 g fatty acids)							
Kangaroos								
This study	18 ± 0.4	3 ± 0.5	14 ± 0.8	28 ± 2.4	22 ± 2.2	5 ± 0.6	9 ± 1.5	1.1 ± 0.12
Previous study	15 ± 1.0	<1	16 ± 0.5	29 ± 1.8	21 ± 1.1	7 ± 0.4	7 ± 1.3	1.1 ± 0.12
Sheep	23 ± 0.9	4 ± 0.1	17 ± 0.4	45 ± 0.8	7 ± 0.4	4 ± 0.4	<1	0.3 ± 0.02
Beef	24 ± 0.7	4 ± 0.1	16 ± 0.3	40 ± 0.5	9 ± 0.3	3 ± 0.2	2 ± 1.1	0.3 ± 0.04

Results are mean ± SEM for 10 analyses of kangaroo, and 4 each of sheep meat and beef. P/S ratio is the sum of all polyunsaturated fats divided by the sum of all saturated fats.

only 30% of the lipid of kangaroo meat was in the form of triacylglycerol compared with 60% in the other species examined. The majority of kangaroo meat lipid was present as phospholipids.

Analysis of fatty acids is given in Table 2. For kangaroo meat, the distribution was similar to previously published results (1), and quite different from the distribution of the sheep meat and beef fatty acids. In kangaroo meat lipids, considerably greater amounts of the fatty acids, linoleate and arachidonate, account for a much higher ratio of polyunsaturated-to-saturated fatty acids compared with that of sheep meat and beef lipids.

These results confirm and extend analysis reported previously for kangaroo meat. The differences from the lipids of sheep meat and beef are now clearly documented. Even when collected from a harsher environment with lower annual rainfall, the kangaroo (*M. giganteus*) maintains a high content of polyunsaturated fatty acids in its meat lipids. In ruminants such as sheep and cattle, such unsaturated dietary fatty acids are hydrogenated in the rumen (5,6), and therefore are present in much reduced proportions in meat lipids. Our analytical technique was not sufficiently sensitive to detect small amounts of other long chain unsaturated fatty acids, but using a support-coated open tubular column, Sinclair and Slattery (7) have reported the presence of 22:4, 20:5, 22:5 and 22:6 in kangaroo meat lipids, also in greater proportions than in sheep meat and beef.

Kangaroo meat for human consumption in Australia will be obtained from the Eastern gray kangaroo (*M. giganteus* described here and previously), and also the red kangaroo (*Megaleia rufa*), which inhabits the inland plains within the 20-in. isohyet (8). Distributions of these two species sometimes overlap within the continent, but even so, they select different plants on which to feed (8). Hence, the results of our analyses on the Eastern gray kangaroo should not be extrapolated to all kangaroo meat until meat from *Megaleia rufa* is studied. However, it is clear that at least as far as lipids are concerned, the qualities of kangaroo meat make it attractive for human consumption.

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Alkane Contamination of Lipids Extracted from *Lagenidium giganteum* and *Lagenidium callinectes*

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ABSTRACT

Alkanes associated with the mycelia of the fungi *Lagenidium giganteum* and *L. callinectes* were analyzed. Careful studies showed that these substances were contaminants derived from glass ware, media and solvents. No alkane biosynthesis or transformation was detected, indicating little or no alkane metabolism. The importance of using clean techniques, high-purity solvents and proper media controls was emphasized.

Lagenidium giganteum Couch, a mosquito-parasitizing Oomycete, must be supplied with exogenous sterols to produce the infective zoospores (1). The related species, *L. callinectes* Couch, a halophilic parasite of crustacea ova, synthesizes sterols and readily produces zoospores. During the course of our sterol analyses, many unknown peaks were present in our gas chromatograms which were identified as alkanes by gas chromatography-mass spectrometry (GC-MS). These alkanes were either contaminants or metabolites and were of interest because of suggestions made by Weete (2) that spore-coat alkanes were in some way related to the virulence of a fungus in parasitizing a host.

The subject of fungal alkanes has been reviewed by Weete (2). Recently, Fisher et al. (3) reported on the alkanes of spore coats in 4 different fungi. To our knowledge, no work has been published relative to Oomycetes. This communication presents our findings on the alkanes of 2 members of that class and our observations on the methods used for their analysis.

MATERIALS AND METHODS

Growth of Fungi

Initial experiments employed undefined media: *L. giganteum* was grown on peptone, yeast extract and glucose broth (PYG) according to established procedures (4). *L. callinectes*, an obligate halophile, was grown the same way, but in 2% artificial sea water (Rila-TM, Carolina Biological Company) + PYG. Both organisms were grown in Erlenmeyer flasks that had been washed by a dish-washing machine and both media were made using distilled, deionized water (house line) that had been stored in polyethylene carboy water dispensers.

Results from undefined media dictated the use of defined media: both fungi were grown as just described but in a modified Gleason's (5) broth (GLE), except that L-glutamate was replaced with filter-sterilized L-glutamine and *L. callinectes* received 2% Rila salt supplement (R-GLE). The inoculum used for analyses were tufts of vegetative mycelia that had been cycled through 3 successive growths from these defined media in order to reduce carryover of any chemical contaminants. Special precautions were taken to ensure that no outside contamination was introduced into the media. Glassware was first acid-washed (6 N HCl), rinsed 5 times with distilled, deionized water, once with methanol (Fisher grade), then chloroform (Mallinckrodt), and held at 110 C until use. Chloroform-rinsed aluminum foil caps were substituted for the styrene foam plugs and the distilled, deionized water was drawn fresh, rather than from the stored carboys.

Isolation and Fractionation of Lipids

Undefined medium. The fungi (ca. 500 mg) were collected by filtration on Whatman filter paper and were immediately ground in equal weights of sand (Baker & Adamson, Allied Chem.) with chloroform/methanol (2:1, v/v, Fisher). The extract was filtered through glass wool and concentrated to a small volume with a rotary evaporator at 30 C under an oil pump vacuum. The residue was dissolved in a small vol of chloroform which was applied to a 10-g Sephadex G-25 (Pharmacia) column and eluted with chloroform/methanol (19:1, v/v, Fisher) saturated with water according to the method of Rouser et al. (6). The first fraction containing neutral lipids, glycolipids and phosphatides was collected, concentrated by rotary evaporation and the residue dissolved in 5 mL chloroform. This was placed on a 15-g silicic acid

column (Bio-Rad 100-200 mesh) and eluted with chloroform (6). The first fraction (ca. 175 mL) which contained neutral lipids was collected, concentrated by rotary evaporation and dissolved in 5 mL hexane (Baker). This was placed on a 12-g, 7% hydrated Florisil (Floridan Co.) column according to Carroll and Serdarewich (7) and eluted with hexane to collect the hydrocarbons. Butylated-hydroxytoluene (BHT) (Sigma) was added as an antioxidant to each fraction which was concentrated to ca. 2 mL, first by rotary evaporation under reduced pressure and finally to ca. 0.2 mL vol under a stream of nitrogen (Seaford). The fractions were stored at -15 C in glass vials with Teflon-faced septa under nitrogen until analyzed.

Defined medium. The extraction and fractionation procedure was modified as follows: all glassware, Teflon stirring bars, spatulas, glass wool and sand were washed as per the procedure described under growth in defined media above; high pressure liquid chromatography grade (Burdick and Jackson) methanol, hexane and chloroform (no ethanol preservative) were used throughout; the vacuum rotary evaporator step used a water aspirator and no BHT was used.

The fungus was collected on glass wool, washed with distilled,deionized H_2O, placed in a beaker with a Teflon-coated stir-bar and 100 mL chloroform/methanol (2:1) and stirred for 30 min. The extraction was repeated with another 100 mL of chloroform/methanol. The combined extracts were washed with portions of H_2O to remove nonlipid polar compounds (Bligh and Dyer [8], modified by Kates [9]). NaCl was added where necessary to break emulsions. The Sephadex column fractionation step was omitted, otherwise fractionation was as described for undefined medium.

Substrate or media controls (no fungal growth) were treated identically, except that water back-extraction was unnecessary as the aqueous media served this purpose. Both experimental and control samples were subjected to the same fractionation procedures.

Further Supplements to Defined Media

Both fungi were studied for possible alkane metabolism by growth with 6 μCi [1-^{14}C] hexadecane in 3.85 mg carrier or with 4 mg heneicosane or octacosane/flask. As Kolattukuddy (10) had reported that palmitic acid was a precursor to alkane formation, we tested fungi with 5 μCi [1-^{14}C] palmitic acid (1 mg carrier) or 2.5 μCi [U-^{14}C] glucose/flask. No particular care was used in glassware preparation for labeled compound studies.

Treatment of supplemented medium. Any excess medium was decanted and the fungus was centrifuged at 5,000 G for 15 min, rinsed several time with distilled H_2O and then extracted as for defined media.

Organisms given radioisotopes were collected, washed thoroughly with H_2O and then treated with ca. 50-mL portions of chloroform/methanol (2:1, v/v, Fisher). The mycelia were stirred for 2 hr, filtered through filter paper, and the extracts back-extracted with 25-mL portions of H_2O. Any further excess water was removed by use of Whatman phase separator filters. The extracts were concentrated to ca. 0.5 mL under nitrogen (Seaford).

Analyses

Gas chromatography. All fungal extracts and controls, except those with radioisotopes, were analyzed with a Packard 417 gas chromatograph using 1.5% OV-17, 1.95% OV-210 on 80/100 Gas Chrom Q. A temperature program was used which was held at 100 C for 4 min, then increased at 8 C min^{-1} to a final 270 C for 15 min. Injector temperature was 250 C, and flame ionization detector temperature was 280 C; N_2 was the carrier gas at 20 mL min^{-1}.

Extracts and controls were also analyzed by MS using a Hewlett-Packard 2700 A GC and 5930 mass spectrometer with GC operation parameters as before.

Autoradiography. Radioactive extracts were subjected to thin layer chromatography on Whatman LK6D-linear K plates (250 μm silica gel on glass with channels). Extracts before and after transesterification (11) were applied to the plates along with the nonradioactive standards: 1-docosene, 5-cholesten-3β-ol palmitate, methyl palmitate, tripalmitin, dipalmitin, monostearin and cholesterol. The plate was developed with petroleum ether/ethyl ether/acetic acid (80:20:1, v/v/v), adapted from Mangold and Malins (12). The plate was visualized by iodine (13) and labeled compounds were identified by standard autoradiographic methods (14).

RESULTS AND DISCUSSION

Preliminary analyses of fungi grown on undefined medium revealed numerous n-alkanes and plasticizers in the neutral lipid extracts. Dioctyl phthalates and adipates were derived from non-HPLC-grade solvents. The undefined media employed also possessed similar contaminants so that different growth and extraction methods were required. BHT was omitted and the stringent cleaning methods employed for pesticide analyses in conjunction with defined

media were used to substantially decrease the number of contaminants.

Alkanes found in *L. giganteum* grown on defined medium corresponded to those present in the medium control. Similar results were obtained with *L. callinectes*. Added heneicosane and octacosane were recovered from extracts of both organisms; however, no new alkanes were detected nor were the levels of those already present altered, indicating that these substances were not metabolized. No metabolism of ^{14}C hexadecane was observed. Neither ^{14}C glucose nor ^{14}C palmitate were converted to alkanes. Thus, alkane biosynthesis or metabolism, at least from these substances, appeared to be absent.

Several investigators have reported that hydrocarbon gas chromatograms change when fungi are grown on different media (3,15,16). Our study indicates that *Lagenidium* spp. may absorb alkanes from media and suggests that reports of alkanes in other organisms may really represent alkanes present as contaminants in the growth broth, possibly further metabolized by these organisms. The application of stringent cleaning procedures, ultra-pure solvents, defined culture media, and careful manipulation reduced some 40 GC peaks (consisting of C_{19}, C_{20}, C_{21} . . . C_{36} n-alkanes) that we detected initially to 8-10 small peaks (C_{26} . . . C_{35} n-alkanes). Other investigators have used cochromatography and GC-MS in lipid analysis (16) without mentioning control procedures employed. Oró et al. (17) studied alkanes of fungal spores, but no mention was made of solvent purity or cleaning precautions used with glassware. Jones (15) studied lipids of soil microorganisms by GC, but did not identify any of the large number of compounds isolated –he suspected that many peaks found were impurities.

This investigation shows the importance of using HPLC-grade solvents, solvent-cleaned glassware and media test controls because alkanes and plasticizers are so easily introduced. The organisms studied by us apparently are unable to synthesize alkanes from glucose or palmitate, nor do they metabolize some n-alkanes. Thus, the alkanes observed in *Lagenidium* spp. are essentially contaminants.

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Phospholipid Studies of Marine Organisms: 2.[1] Phospholipids, Phospholipid-Bound Fatty Acids and Free Sterols of the Sponge *Aplysina fistularis* (Pallas) forma *fulva* (Pallas) (= *Verongia thiona*)[2]. Isolation and Structure Elucidation of Unprecedented Branched Fatty Acids

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ABSTRACT

The free sterols and phospholipids of the demosponge *Aplysina fistularis* were isolated and analyzed. The free sterols consisted mainly of the unusual 26-methylated sterols aplysterol (53%) and 24(28)-dehydroaplysterol (7%) together with 7 commonly occurring sterols. The major phospholipids were phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine and diphosphatidylglycerol. The major fatty acyl components of the phospholipids consisted of 85% C_{14}-C_{20} acids, including the unprecedented 2,6,10-trimethyl-5-tetradecenoic acid and 11-methyloctadecanoic acid. The remaining 15% were C_{27}-C_{30} demospongic acids, including 2 novel acids tentatively assigned the structures 5,9,23-octacosatrienoic acid and 5,9,23-nonacosatrienoic acid, and 3 novel acids proven to be 5,9,21-octacosatrienoic acid, Z,Z-20-methyl-5,9-hexacosadienoic acid and Z,Z-22-methyl-5,9-octacosadienoic acid. The biosyntheses of the novel demospongic acids are proposed to occur by chain elongation of monoenoic or branched precursors followed by desaturation. The large quantities of typically bacterial phospholipids and fatty acids found implied the presence of bacteria in the sponge, in agreement with microscopic studies. Analysis of the phospholipid-bound fatty acids in a sponge cell-enriched fraction indicated that the demospongic acids, including the 2 branched structures, were the major acids of the sponge cells. The presence in *A. fistularis* of demospongic acids containing membrane disordering groups—methyl branches or double bonds—on the $\omega 7$ carbon is proposed to be due to the need by the sponge for membranes possessing fluidity near the middle of the phospholipid bilayer. It is also proposed that the C_{26} methyl group of aplysterol causes disordering of the phospholipid bilayer in the same region, and thus also evolved in response to this need.

INTRODUCTION

Research in this and other laboratories over the past decade has revealed that a large number of diverse side-chain-alkylated sterol molecules exist in marine invertebrates and algae (3-6). We have proposed (7) that the most plausible biological role for many of these unusual sterols is one of a membrane component, and based this idea on the high concentrations of some of these sterols in their source organisms, and on their structural similarities to the known membrane component, cholesterol. Model membrane studies (8,9) using egg lecithin vesicles have shown that cholesterol contains the optimum structural features for imparting a high viscosity to these phospholipid bilayers (a typical measure of sterol effectiveness).

We are currently considering (1) the hypothesis that marine organisms have evolved alkylated sterols to allow an optimal interaction between these molecules and the fatty acyl chains of the phospholipids present in the membranes of these organisms, in analogy with

Abbreviations: PC: phosphatidylcholine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PS: phosphatidylserine; DPG: diphosphatidylglycerol; BHT: butylated hydroxytoluene.

[1] For part 1, see ref. 1.

[2] Phylum porifera, class demospongia, subclass ceractinomorpha, order verongida, family aplysinidae (2). Formerly known as *Verongia thiona* de Laubenfels.

[3] Zoecon Corp., 975 California Ave., Palo Alto, CA 94304, where all of the GC-MS analyses of the fatty acid mixtures were made.

[4] Major diagnostic fragments from peak 23: M^+ 471 (2.9%); m/z 442 (C_{26}, 0.2%); m/z 428 (C_{25}, 0.2%); m/z 414 (C_{24}, 0.1%); m/z 400 (C_{23}, 0.3%); (absence of C_{22} peak); m/z 374 (C_{21}, 0.2%); m/z 360 (C_{20}, 0.2%); m/z 234 (C_{11}, 1.0%); m/z 220 (C_{10}, 0.7%); m/z 206 (C_9, 0.5%); m/z 194 (C_8, 0.4%); m/z 180 (C_7, 24.9%); m/z 166 (C_6, 1.2%); m/z 152 (C_5, 0.8%); m/z 140 (C_4, 1.3%); m/z 126 (C_3, 18.7%).

Major diagnostic fragments from peak 25: M^+ 485 (0.9%); m/z 456 (C_{27}, 0.1%); (absence of C_{26}, C_{25} peaks); m/z 414 (C_{24}, 0.3%); m/z 400 (C_{23}, 0.1%); m/z 402 (C_{23}, 0.1%); (absence of C_{22} peak); m/z 374 (C_{21}, 0.1%); m/z 360 (C_{20}, 0.1%); m/z 234 (C_{11}, 0.5%); m/z 220 (C_{10}, 0.3%); m/z 206 (C_9, 0.1%); m/z 194 (C_8, 0.2%); m/z 180 (C_7, 18.0%); m/z 166 (C_6); m/z 152 (C_5), m/z 140 (C_4); m/z 126 (C_3, 15.0%).

the "perfect fit" between cholesterol and egg lecithin. This hypothesis predicts that the phospholipids of such a marine organism will contain unique structural features which reflect their complementarity to an alkylated sterol. To test this prediction, we have analyzed both the sterols and the phospholipids of the marine demosponge *Aplysina fistularis* (Pallas) forma *fulva* (Pallas) which has been reported (10) to contain as a major sterol the side-chain-alkylated aplysterol (Fig. 1). This analysis has led us to propose the structures of 2 novel long chain fatty acids, and has allowed us to firmly identify 3 other novel long chain acids, including 2 possessing an unprecedented monomethyl branched chain. Spectroscopic measurements, chemical degradations and synthetic studies were used to elucidate the structures of these branched fatty acids, and their presence in the sponge as sponge cell, and not bacterial products, was experimentally proven. These results, and their relevance to the hypothesis just stated, will be discussed in this paper.

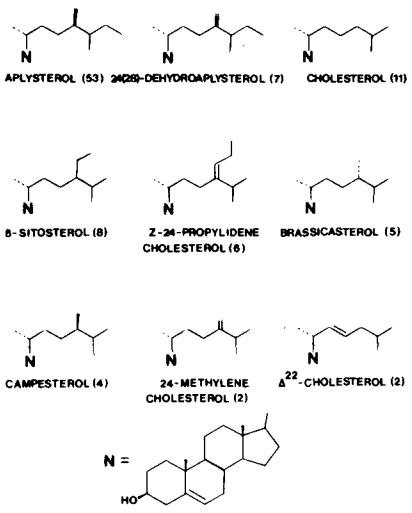

FIG. 1. The major free sterols found in *Aplysina fistularis*. Weight fractions (as percentage of total free sterols) are given in parentheses.

EXPERIMENTAL PROCEDURES

Extraction and Isolation of Phospholipids

Aplysina fistularis sponge colonies were collected by hand at low tide from a depth of 1-2 m at Seal Point, La Jolla, CA, in July 1979, and were cleaned and frozen immediately. The total lipids were extracted by blending the sponge tissue in a Sorvall Omnimixer at 0 C for 1 min with chloroform/methanol (1:1, v/v) followed by filtration. Water was removed azeotropically, and the total lipids were purified (11) by filtration through Sephadex G-25 (100-300 μ). The phospholipids were separated from the neutral lipids and glycolipids by chromatography on ammonium hydroxide-treated silicic acid (100-200 mesh) using the procedure of Privett et al. (12), which was found to be the best for removing pigmented polar impurities from the phospholipids. The phospholipids thus obtained were kept under argon at -10 C in chloroform/methanol (1:1) containing 0.002% BHT.

Analysis of Free Sterols

The free sterols were isolated from the neutral lipids by preparative thin layer chromatography (TLC) on 250 μ layers of Silica Gel G, using n-hexane/diethyl ether (8:2, v/v) as a developing solvent. The sterols were then identified by gas chromatographic comparison with known sterol mixtures, using a Hewlett-Packard Model 402 gas chromatograph equipped with a 6 m x 2 mm silanized glass column packed with 3% OV-17 on 120/140 mesh GasChrom Q, and a flame ionization detector. The sterols were chromatographed at 260 C with helium as the carrier gas at 40 mL/min. The peaks were integrated by the cut-and-weigh method. The identities of the major sterols were verified by gas chromatography-mass spectrometry (GC-MS) using a Varian MAT-44 mass spectrometer interfaced with a Varian Aerograph GC containing a 3% OV-17 column. The relative amounts (Fig. 1) of aplysterol and 24(28)-dehydroaplysterol were discerned by argentic chromatography (13).

Analysis of Phospholipids

The phospholipids were separated into classes by TLC on 250 μ layers of Silica Gel H (predeveloped in diethyl ether and dried overnight at 120 C) by double development in chloroform/methanol/28% ammonium hydroxide (65:35:8, v/v/v). The identities of all of the major phospholipid classes were established by codevelopment with known phospholipids and by the use of Dragendorff, ninhydrin, and periodate-Schiff spray reagents to identify the presence of choline, free amino groups and vicinal glycol groups, respectively (14,15). The presence of plasmalogens was discerned through the use of a 2,4-dinitrophenylhydrazine (DNP) spray reagent and by 2-dimensional TLC involving treatment of the lipids with mercuric chloride between developments (14). A quantitative analysis of the phospholipids was made by visualizing the spots on a developed plate with a rhodamine 6G spray reagent (15), collection and solvent elution of each spot (16), and spectrophotometric phosphorus assay of

each eluted sample (17). Corrections were made for residual phosphate present in the silica gel and in the eluting solvents (16).

Analysis of Fatty Acids

The fatty acyl components of the phospholipids were obtained as their methyl esters by sequential treatment of the phospholipids with methanolic sodium methoxide and methanolic hydrogen chloride (18), followed by purification by column chromatography. The resulting methyl esters were analyzed by gas chromatography (GC) using a Carlo Erba Series 4160 Fractovap chromatograph equipped with a Model 400 LT programmer, a 15 m x 0.32 mm fused silica column coated with SE-54 (J&W Scientific, Inc.), a cooled on-column injection system, and a flame ionization detector. An optimal resolution of the esters was achieved using a linear temperature program from 70 C to 280 C at 3.0 C/min, although routine analyses were performed, with comparable resolution, using a program of 130 C to 280 C at 2.5 C/min. Peaks were recorded using a Linear Instruments strip chart recorder, and were integrated electronically using a Waters Associates Data Module. Methyl heptadecanoate and methyl heptacosanoate were used as internal standards for quantitative analyses.

An aliquot of the fatty acid methyl esters was hydrogenated for GC analysis by stirring overnight in methanol with platinum (IV) oxide under a hydrogen-filled balloon.

Equivalent chain length (ECL) values were obtained for each of the major fatty acid methyl esters from a plot of the retention temperatures of known saturated methyl ester standards vs their chain lengths (19). The identities of the fatty acids were determined by comparing their ECL values with those of known compounds and by obtaining the mass spectra of their methyl esters and of their pyrrolidide amides (20) by GC-MS using a Hewlett-Packard 5985A GC/MS/DS system equipped with an SE-52-coated glass capillary column and a platinum-iridium transfer line with an open split connection. Helium was used as the carrier gas for all GC-MS analyses.

The N-acyl pyrrolidides were formed from the methyl esters upon treatment with pyrrolidine/acetic acid (10:1, v/v) at 100 C for 1 hr in a capped vial, followed by partitioning between diethyl ether and cold 5% HCL. The ether solution was then washed with cold water, dried, and the crude pyrrolidides obtained by removal of the solvent were purified by preparative TLC (Silica Gel G, single development in hexane/diethyl ether [1:4, v/v]). Gas chromatographic analysis of the pyrrolidides required a temperature program of 190-285 C at 2.0 C/min to allow a resolution comparable to that obtained with the methyl esters.

The 2 novel branched dienoic methyl esters were isolated for spectroscopic and degradative studies by first partitioning the total esters according to their degrees of unsaturation by TLC (double development in hexane/diethyl ether [9:1, v/v]) on 750 μ layers of Silica Gel G impregnated with 15% (by wt) silver nitrate. The developed bands were recovered by the method of Hill et al. (21), and the novel dienes were isolated from the second most polar TLC fraction by high performance liquid chromatography (HPLC) on a 50 cm x 9 mm Whatman ODS-2 reversed phase column, using methanol as the eluting solvent at a rate of 1.5 mL/min. HPLC components included a Waters M-6000A pump, a Valco loop injector and a Waters R401 refractometer for detection.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra of the 2 novel dienes were obtained on a 360 MHz instrument at the Stanford Nuclear Magnetic Resonance Laboratory. Infrared spectra were obtained from films held between salt plates using a Beckman Acculab II spectrophotometer, and rotations were measured using an Autopol III automatic polarimeter (Rudolph Research).

Degradation studies to discern methyl branching in the 2 dienes were carried out by ozonizing 0.2-1.0 mg aliquots of each of the isolated methyl esters in dichloromethane at -78 C for 2-5 min, followed by degradation of the ozonide with triphenylphosphine (22), oxidation of the resulting aldehydes with silver (II) oxide (23), methylation of the resulting carboxylic acids with diazomethane, and isolation of the monoester product by preparative TLC. The mass spectral and gas chromatographic properties of the pyrrolidides of these monoesters were then compared with those of known synthetic pyrrolidides.

Two monomethyl branched esters, methyl 11-methylheptadecanoate (**1**) and methyl 13-methylnonadecanoate (**2**), were synthesized as shown in Figure 2 for comparison with the degradation products obtained from the 2 dienoic esters. The reaction of 2-iodooctane (**3**) (prepared from 2-octanol [24]) with triphenylphosphine in refluxing benzene overnight produced the salt, 2-octyltriphenylphosphonium iodide (**4**), in 17% yield. This salt was readily deprotonated by ethereal phenyllithium in tetrahydrofuran at -78 C to form 2-octyltriphenylphosphoranylidene, **5**, which was reacted in situ at -78 C with methyl 10-oxodecanoate (**6**) in one case, and with methyl 12-oxododeca-

noate (7) in another, to form methyl 11-methyl-10-heptadecenoate (8) and methyl 13-methyl-12-nonadecenoate (9) in 27% and 47% yields, respectively. The C_{10} aldehyde ester 6 was synthesized by ozonization of methyl undecylenate (10) (25), and the C_{12} aldehyde ester 7 was formed by oxidation of methyl 12-hydroxydodecanoate (11) by the dipyridine adduct of chromic anhydride (26). Hydrogenation of the esters 8 and 9 produced quantitatively the target esters 1 and 2.

^{1}H-NMR spectra of each of the synthetic compounds were obtained using a Varian T-60 60 MHz spectrometer. Mass spectra of each of the final synthetic compounds were obtained using a Ribermag R10-10B mass spectrometer.

Analysis of the Fatty Acids of Phospholipid Classes

The fatty acid content of individual phospholipid classes was determined by separating the phospholipids by TLC, visualizing them with a rhodamine spray, then scraping each band into a test tube, transmethylating the silica-bound phospholipids by digestion with methanolic boron trifluoride (27), and analyzing the resulting methyl esters by gas chromatography.

Assessment of Bacterial Content of *A. fistularis*

Estimations of the relative cell numbers, surface densities, and cell volumes of bacterial and identifiable sponge cells in the tissues of *A. fistularis* were made by following established stereological methods (28) using a 30 cm x 32 cm composite transmission electron micrograph of a section of the sponge body wall located near an excurrent pore, taken at 4,050X magnification by Janice E. Thompson of Scripps Institution of Oceanography.

Assessment of Cellular Origin of the Long Chain Branched Acids

A comparison was made between the fatty acid contents of the phospholipids from a suspension of *A. fistularis* cells and the phospholipids of a fraction of that suspension enriched in spherulocytes, a type of sponge cell which occurs in *A. fistularis*. Suspensions of sponge cells were prepared from a fresh sponge sample collected on December 9, 1980, by pronase digestion of 5 g pieces in 50 mL of calcium- and magnesium-free seawater (CMF-SW) at room temperature, followed by centrifugation and repeated washing of the pellet with fresh CMF-SW. Isolation of the spherulocyte-enriched cell fraction was achieved by centrifugation at 2,000 rpm of an aliquot of this cell suspension on a stepwise (5%) gradient of

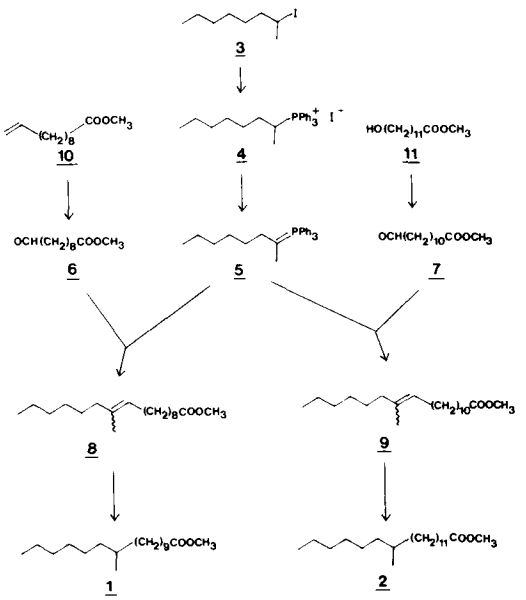

FIG. 2. The synthetic routes to methyl 11-methylheptadecanoate (1) and methyl 13-methylnonadecanoate (2).

0-30% Ficoll in CMF-SW, for 5 min at 4 C. The bright yellow bands of cells which formed at the 20%/25% and 25%/30% Ficoll interfaces were combined and examined by light microscopy for enrichment in spherulocytes. This enriched fraction was washed with CMF-SW, then it and an aliquot of the nonenriched cell suspension were each extracted, and their phospholipids were isolated and transmethylated. The resulting methyl esters were then analyzed by GC.

RESULTS

Analysis of Free Sterols

Analysis by GC, GC-MS and argentic chromatography revealed that 9 major sterols exist in *A. fistularis*. The structures of these sterols and their relative proportions are given in Figure 1.

Analysis of Phospholipids

Analysis of the purified phospholipids by TLC indicated that the 6 phospholipid classes listed in Table 1 comprise ca. 83% of the mixture. The remaining 17% was distributed among several minor unidentified spots on the chromatogram. The DNP spray reagent and 2-dimensional TLC indicated only minor amounts of plasmalogens in the PC and PE spots. It was difficult to achieve reproducibility

in the analyses using TLC and phosphate assay; therefore, the standard errors in the quantitation of the phospholipids (Table 1) were on the order of 10-15%.

Analysis of Fatty Acids

Capillary GC analysis (Fig. 3) of the fatty acid methyl esters from the phospholipids of *A. fistularis* indicated the presence of ca. 84 detectable peaks, of which 26 each comprised 0.4% or more of the total. A GC analysis of a hydrogenated aliquot of the methyl esters indicated, according to changes in the ECL values of the major esters, that unsaturated, unbranched acids containing 16, 17, 18, 19, 20, 28, 29 (minor), and 30 carbon atoms were present. This analysis also implied that unsaturation existed in an iso-branched C_{17} acid and that branching (or some other functionality) existed in 2 unsaturated long chain acids. The latter observation was based on the appearance of 2 peaks having ECL values of 26.50 and 28.48 in the hydrogenated mixture.

Analysis of the methyl esters by GC-MS provided molecular weight information, and allowed the assessment of branching in the saturated esters. However, GC-MS analysis of the N-acyl pyrrolidides provided this information as well as information which allowed the positions of double bonds to be assigned in most of the unsaturated acids (20). To locate double bond positions, the spacings between major peaks due to the carbonyl-bearing fragments were considered and, in addition, the relative intensities of these peaks were assessed. The latter consideration aided the identification of double bond positions due to the observed enhancement of peaks corresponding to fragments formed by the cleavages of allylically activated carbon-carbon bonds. Thus, the mass spectrum of the pyrrolidide of 5,9,21-octacosatrienoic acid (peak 22, Fig. 4) exhibited spacings of 12 amu between the C_{20} (m/z 360, 0.1%) and C_{21} (m/z 372, 0.2%), between the C_8 (m/z 194, 0.7%) and C_9 (m/z 206 0.5%), and between the C_4 (m/z 140, 1.1%) and C_5 (m/z 152, 0.7%) fragments, as expected (20), and also exhibited peaks of higher-than-average intensities corresponding to the C_3 (m/z 126, 19.4%), C_7 (m/z 180, 24.8%), C_{11} (m/z 234, 1.0%), C_{19} (m/z 346, 0.5%) and C_{23} (m/z 400,

TABLE 1

The Major Phospholipids of *Aplysina fistularis*

Phospholipid class	Mol %[a]
Phosphatidylcholine (PC)	32
Phosphatidylglycerol (PG)	15
Phosphatidylinositol (PI)	14
Phosphatidylethanolamine (PE)	12
Phosphatidylserine (PS)	7
Diphosphatidylglycerol (DPG)	3

[a]Average of 4 replicates; percentages are based on relative phosphate content.

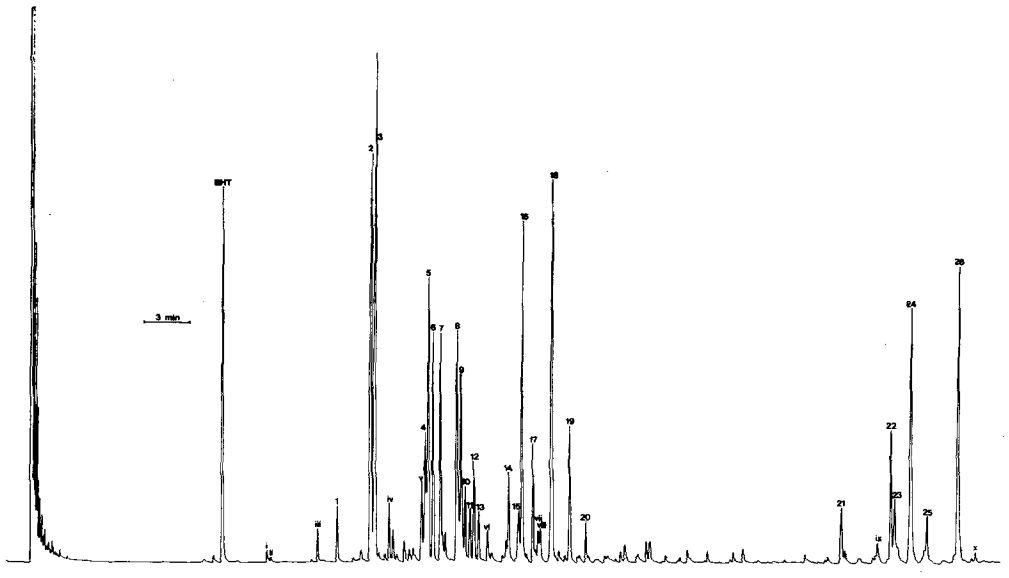

FIG. 3. Capillary GC analysis of the fatty acid methyl esters from the phospholipids of *Aplysina fistularis*. Temperature programmed from 70 C at 3.0 C/min. Other conditions are as described in the text. See Table 2 and text for identities of numbered peaks.

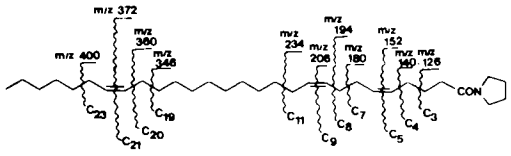

FIG. 4. The major diagnostic mass spectral fragmentations of N-(5,9,21-octacosatrienoyl) pyrrolidine (peak 22, Fig. 3 and Table 2).

0.2%) fragments which are formed by the cleavages of allylic carbon-carbon bonds (Fig. 4). The peak corresponding to the C_7 fragment was especially enhanced by virtue of its arising from the cleavage of a double-allylically activated bond. In fact, a prominent m/z 180 peak was found to be diagnostic of $\Delta^{5,9}$ unsaturation in these fatty acyl pyrrolidides. Consideration of the relative intensities of peaks in the mass spectra of the pyrrolidides therefore aided the identification of double bond positions, particularly in the spectra of the long chain compounds which often yielded very small peaks.

The structures assigned to the major (>0.4%) fatty acids from the phospholipids of *A. fistularis*, their ECL values, and their relative amounts, are listed in Table 2 with the hitherto unknown acids denoted with asterisks. One novel short chain acid in the mixture, a C_{17} monoene (peak 10, ECL 16.51), is assigned the structure 2,6,10-trimethyl-5-tetradecenoic acid based on the following mass spectral properties (see Fig. 5) of its pyrrolidide: (a) M^+ 321; (b) a diminished m/z 250 (C_{12}, 2%) fragment concurrent with enhanced m/z 236 (C_{11}, 10%) and m/z 264 (C_{13}, 8%) fragments, implying the presence of a methyl branch at C_{12}; (c) an enhanced m/z 208 (C_9, 43%) fragment concurrent with the absence of any C_7 fragment and a spacing of 12 amu between the m/z 166 (C_6) and m/z 154 (C_5) fragments, implying a double bond between the C_6 and a methyl-substituted C_7 atom; and (d) a base peak at m/z 126 (C_3) due to an α-cleavage next to the 2-methyl substituent. The only other novel short chain acid (peak 18, ECL 18.40) was assigned the structure 11-methyloctadecanoic acid, based on (a) M^+ 312 (14.8%) for the methyl ester, M^+ 351 (7.5%) for the pyrrolidide; and (b) a diminished C_{11} fragment (m/z 199 [7.0%] for the ester, m/z 238 [2.6%] for the pyrrolidide) concurrent with enhanced C_{12} (m/z 213 [16.7%] for the ester, m/z 252 [6.1%] for the pyrrolidide) and C_{10} (m/z 185 [19.3%] for the ester, m/z 224 [8.8%] for the pyrrolidide) fragments. This compound is known (29,30) but its occurrence in nature is, to our knowledge, unprecedented.

The unsaturated iso-branched C_{17} acid, existence of which was implied from the analysis of the hydrogenated esters, was identified by GC-MS as the known (31) 15-methyl-9-hexadecenoic acid (peak 8, ECL 16.35).

The major long chain acids (peaks 21-26 in Table 2) were indicated by GC-MS to contain $\Delta^{5,9}$ unsaturation. The only major C_{30} acid occuring in *A. fistularis* is 5,9,23-triacontatrienoic acid (peak 26, ECL 29.43). Two trienoic acids having 28 and 29 carbons each (peaks 23 and 25, respectively) were suspected of being homologs of this acid by virtue of their ECL values (27.43 and 28.43, respectively). Indeed, mass spectral analysis[4] of the pyrrolidides of these trienes indicated that the C_{28} triene is either the $\Delta^{5,9,22}$ or the $\Delta^{5,9,23}$ compound, and that the C_{29} triene is either the $\Delta^{5,9,22}$, the $\Delta^{5,9,23}$, or the $\Delta^{5,9,24}$ compound. Unfortunately, the low intensities of the fragments produced by these relatively minor components prevents an unambiguous assignment of their structures to be made from GC-MS analysis. However, the remaining C_{28} trienoic acid (peak 22, ECL 27.35) could be identified unambiguously (Fig. 4) by GC-MS as 5,9,21-octacosatrienoic acid.

The 2 remaining major long chain acids (peaks 21 and 24) were inferred to be 2-carbon homologs according to their ECL values (25.90 and 27.90, respectively). Analysis of the reduced esters, already discussed, implied that these were the acids that produced the ECL 26.50 and ECL 28.48 peaks upon hydrogenation. The mass spectra of the unsaturated pyrrolidides indicated that peak 21 was a $\Delta^{5,9}$-C_{27} diene, and that peak 24 was a $\Delta^{5,9}$-C_{29} diene, as shown by their molecular ions (m/z 459 and m/z 487, respectively) and by the fragments diagnostic for $\Delta^{5,9}$ unsaturation, as shown in Figure 6. In addition, a relatively diminished C_{22} fragment peak (m/z 388, 0.5%) concurrent with relatively enhanced C_{21} and C_{23} fragment peaks (m/z 374, 1.8% and m/z 402, 1.4%) in the mass spectrum of the C_{29} diene (Fig. 6A) and a similar pattern centered at the C_{20} fragment peak in the spectrum of the C_{27} diene (Fig. 6B; m/z 374 [C_{21}, 0.6%], m/z 360 [C_{20}, 0.4%], m/z 346 [C_{19}, 0.6%]) suggested the presence of a methyl group attached to the ω7 carbon of each acid.

To further verify the implied structures of the 2 long chain dienes, each was isolated as the methyl ester by HPLC of the diene-containing fraction obtained from argentic chromatography of the total esters. The purified C_{29} ester thus obtained exhibited $[\alpha]_D^{28.4}$ + 14.2° (c= 0.020 in $CHCl_3$). Efforts to accurately measure the rotation of the C_{27} ester failed due to lack

TABLE 2

The Major Fatty Acids from the Phospholipids of *Aplysina fistularis*[a]

Peak no.[b,c]	ECL[d]	Identity[c,e]	Percent (by wt.) in phospholipids[f]	Distribution[g] in phospholipid classes				
				PI	PS	PC	PG	PE/DPG
1	14.00	Tetradecanoic (n-14:0; myristic)	0.5 ± 0.1	1.5	–	–	–	–
2	14.63	13-Methyltetradecanoic (iso-15:0)	11.9 ± 0.4	11.8	1.4	19.7	11.2	1.1
3	14.71	12-Methyltetradecanoic (anteiso-15:0)	11.3 ± 0.7	15.6	3.0	18.1	10.7	1.6
4	15.60	13-Methylpentadecanoic (anteiso-16:0)	0.5 ± 0.0	1.0	–	–	–	–
5	15.73	9-Hexadecenoic (Δ^9-16:1; Palmitoleic)	10.4 ± 0.1	–	0.7	13.4	13.1	2.8
6	15.83	11-Hexadecenoic (Δ^{11}-16:1)	2.1 ± 0.1	–	1.2	–	5.1	–
7	16.00	Hexadecanoic (n-16:0; palmitic)	2.6 ± 0.1	9.0	1.3	–	8.4	1.2
8	16.35	15-Methyl-9-hexadecenoic (Δ^9-iso-17:1)	8.2 ± 0.2	–	0.7	12.6	8.9	2.8
9	16.43	10-Methylhexadecanoic (10-Me-16:0)	4.7 ± 0.2	3.0	1.2	–	8.7	2.0
10*	16.51	2,6,10-Trimethyl-5-tetradecenoic (2-Me,6-Me,10-Me-Δ^5-14:1)*	0.7 ± 0.0	–	–	1.6	–	–
11	16.60	15-Methylhexadecanoic (iso-17:0)	0.7 ± 0.1	0.6	–	–	–	0.6
12	16.70	14-Methylhexadecanoic (anteiso-17:0)	0.5 ± 0.1	–	–	–	–	–
13	16.78	9-Heptadecenoic (Δ^9-17:1)	0.9 ± 0.1	0.6	0.5	1.4	2.3	0.5
14	17.45	5,9-Octadecadienoic ($\Delta^{5,9}$-18:2)	3.5 ± 0.1	–	–	7.7	–	1.0
15	17.68	3,7,11,15-Tetramethylhexadecanoic (phytanic)	2.7 ± 0.1	–	–	1.2	–	17.5
16	17.74	11-Octadecenoic (Δ^{11}-18:1; vaccenic)	8.9 ± 0.2	1.3	–	9.3	14.4	1.5
17	18.00	Octadecanoic (n-18:0; stearic)	0.7 ± 0.1	5.3	1.3	–	1.2	–
18*	18.40	11-Methyloctadecanoic (11-Me-18:0)*	7.4 ± 0.2	34.0	5.7	4.2	3.2	1.0
19	18.80	11-Nonadecenoic (Δ^{11}-19:1)	1.0 ± 0.0	1.6	–	1.1	0.6	2.0
20	19.18	5,8,11,14-Docosatetraenoic ($\Delta^{5,8,11,14}$-20:4; arachidonic)	0.4 ± 0.1	–	6.7	–	–	–
21*	25.90	20-Methyl-5,9-hexacosadienoic (20-Me-$\Delta^{5,9}$-26:2)*	0.5 ± 0.1	–	2.2	–	–	2.4
22*	27.35	5,9,21-Octacosatrienoic ($\Delta^{5,9,21}$-28:3)*	2.1 ± 0.2	1.0	8.3	–	–	3.6
23*	27.43	5,9,23-Octacosatrienoic ($\Delta^{5,9,23}$-28:3)*[h]	0.4 ± 0.2	–	3.1	–	–	3.0
24*	27.90	22-Methyl-5,9-octacosadienoic (22-Me-$\Delta^{5,9}$-28:2)*	6.1 ± 0.1	1.6	20.0	0.6	1.1	17.8
25*	28.43	5,9,23-Nonacosatrienoic ($\Delta^{5,9,23}$-29:3)*[h]	0.6 ± 0.1	–	1.4	–	–	1.2
26	29.43	5,9,23-Triacontatrienoic ($\Delta^{5,9,23}$-30:3)	5.6 ± 0.1	1.5	24.5	0.6	1.0	18.4

[a]Identified minor (<0.4%) acids (Fig. 3): (i) iso-13:0 (ECL 12.62), (ii) anteiso-13:0 (ECL 12.70), (iii) iso-14:0 (ECL 13.62), (iv) n-15:0 (ECL 15.00), (v) iso 16:0 (ECL 15.60), (vi) n-17:0 (ECL 17.00), (vii) 19:1 (ECL 18.10), (viii) 19:1 (ECL 18.15), (ix) $\Delta^{5,9}$-28:2 (ECL 26.90), (x) $\Delta^{5,9}$-31:2 (ECL 29.90).

[b]Peak numbers correspond to numbered peaks in Fig. 3.

[c]An asterisk after the peak number and the name indicates that the acid is hitherto unknown in nature.

[d]Equivalent chain length values are those of the methyl esters of these acids.

[e]Shorthand notation of the acid ([methyl group position]-Me-Δ[double bond positions]-[# of carbons in straight-chain backbone]:[# of C=C double bonds]), and common name (if known) given in parentheses.

[f]Averages from 4 determinations; listed with standard deviations.

[g]Weight percent (averages from 3 determinations); "–" implies that the acid only occurred in a minor (<0.4%) amount.

[h]Tentative assignment; see text.

of material, although it also exhibited a positive rotation. The ^{1}H-NMR spectra of the 2 compounds were identical in all respects, exhibiting in deuterated chloroform a singlet (3H) at 3.66 ppm, a triplet (2H, J=7.63 cps) at 2.324 ppm, and a large singlet at 1.257 ppm, corresponding to the methoxycarbonyl, C_2-methylene and saturated methylene protons, respectively, as shown in the spectrum of the C_{29} ester (Fig. 7). In addition, a single monomethyl branch was indicated by a doublet (3H, J=6.51 cps) at 0.833 ppm, upfield from the terminal methyl signal (3H, t, J-6.9 cps) at 0.881 ppm. The position of one of the double bonds at C_5 was verified by the presence of a pentet (2H, J=7.0 cps) at 1.690 ppm assignable to the C_3 methylene group, in agreement with literature precedents (32). The ^{1}H-NMR signals for the allylic methylene groups consist of an uninterpretable multiplet (6H) centered at 2.072 ppm and a quartet (2H, apparent J=7.0 cps) at 2.015 ppm, which was assigned to the C_{11} methylene group because of its upfield position. Coupling of these protons with the 2 C_{12} protons (expected J=6.5-7.5 cps [33]) and with the single C_{10} proton (expected J=7.0 cps [33]) would explain this splitting pattern. This quartet is more clearly defined, at 1.983 ppm, when the spectrum is run in deuterated benzene.

The olefinic protons produce an uninterpretable multiplet in the ^{1}H-NMR spectrum

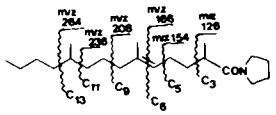

FIG. 5. The major diagnostic mass spectral fragmentations of N-(2,6,10-trimethyl-5-tetradecenoyl) pyrrolidine (peak 10, Fig. 3 and Table 2).

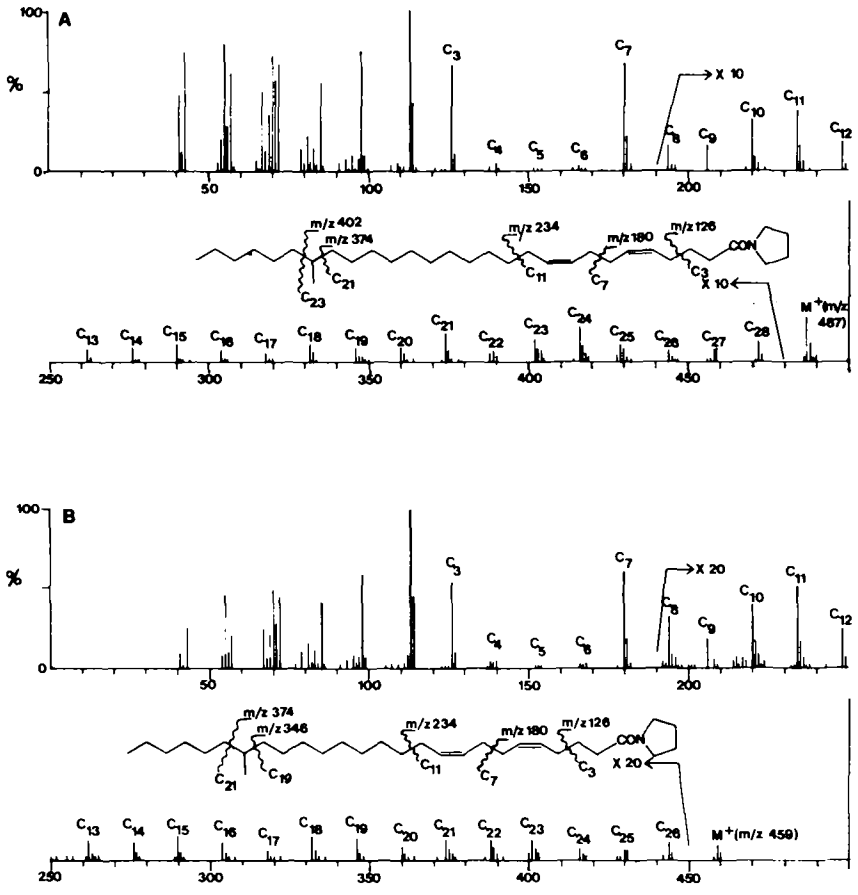

FIG. 6. (A) Mass spectrum of N-(22-methyl-5,9-octacosadienoyl) pyrrolidine (peak 24, Fig. 3 and Table 2). (B) Mass spectrum of N-(20-methyl-5,9-hexacosadienoyl) pyrrolidine (peak 21, Fig. 3 and Table 2).

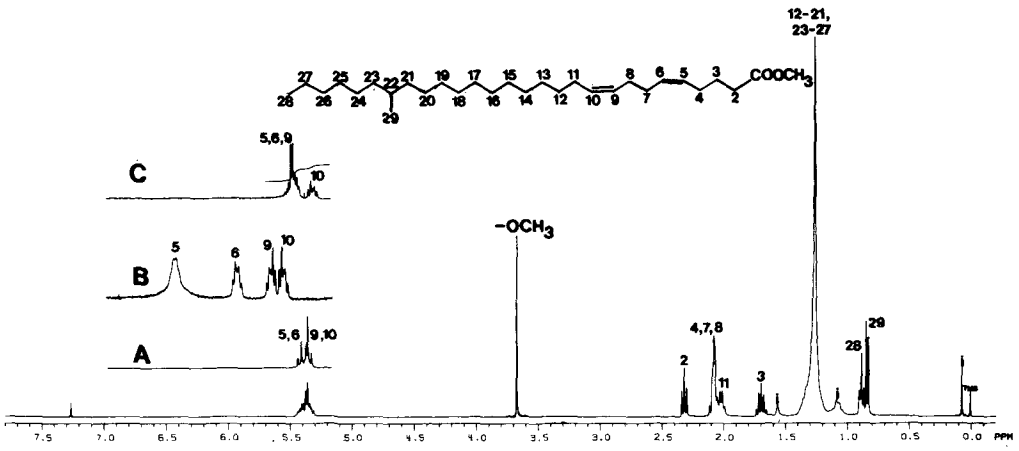

FIG. 7. 360 MHz ^{1}H-NMR spectrum of methyl 22-methyl-5,9-octacosadienoate measured in d_1-chloroform (+TMS reference). (A) Olefinic proton signals upon irradiation of 2.0-2.2 ppm region. (B) Olefinic protons in the presence of 0.5 equivalent of Yb $(fod)_3$. (C) Olefinic protons from spectrum measured in d_6-benzene. Numbers given above peaks indicate carbons bearing the protons responsible for those signals. i = impurity.

(4H) centered at 5.375 ppm. However, irradiation of the allylic methylene signals collapses this signal into 2 apparent doublets (Fig. 7A) at 5.419 ppm (apparent J=10.92 cps) and at 5.335 ppm (apparent J=10.70 cps), thus implying (32) a Z geometry for both double bonds. Support for this geometry was obtained from the spectra recorded in the presence of 0.5 equivalents of tris (6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)ytterbium $(Yb(fod)_3)$. This shift reagent resolved the olefinic proton signals into 4 multiplets (Fig. 7B). The 2 best-resolved multiplets, upfield at 5.645 ppm and at 5.550 ppm, correspond to the C_9 and C_{10} protons, respectively, and are each split into a pattern corresponding to that expected (33) from an ABX_2 system having J_{AB} = 10.50 cps and J_{AX}=6.75 cps, as measured from the spectrum. This AB coupling constant could only arise if the Δ^9 double bond has Z geometry (33). (This coupling data can be verified from the spectrum run in deuterated benzene, in which the C_{10} proton is clearly defined as an ABX_2 multiplet [J_{AB}=10.8 cps, J_{AX}=7.3 cps] at 5.315 ppm [Fig. 7C].) The apparent quartet at 5.919 ppm, which corresponds to the C_6 proton, is also split into an ABX_2 pattern (J_{AB}=10.50 cps, J_{AX}=7.35 cps), although the signals are broadened by the shift reagent. The signal at 6.422 ppm, due to the C_5 proton, has been broadened too much for reliable analysis. However, the presence of only 0.4 equivalents of the shift reagent allows this signal to be resolved into an apparent quartet corresponding to an ABX_2 pattern (J_{AB}=10.3 cps, J_{AX}=7.2 cps) at 5.874 ppm. Therefore, data from the chemical shift experiments indicate that both the Δ^5 and Δ^9 double bonds of the C_{27} and the C_{29} dienes occur in the Z geometry. This assignment is supported by the infrared spectra of these esters, which exhibit a notable absence of absorptions in the 950-1000 cm^{-1} region, thus indicating (34) the absence of *trans* C=C vibrations. However, a strong absorption at 760 cm^{-1} from the C_{29} diene and one at 820 cm^{-1} from the C_{27} diene correspond (34) to *cis* C=C bending vibrations.

To verify the positions of the monomethyl branches in the C_{27} and C_{29} dienes, the monofunctional products from the ozonolysis and oxidation of each of them were subjected to GC and to GC-MS analyses. The sole C_{20} methyl ester derived from the C_{29} diene (ECL 19.48) was indicated, by the mass spectrum of its pyrrolidide (Fig. 8A), to possess a single methyl group on its ω7 carbon by virtue of enhanced C_{12} and C_{14} fragments and a diminished C_{13} fragment peak. In like manner, the presence of an ω7-methyl group on the C_{18} ester (ECL 17.45) obtained from the C_{27} diene was also implied (Fig. 8B). Furthermore, the C_{20} and C_{18} degradation products each exhibited GC behavior (as assessed by coinjection) and mass spectra identical to those of the synthetic ω7-methyl compounds, methyl 13-methylnonadecanoate (**2**) and methyl 11-methylheptadecanoate (**1**) (and their pyrrolidides), respectively. The presence of the methyl branch on the ω7 carbon of the 2 synthetic products was verified by the ^{1}H-NMR spectra of their synthetic precursors, methyl13-methyl-12-nonadecenoate (**9**) and methyl 11-methyl-10-heptadecenoate (**8**) (Fig. 2), which exhibited singlets at 1.58 ppm and doublets (J≈2 cps)

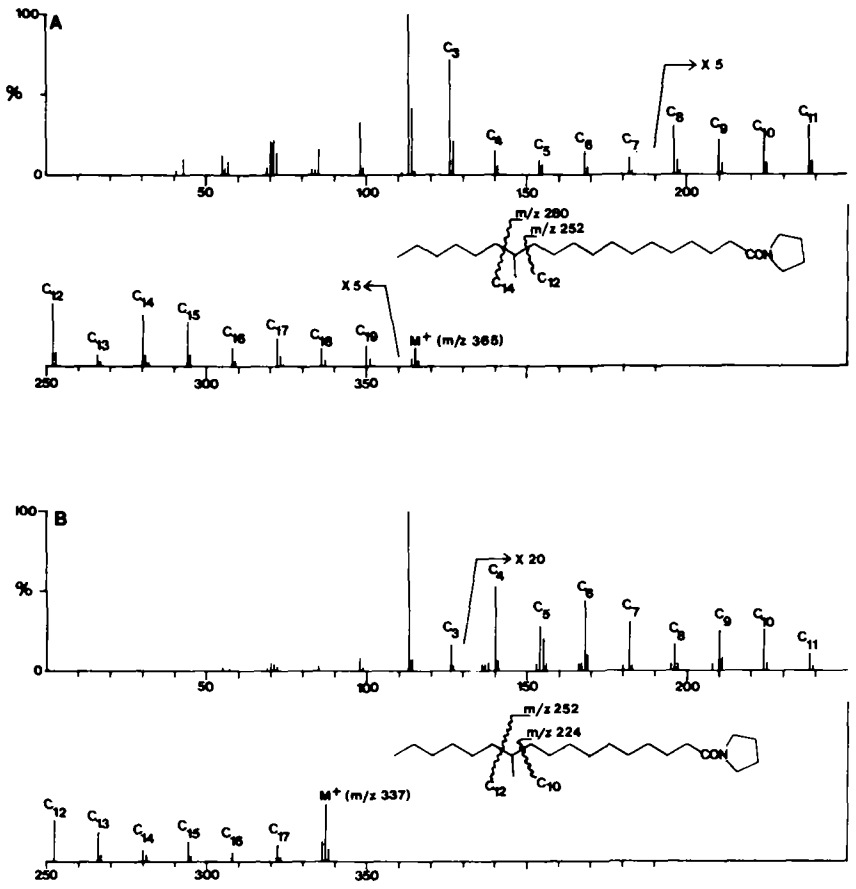

FIG. 8. (A) Mass spectrum of the pyrrolidide of the monofunctional degradation product from methyl 22-methyl-5,9-octacosadienoate. (B) Mass spectrum of the pyrrolidide of the monofunctional degradation product from methyl 20-methyl-5,9-hexacosadienoate.

at 1.66 ppm corresponding to the vinylic methyl group (E and Z isomers, respectively) expected only if the Wittig reaction which formed them proceeded as shown in Figure 2.

In addition to the major fatty acids listed in Figure 3 and Table 2, several minor fatty acids were identified by GC and by GC-MS (Table 2, footnote a). Of particular interest are the 2 minor long chain acids (peaks ix and x, Fig. 3). Mass spectral evidence indicates that these acids are a $\Delta^{5,9}$-C_{28} diene and a $\Delta^{5,9}$-C_{31} diene, by virtue of prominent molecular ions (m/z 483 and m/z 515, respectively) and prominent C_7 fragments (m/z 180). Unfortunately, the low concentration of these acids prevented further mass spectral analysis. However, their ECL values (26.90 and 29.90) strongly suggest that these acids may be C_{28} and C_{31} homologs of the ω7-methyl C_{27} (ECL 25.90) and C_{29} (ECL 27.90) dienes (peaks 21 and 24, respectively).

Analysis of Fatty Acid Contents of Phospholipid Classes

Fatty acid methyl esters were obtained from the phospholipid fractions identified as PI, PS, PC, PG and from a fraction containing both PE and DPG (these 2 phospholipids could not be accurately resolved by preparative TLC). The results of the GC analyses of these esters are given in Table 2. These results indicate that PI, PC and PG contain almost exclusively short chain acids, that PS contains a majority of long chain acids and that the PE/DPG fraction contains an equal complement of long and short chain acids.

Assessment of the Bacterial Content in *A. fistularis*

The transmission electron micrograph of *A. fistularis* indicated the presence of numerous spherulocytes and archaeocytes (both

sponge cells) as well as bacterial cells. Stereological analysis indicated that spherulocytes make up ca. 30% of the tissue volume, that archaeocytes make up ca. 28% of the tissue volume, and that bacterial cells comprise about 5% of the tissue volume. However, consideration of the average surface densities of each cell type (28), and the relative number of each type of cell per unit volume reveals that the relative surface area contributions of the spherulocytes, archaeocytes and bacteria are ca. 1.2, 1.4 and 1.0, respectively.

Assessment of Sponge Cell Contribution to Long Chain Branched Acids

Microscopic examination of the cell fraction obtained by centrifugation of *A. fistularis* cells on a Ficoll gradient indicated a significant enrichment, though not a virtual isolation of spherulocytes. Gas chromatographic analysis of the fatty acid methyl esters obtained from the phospholipids of this sample indicated a greater proportion of the C_{27}-C_{30} $\Delta^{5,9}$ dienoic and trienoic acids relative to the acid contents of a nonenriched sample. Specifically, the ratio of all C_{14}-C_{20} acids to all C_{27}-C_{30} acids was 3.45 in the nonenriched sample but was 2.02 in the enriched sample. The ratio of C_{15}-C_{19} monomethyl-branched "bacterial-type" acids to all C_{27}-C_{30} acids was 1.88 in the nonenriched sample but only 0.96 in the enriched sample. Finally, the ratio of C_{15}-C_{19} monomethyl-branched acids to the 2 major (C_{27} and C_{29}) branched long chain dienes was 4.52 in the nonenriched sample, but was only 2.11 in the enriched sample.

DISCUSSION

As expected from previous studies (10), the free sterols of *A. fistularis* (Fig. 1) contain a large proportion of the 26-methylated sterols aplysterol and 24(28)-dehydroaplysterol. These 2 sterols comprise 60% of the total sterol mixture. The 7 major sterols comprising the remaining 40% of the mixture are ubiquitous in the marine environment (3-6), and are probably dietary in origin. However, the aplystane side chain of aplysterol and dehydroaplysterol is found almost exclusively in sponges of the genus *Aplysina* (35); therefore, these 2 sterols are probably synthesized by the sponge (possibly by transformation of a dietary precursor). Cholesterol is present as only 11% of the total sterol mixture, and therefore, probably plays a relatively insignificant role as a membrane component.

The major phospholipids present in *A. fistularis* (Table 1) are almost exclusively diacylglycerophosphate derivatives possessing head groups known throughout the plant and animal kingdom. The presence of PC, PE and PS in sponges is well precedented (36). However, to our knowledge, PI, PG and DPG have not been reported to occur in sponges, although PI is known to occur in other marine invertebrates (36). Of particular interest are the high proportions of PG and DPG found in *A. fistularis*. These phospholipids are generally found only in very small amounts in most eukaryotes, in which they occur in the mitochondria (37). However, PG and DPG, as well as PI, are very commonly found as the major phospholipids of bacterial membranes (38,39). This phospholipid analysis therefore implies the presence of significant numbers of bacteria in the sponge.

Electron microscopic studies of mediterranean species of *Aplysina* (=Verongia) have indicated that these sponges harbor large quantities (as much as 37% of the total volume) of bacteria in a tight, specific association (40, 41). Our analysis of the phospholipids of *A. fistularis* suggests that a similar association exists in this sponge, and our analysis of an electron micrograph of *A. fistularis* indeed indicates that bacterial cells occupy at least 5% of the sponge volume. More importantly, when the relative surface areas of the bacterial cells were estimated and compared with the relative areas of the 2 major identifiable sponge cells (the spherulocytes and archaeocytes), we found that the bacterial cells contributed almost as much surface area as each of the sponge cell classes. This implies that the plasma membranes of the bacteria possess as much relative surface area as the plasma membranes of each sponge cell class, and therefore, we would expect the bacterial phospholipids to contribute proportionately to the total phospholipids extracted from the sponge.

The fatty acid content of the phospholipids of *A. fistularis* (Table 2) also strongly suggests the presence of bacteria in this sponge. Data concerning the precedented occurrences of each of the major fatty acids are summarized in Table 3. Seven of the major acids (peaks 2, 3, 4, 8, 9, 11, and 12), representing 37.8% of the total, are known to occur almost exclusively in bacteria. These include the iso- and anteiso-branched acids (peaks 2, 3, 4, 11 and 12, as well as i, ii, iii, and v), which have been found in particular abundance in many species of *Bacillus* (43) and the Δ^9-iso-17:1 acid (peak 8), which is known to occur in a marine anaerobic bacterium (31). 10-Methylhexadecanoic acid (peak 9) is known to occur in mycobacteria (45), *Staphylococcus aureus* (46), and *Microbispora parva* (47). The similarity between the

TABLE 3

Possible Origins of the Major Fatty Acids from the Phospholipids of *Aplysina fistularis*[a]

Peak no.[a]	Acid[b]	Precedented origin	Hypothetical origin in *A. fistularis*
1	n-14:0	Ubiquitous in marine plants, animals and bacteria (42)	Diet, bacteria and/or sponge
2	iso-15:0	Typical bacterial acid (esp. *Bacillus* spp. (43)	Bacteria
3	anteiso-15:0	Same as peak 2	↓
4	anteiso-16:0	Same as peak 2	
5	Δ^{9}-16:1	Known in marine bacterium (31), ubiquitous in marine animals and plants (42)	Diet, bacteria and/or sponge
6	Δ^{11}-17:1	Ubiquitous, known in bacteria (44)	↓
7	n-16:0	Ubiquitous (42)	
8	Δ^{9}-iso-17:1	Known in marine bacterium (31)	Bacteria
9	10-Me-16:0	Known in mycobacteria (45), *Staphylococcus aureus* (46) and *Microbispora parva* (47)	Bacteria
10	2-Me,6-Me,10-Me-Δ^{5}-14:1	Unprecedented; similar to phytanic and pristanic acids (48)	Diet
11	iso-17:0	Same as peak 2	Bacteria
12	anteiso-17:0	Same as peak 2	↓
13	Δ^{9}-17:1	Widely occurring in bacteria, including marine bacteria (31,49)	
14	$\Delta^{5,9}$-18:2	Known in conferous plants (50); $\Delta^{5,9}$ unsaturation is typical for sponges (51)	Sponge
15	Phytanic	Known fatty acid component of marine food webs (48)	Diet
16	Δ^{11}-18:1	Known in bacteria and phytoplankton (44,52)	Bacteria and/or diet
17	n-18:0	Ubiquitous (42)	Diet, bacteria and/or sponge
18	11-Me-18:0	Unprecedented; branching pattern typical of bacterial acids	Bacteria
19	Δ^{11}-19:1	Same as peak 13	Bacteria
20	Arachidonic	Known in phytoplankton (52)	Diet
21	20-Me-$\Delta^{5,9}$-26:2	Unprecedented; typical demospongic acid	Sponge
22	$\Delta^{5,9,21}$-28:3	↓	↓
23	$\Delta^{5,9,23}$-28:3[c]		
24	22-Me-$\Delta^{5,9}$-28:2		
25	$\Delta^{5,9,23}$-29:3[c]		
26	$\Delta^{5,9,23}$-30:3	Known in the marine demosponge *Chondrilla nucula* (52)	

[a]Peak numbers refer to Fig. 3 and Table 2.

[b]Shorthand notation or common name; see Table 2 for full names.

[c]Tentative assignment; see text.

mid-chain branching pattern of this acid and that of the unprecedented 11-methyloctadecanoic acid (peak 18) implies that the latter, which comprises 7.4% of the total fatty acids, may have a similar bacterial origin.

The polymethyl branched acid, phytanic acid (peak 15), is a known component of marine food webs, and presumably arises by degradation of the isoprenoid side chain of chlorophyll (48). The similarity between this acid and the other major polymethyl branched acid in *A. fistularis*, 2,6,10-trimethyl-5-tetradecenoic acid (peak 10), leads us to believe that this heretofore unknown acid is, like phytanic acid, dietary in origin.

Research on the fatty acids of sponges by Litchfield and coworkers (53-55) has led to the characterization of several novel C_{24}-C_{30} fatty acids, most of which featured $\Delta^{5,9}$ diunsaturation and, in some cases, one more double bond near the chain terminus, as major components of many marine demosponges. The widespread occurrence of this class of so-called "demospongic acids," and their biosynthesis by the sponges studied, is now well known (51,56). Our analysis indicates that *A. fistularis* also contains demospongic acids (peaks 21-26, Table 2) as 15.2% of the total acids and that all but one acid is unprecedented. Only the 5,9,23-triacontatrienoic acid (peak 26), which comprises 5.6% of the *A. fistularis* mixture, has been found previously, in the demosponge *Chondrilla nucula* as a major (34%) fatty acid (53). The only precedented octacosatrienoic acid reported in sponges is the $\Delta^{5,9,19}$ compound found in *Xestospongia halichondroides* (55). Mass spectrometry indicates that neither of the octacosatrienoic acids in *A. fistularis* (peaks 22 and 23) have Δ^{19} double bonds but that the major triene (peak 22) is 5,9,21-octacosatrienoic acid. Incomplete mass spectral data, biosynthetic precedents for the occurrence of double bonds at odd carbon positions, and the homology with the $\Delta^{5,9,23}$-30:3 acid implied by its ECL value suggest that the minor 28:3 acid (peak 23) is 5,9,23-octacosatrienoic acid.

Demospongic acids containing 29-carbon chains are unprecedented. Thus, the nonacosatrienoic acid in *A. fistularis* (peak 25) is also a new structure. Again, incomplete mass spectral data, biosynthetic precedents, and an ECL value which suggests homology with the $\Delta^{5,9,23}$-30:3 acid implies that this acid is 5,9,23-nonacosatrienoic acid.

Two other unprecedented demospongic acids (peaks 21 and 24) present in *A. fistularis* are Z,Z-20-methyl-5,9-hexacosadienoic acid and Z,Z-22-methyl-5,9-octacosadienoic acid, which are particularly unusual for possessing methyl branches at their $\omega 7$ carbons. The structures of these acids, which have been rigorously proven using spectroscopic and chemical methods, have never before been found in nature, although their monomethyl branching would suggest a bacterial origin. However, our comparison between the fatty acids from a spherulocyte-enriched cell suspension and the fatty acids from a nonenriched cell suspension clearly indicated that enrichment in the sponge cells produced a concomitant enrichment in these 2 fatty acids relative to the known bacterial fatty acids. In fact, all of the demospongic acids were enriched in the sponge cell-enriched sample, thus confirming that these fatty acids are major components of the sponge cell phospholipids.

An analysis of the major fatty acids in the isolated phospholipid classes (Table 2) indicates that the demospongic acids predominate in PS and in the PE/DPG fractions. This result agrees with analyses from 4 other sponges that have consistently shown that the demospongic acids are found primarily in the free amino-containing phospholipids PS and PE (53). Our analysis also indicates, as expected, that the bacterial-type fatty acids predominate in PI, PC and in PG. Interestingly, a large proportion of phytanic acid (peak 15) occurs in the PE/DPG fraction.

Incorporation studies using ^{14}C-acetate in the sponge *Microciona prolifera* have indicated that this sponge synthesizes its demospongic acids by the chain elongation of short chain length precursors followed by $\Delta^{5,9}$ desaturation (56). Using these results as a precedent, Litchfield et al. (53) proposed a biosynthetic route to 5,9,23-triacontatrienoic acid from 9-hexadecenoic acid. Using the same precedent, we propose that 5,9,23-triacontatrienoic acid (peak 26) and 5,9,21-octacosatrienoic acid (peak 22) in *A. fistularis* arise from 9-hexadecenoic acid (peak 5) and/or 11-octadecenoic acid (peak 16) in the same manner:

$$\Delta^{9}\text{-}16{:}1 \rightarrow \Delta^{11}\text{-}18{:}1 \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow$$
$$\Delta^{21}\text{-}28{:}1 \rightarrow \Delta^{9,21}\text{-}28{:}2 \rightarrow \Delta^{5,9,21}\text{-}28{:}3$$
$$\downarrow$$
$$\Delta^{23}\text{-}30{:}1 \rightarrow \Delta^{9,23}\text{-}30{:}2 \rightarrow \Delta^{5,9,23}\text{-}30{:}3$$

In a similar fashion, 5,9,23-octacosatrienoic acid (peak 23), existence of which is implied in *A. fistularis*, can arise from 11-hexadecenoic acid (peak 6):

$$\Delta^{11}\text{-}16{:}1 \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow$$
$$\Delta^{23}\text{-}28{:}1 \rightarrow \Delta^{9,23}\text{-}28{:}2 \rightarrow \Delta^{5,9,23}\text{-}28{:}3$$

These hypothetical precursors (peaks 5, 16 and 6) are present in *A. fistularis*. The implied

existence of 5,9,23-nonacosatrienoic acid suggests a biosynthesis from an 11-heptadecenoic acid starting material, but this potential precursor was not detected in the mixture:

$$\Delta^{11}\text{-}17{:}1 \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow$$

$$\Delta^{23}\text{-}29{:}1 \rightarrow \Delta^{9,23}\text{-}29{:}2 \rightarrow \Delta^{5,9,23}\text{-}29{:}3$$

The most plausible biosynthetic route to the ω7-branched dienoic acids, and one which agrees with the known route to demospongic acids (56), is one involving chain elongation of 10-methylhexadecanoic acid (peak 9):

$$\text{10-Me-16:0} \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow$$

$$\text{20-Me-26:0} \rightarrow \text{20-Me-}\Delta^{9}\text{-26:1} \rightarrow \text{20-Me-}\Delta^{5,9}\text{-26:2}$$

$$\downarrow$$

$$\text{20-Me-28:0} \rightarrow \text{22-Me-}\Delta^{9}\text{-28:1} \rightarrow \text{22-Me-}\Delta^{5,9}\text{-28:2}$$

This biosynthetic route would also explain the presence of the proposed C_{31} homolog of these acids (peak x), although it does not readily account for the biosynthesis of the proposed C_{28} homolog (peak ix).

Consideration of the major demospongic acids of *A. fistularis* reveals that almost all of these acids contain either a double bond or a methyl branch on the ω7 carbon. Since the presence of a methyl branch on a fatty acyl chain induces fluidity in phospholipid bilayers to nearly the same degree as does unsaturation (30,57), this observation suggests that *A. fistularis* has evolved the syntheses of these ω7-substituted acids as a means for increasing the fluidity of its membranes in a region of the bilayer located near the ends of the fatty acyl chains. Furthermore, this observation leads us to propose that *A. fistularis* has evolved a fatty acyl synthetase which will elongate and desaturate *any* short chain acid which possesses a "fluidizing group"—a double bond or a methyl group—on its ω7 carbon to produce $\Delta^{5,9}$ ω7 substituted demospongic acids. Such a relatively nonspecific enzyme system would allow this sponge to utilize its supply of short chain starting materials, especially those from its associated bacteria, to fullest advantage.

Our analysis of the free sterols of *A. fistularis* has indicated that this sponge contains as its major sterol aplysterol—a product of C_{26} biomethylation. The potential disordering effect of the side chain alkylation of yeast sterols on phospholipid bilayers has been pointed out (8). The apparent need of *A. fistularis* for fluidity deep within its phospholipid bilayer, as already suggested, may have likewise led to the evolution of a "fluidizing group"—a methyl group at C_{26} of the side chain—on the sterols produced by this sponge. Thus, we propose on the basis of our findings that *Aplysina fistularis* has evolved membranes which possess a "zone" of membrane fluidity near the center of the phospholipid bilayer, due to the fluidizing effects of 26-methylated sterols and of ω7 double bonds and ω7-methyl groups in the fatty acyl chains.

Regarding the specific dimensions of this proposed zone, and the relative positions of the demospongic acids and aplysterol in the membrane, space-filling models indicate that the total length of 22-methyl-5,9-octacosadienoic acid (peak 24, Table 2), for example, when fully extended, is ca. 37.7 Å, and the distance from the carboxyl group to the ω7-methyl group is about 30.0 Å, whereas the length of aplysterol (Fig. 1) is only about 21.2 Å. This implies that very little interaction would occur within the membrane between the sterol side chain and the ω7 groups on the demospongic acids. However, a configuration for carbons 1-10 of the acid in which the carbonyl, the Δ^5, and the Δ^9 double bonds are "stacked" with their nodal planes parallel to allow through-space π-π interactions (Fig. 9) would shorten the acyl chain to an effective length (measured from the carboxyl group) of about 27.2 Å. The ω7-methyl group in such a configuration is only 20.3 Å from the carboxyl group. It is intriguing to speculate that such a configuration would allow the fatty acyl group to curl around the polar end of the sterol to allow hydrogen bonding between the carbonyl and the 3β-hydroxy group of the sterol. Such a juxtaposition, indicated schematically in Figure 9,

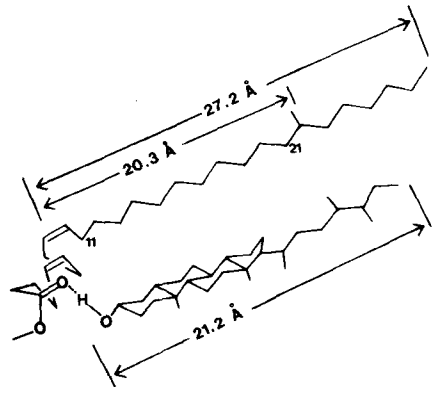

FIG. 9. A hypothetical folded configuration of a $\Delta^{5,9}$-ω7-substituted demospongic acid (in this case, 22-methyl-5,9-octacosadienoic acid), indicating approximate dimensions and the potential "fit" between the acid in this configuration and aplysterol (not drawn to scale). The dotted line indicates hydrogen bonding between the fatty acyl carbonyl and the 3β-hydroxyl group of the sterol.

would allow the rigid ring system of the sterol to lie alongside the presumably rigid extended C_{11}-C_{21} portion of the acid, and would bring the $\omega 7$ group of the acid and the terminal groups of the sterol side chain into close proximity within the interior of the membrane, where they would both presumably cause disordering. Such an unusual feature of the membrane may allow the sponge to adapt more proficiently to variations in the temperature and/or hydrostatic pressure in its environment, it may represent a response to stress from the sponge's biotic environment, or it may reflect a specialized requirement of certain membrane-bound enzymes for internal fluidity.

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Incorporation into Lipid Classes of Products from Microsomal Desaturation of Isomeric *trans*-Octadecenoic Acids

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ABSTRACT

The microsomal desaturation of positional isomers of *trans*-octadecenoic acids is effected by the Δ9-desaturase and, with concomitant geometric isomerization, *cis,trans*- and *cis,cis*-octadecadienoic acids of unusual structure are formed. Incorporation of the substrates and their products into lipids varied from 50.5% for incubations with 14-18:1 to 81.0% for 6-18:1. A detailed study of the composition of each of the major lipid classes, i.e., phospholipids, triacylglycerol and cholesteryl esters, as well as the composition of the free fatty acid fraction, revealed a complex picture. Generally, the *c,c*-18:2 products were enriched in the phospholipid fraction, whereas the *c,t*-18:2 appeared preferentially in cholesteryl esters. The 18:1 substrates themselves did not show marked preferences for any of the lipid classes. Phospholipase A_2 action on phosphatidylcholine and phosphatidylethanolamine demonstrated enrichment of the *c,c*- and the *c,t*-18:2 products in the 2-position, whereas the 18:1 substrates were preferentially inserted into the 1-positions. The *c,c*- and *c,t*-18:2 formed by desaturation of *t*11-18:1 varied from this pattern, probably due to their conjugated double bond structures. Linoleic acid, *c*9,*c*12-18:2, formed during desaturation of *t*12-18:1, surprisingly showed enrichment in the 1-position of phosphatidylcholine. Incubation experiments with *t*5- and *t*6-isomers using liver microsomes from rats fed a corn-oil-supplemented diet showed conversion and incorporation rates similar to the rates obtained with microsomes from EFA-deficient rats. The fatty acid composition of lipid classes and the distributions of products and substrate between the 1- and 2-positions of phosphatidylcholine also agreed with results obtained using microsomes from EFA-deficient rats.

INTRODUCTION

The metabolic fate of *trans* fatty acids has attracted much attention in recent years due to increasing consumption of partially hydrogenated fats and oils containing, in some cases, considerable amounts of *trans*-unsaturated acids (1,2). Incorporation into lipids (3,4), catabolism and anabolism (5,6), absence of EFA activity (7) and effect on the physical properties of lipid membranes of *trans* acids (8,9) have been reported by many investigators and have been reviewed by Houtsmuller (10).

Mahfouz et al. of this laboratory (11) and Pollard et al. (12) demonstrated independently that positional isomers of *t*-octadecenoic acids (*t*-18:1) are desaturated in rat liver microsomal systems by Δ9-desaturase. The products of desaturation are a series of unusual *c,c*- and *c,t*-octadecadienoic acids (*c,c*- and *c,t*-18:2) that, in some cases, are desaturated again by Δ6-desaturase to polyunsaturated acids of unusual and perhaps unnatural structure (12). Chain elongations of some of the positional isomers of *t*-18:1 acids have been shown to take place in liver microsomal suspensions, although the rates of elongation are much slower than the rates of Δ9-desaturation (13). In an effort to assess the effects of *t*-18:1 isomers on essential fatty acid metabolism, these isomers were shown to exert inhibitory effects in vitro on the Δ5-, Δ6- and Δ9-desaturases (14). Thus, these studies indicate that products of partial hydrogenation of fats and oils may interfere with the metabolism of essential fatty acids (7), and the possibility remains that they may, themselves, serve as substrates for biosynthesis of oxidized polyunsaturated acids of unusual structure with biopotencies that cannot be predicted (15).

A number of studies have shown the deposition of *t*-unsaturated acids in tissues (7,16,17). Wood and Chumbler and Reichwald-Hacker et al. have shown that differential incorporation into tissue lipids of *c*- and *t*-positional isomers of 18:1 acids can occur (18,19). In a subsequent paper by Reichwald-Hacker et al., specificities in the incorporation of individual *c*- and *t*-18:1 acids into the acyl moieties of the major lipid classes of liver, heart and serum were demonstrated (20). In vitro studies have indicated that the acyltransferases, in addition to selectivity for the hydroxyl position in lysophospholipids, recognize geometric and positional isomers of unsaturated substrate acyl chains (21,22).

This study investigated the incorporation into microsomal lipids of the unusual 18:2 acids formed in situ from the desaturation

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of a series of isomeric *t*-18:1 acids, and positional specificities in the incorporation into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were determined.

MATERIALS AND METHODS

Reagents and Substrates

ATP, CoA, NADH and bovine serum albumin V fraction were all obtained from Sigma Chemical Co. (St. Louis, MO). *trans*-Positional isomers of [1-^{14}C]- and unlabeled 18:1 acids were synthesized by Valicenti, Pusch and Holman in this laboratory (23). The purity of all the unlabeled and labeled acids was more than 90% (11).

The methyl esters of stearic, oleic, elaidic and linoleic acids used as carriers in $AgNO_3$-TLC (vide infra) were purchased from Nu-Chek-Prep (Elysian, MN). Isomerized linoleic acid methyl ester (mixed *c*9,*t*12- and *t*9,*c*12-18:2 isomers) was prepared by Pusch (unpublished) and was used as a standard to locate the *c*,*t*-18:2 products on plates. Egg lecithin, egg lysolecithin and egg PE were obtained from Supelco (Bellefonte, PA), and phospholipase A_2 purified from bee venom was purchased from the Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade or of the highest purity commercially available.

Preparation of Microsomes

Weanling male Sprague-Dawley rats kept on an EFA-deficient diet or a similar diet supplemented with 5% corn oil for 3-6 months were sacrificed and the individual livers were quickly removed and rinsed in cold homogenization solution consisting of 0.15 M KCl, 0.005 M $MgCl_2$, 0.004 M EDTA, 0.25 M sucrose, 0.0015 M glutathione and 0.05 M potassium phosphate buffer (pH 7.4) (24). Each entire liver was homogenized in a Teflon pestle homogenizer in 3 vol of the same solution. The microsomal fraction sedimenting between 12,000 x g and 105,000 x g was resuspended in homogenization solution to provide microsomes equivalent to 1 g of original liver/1 ml. The microsomes were stored in screw-capped vials under nitrogen at -70 C. Protein was determined by the biuret reaction. Microsomes prepared from each animal were used in incubations to compare all isomers.

Incubation Conditions

Each incubation carried out in 1 ml of a 0.15 M KCl/0.25 M sucrose solution contained ATP (5.0 mM), CoA (0.2 mM), NADH (0.7 mM), $MgCl_2$ (5.0 mM), glutathione (1.5 mM), NaF (45 mM) and nicotinamide (0.5 mM). The pH was adjusted to 7 by phosphate buffer (100 mM); 120 nmol *t*-18:1 acid containing an amount of radioactively labeled acid equivalent to 0.1 μCi was added to the incubation mixture as the sodium salt bovine serum albumin complex suspended in potassium phosphate buffer at pH 7.4. The incubation was started by the addition of 4 mg microsomal protein suspended in 0.15 M KCl/0.25 M sucrose. The incubations were done in a Dubnoff metabolic shaker at 37 C for 20 min with exposure to air. These conditions were similar, but not identical, to those used by Mahfouz et al. (11).

Analysis of Desaturation and Isomerization Products

The incubations were terminated by the addition of 5% HCl in methanol. The lipids were extracted with chloroform/methanol (2:1, v/v) and the extract was dried under a stream of nitrogen and esterified with 10% HCl in methanol at 80 C for 2 hr. The tubes were evaporated to dryness, unlabeled carriers added (the methyl esters of 18:0, *c*-18:1, *t*-18:1, *c*,*t*-18:2 and *c*,*c*-18:2) and the mixture was separated on 10% $AgNO_3$ Silica Gel H plates in petroleum ether/diethyl ether (100:10, v/v). The separated bands were made visible by spraying with 0.1% 2,7-dichlorofluorescein and viewing under ultraviolet light. The spots were scraped into scintillation vials and counted in a Packard scintillation counter after additon of 15 ml toluene-based scintillation fluid. The analyses, determination of the double bond positions, determination of configurations and the nature of the impurities were described previously (11). Specific activities were not determined on the final product, but were projected to be about 52 mCi/mmol.

Analysis of Lipid Classes

The lipid extract from the incubation medium was evaporated to dryness under nitrogen, redissolved in $CHCl_3$ and a carrier mixture of cholesterol, cholesteryl oleate, triolein, oleic acid and PC (obtained from Nu-Chek-Prep, Elysian, MN) was added. The mixture was spotted on Silica Gel H plates and developed in petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v). Typically, 12-14 incubations with each isomer were extracted and the lipids were separated for 6 of these samples. The spots were made visible under UV light and were scraped into scintillation vials and counted in a scintillation counter for determination of incorporation of radioactivity into lipid classes. Another group of 6 samples was similarly separated into lipid classes, and the phospholipid (PL), free fatty acid (FFA), triacylglycerol (TAG) and cholesteryl ester

(CE) bands were scraped into tubes. The lipids were transesterified with 14% BF_3 (w/v) in methanol at 80 C for 1 hr. One ml of deionized water was added and the methyl esters were extracted with 2 ml of petroleum ether. The methyl esters were separated on $AgNO_3$ Silica Gel H plates as already described and the distribution of desaturase products and substrate was calculated for each of the lipid classes following scintillation counting.

Phospholipase A_2

To investigate the incorporation of labeled substrate and products into the 1- and 2-positions of PC and PE, the PC and PE extracted from the crude lipid mixture were treated with phospholipase A_2. The dried lipid extract from the incubation mixture was spotted on Silica Gel H plates after addition of lyso-PC, -PC and -PE as carriers. The plates were developed in chloroform/methanol/water (65:25:4, v/v/v) and the spots were made visible. The PC and PE spots were scraped into tubes, then 1 ml of water and 1 ml of methanol were added. The PC and PE were extracted twice with 2 ml of diethyl ether. Scintillation counting showed that more than 75% of the activity was recovered. The ether extracts were evaporated to dryness under nitrogen, 1 ml of diethyl ether and 100 μl of a phospholipase A_2 solution in 0.005 M $CaCl_2$ (activity ca. 40 units/ml) were added. The tubes were filled with nitrogen, capped and reacted for 90 min at room temperature (22-24 C) under vigorous shaking. TLC analysis showed more than 95% hydrolysis.

After completion of the reaction, the tubes were evaporated to dryness by a stream of nitrogen. The reaction mixture was redissolved in chloroform/methanol (1:1, v/v) and spotted on Silica Gel H plates. The plates were developed in chloroform/methanol/water (65:25:4, v/v/v), and the lysolipids and FFA were made visible and scraped into tubes. Transesterification and analysis of methyl esters is as already described in Analysis of Lipid Classes. The distributions of desaturase products and substrate for each of the positions 1 and 2 of PC and PE were calculated.

RESULTS AND DISCUSSION

The series of positional isomers of *t*-18:1 acids from *t*4-18:1 to *t*15-18:1 enabled Mahfouz et al. to study the desaturation of these isomers, common in hydrogenated fats, by liver microsomal systems (11). They found that isomeric *t*-18:1 acids are desaturated by Δ9-desaturase. The rates of desaturation of the *t*8-, *t*9- and *t*10-isomers are vey low, whereas the rates increased as the double bond position moved toward either end of the molecule reaching maxima for *t*5- and *t*13-18:1. The products formed are mainly *c,c*-18:2 acid from *t*5-18:1 and mixtures of *c,t*- and *c,c*-18:2 acids from the *t*4-, *t*6-, *t*7-, *t*11-, *t*12-, *t*13-, *t*14- and *t*15-isomers. In a similar study, Pollard et al. also found a series of *t*-18:1 acids to be Δ9-desaturated in liver microsomal systems (12). Their incubations were done with nonsaturating amounts of substrate and over a prolonged time (1 hr vs 20 min). Consequently, the rates of conversion they report are much higher than reported by Mahfouz et al. Pollard et al. also showed that, in some instances, the Δ5- and Δ6-desaturases are operative on the *t*-18:1 isomers and that some of the unusual desaturase products formed were further desaturated by Δ6-desaturase to polyunsaturated acids of unusual structure.

In this study, the same incubation conditions were used as previously reported (11), except for the use of 4 mg microsomal protein/incubation tube instead of 2 mg protein to achieve higher incorporation of labeled material into lipid classes. At this higher level of microsomal protein, the percentages of *t*-18:1 acids converted were higher, whereas the rates of conversion in terms of nmol substrate desaturated/min/mg were less than the rates previously reported (11) under the more ideal conditions, except for *t*12. Table 1 gives the percentage conversion obtained in this study. Only those isomers giving appreciable rates of conversion were included. The ratios of *c,c*- to *c,t*-18:2 products deviated to some extent from the ratios obtained with less microsomal protein, and except for *t*6- and *t*13-18:1, the relative amounts of *c,c*-isomers were higher.

The percentage desaturation listed in Table 1, part A, were obtained with incubation systems using rat liver microsomes from EFA-deficient rats, as were results previously reported (11, 12), because EFA deficiency is known to enhance desaturase activity. To test whether normal intake of EFA would influence the proportion of products formed, the *t*5- and *t*6-18:1 isomers were incubated with microsomes prepared from rats fed a diet in which 5% corn oil was substituted for an equal amount of sucrose. The percentage conversions are given in part B of Table 1, and comparison of the results shows that the conversions are somewhat lower, as would be expected (25). The data also demonstrate that the lessened activity is exclusively due to a decrease in the isomerization to *c,c*-18:2 isomers. That is, in the absence of adequate supplies of EFA, the

isomerization of *c,t*-isomers to *c,c*-isomers is enhanced, providing increased proportions of unusual isomers of polyunsaturated acids in partial substitution for the usual ω6 acids.

Incorporation into Lipid Classes

Table 2 shows the incorporation of labeled material–substrate + products–into PL (mainly PC and PE), TAG, CE and FFA. The results showed an overall incorporation of radioactive fatty acids varying from 50.5% for *t*14-18:1 to 81.0% for *t*6-18:1. There were differences in the incorporation with different preparations of microsomes, but these differences did not change the patterns shown in Table 2. For each isomer investigated, except *t*11- and *t*14-18:1, incorporations into lipid classes were of the order: PL = CE > TAG. For the *t*11- and *t*14-isomers, the incorporation into PL was much lower than incorporation into CE. This pattern of incorporation may not so much have reflected substrate preference by the acyltransferases as it reflected the availability of receptor molecules in the microsomal preparations.

In incubations of *t*5-18:1, the results obtained with microsomes from rats fed corn oil and from EFA-deficient rats were very similar (Table 2, part B), whereas when *t*6-18:1 was substrate, the PL and TAG fractions were enriched at the expense of CE.

TABLE 1

Desaturation of *t*-Octadecenoic Acids to *c,t*- and *c,t*-Octadecadienoates by Rat Liver Microsomes[a]

t-18:1 Isomer	% Conversion		
	c,c-18:2	*c,c*-18:2	*c,c*- + *c,t*-18:2
A EFA-deficient rats			
Δ4	32.4 ± 2.8	7.6 ± 0.4	40.0 ± 2.7
Δ5	48.2 ± 0.9	4.5 ± 0.3	52.6 ± 0.8
Δ6	18.9 ± 1.1	20.4 ± 1.4	39.2 ± 1.1
Δ11	10.3 ± 0.3	7.6 ± 0.2	17.9 ± 0.2
Δ12	18.4 ± 1.1	8.8 ± 0.7	27.2 ± 1.4
Δ13	12.5 ± 1.1	13.1 ± 0.5	25.6 ± 0.8
Δ14	8.7 ± 1.2	8.1 ± 0.5	16.8 ± 0.8
B EFA-supplemented rats			
Δ5	39.7 ± 1.4	4.4 ± 1.0	44.0 ± 1.7
Δ6	12.9 ± 1.6	20.9 ± 1.5	33.7 ± 0.4

[a]Values are means and SD (n=6).

TABLE 2

Distribution of Radioactive *t*-18:1 Substrates Plus Their Products between Acyl Lipids and Free Fatty Acids in Rat Liver Microsomes[a]

t-18:1 Isomer	Radioactivity (%)[b]				
	PL	TAG	CE	Total	FFA
A EFA-deficient rats					
Δ4	27.2 ± 1.0	7.4 ± 1.7	17.4 ± 2.1	51.9 ± 1.3	40.5 ± 1.4
Δ5	29.5 ± 1.1	13.8 ± 0.5	28.7 ± 1.2	72.0 ± 0.7	19.1 ± 0.4
Δ6	34.8 ± 0.8	12.0 ± 1.6	34.2 ± 2.2	81.0 ± 0.5	11.5 ± 0.5
Δ11	15.6 ± 0.9	16.9 ± 0.6	39.5 ± 1.6	71.9 ± 1.4	32.6 ± 1.2
Δ12	29.2 ± 0.9	6.5 ± 0.7	31.3 ± 0.8	67.0 ± 0.9	19.0 ± 0.4
Δ13	24.9 ± 1.7	16.9 ± 2.8	21.5 ± 4.2	63.3 ± 1.4	32.9 ± 1.5
Δ14	13.9 ± 3.1	8.5 ± 0.4	28.1 ± 0.5	50.5 ± 2.4	46.0 ± 2.2
B EFA-supplemented rats					
Δ5	29.2 ± 0.9	10.9 ± 1.3	28.7 ± 1.6	68.8 ± 0.4	20.6 ± 0.5
Δ6	40.4 ± 3.1	20.0 ± 2.3	19.0 ± 3.8	79.4 ± 1.0	10.0 ± 0.6

[a]Values are means and SD (n=6).

[b]PL, phospholipids; TAG, triacylglycerols; CE, cholesteryl esters; total, PL + TAG + CE; FFA, free fatty acids.

Fatty Acid Composition of Lipid Classes

While Table 2 shows the insertion into lipid classes of both the substrate and its products, a more detailed analysis of the composition of labeled material of each of the major lipid classes was made to locate the isomers in the lipid classes. Figures 1, 2 and 3 show the incorporation into PL, TAG, CE and FFA of *c,c*-18:2 products, *c,t*-18:2 products and *t*-18:1 substrates, respectively. For each comparison, the results have been expressed as the discrimination index, which is the ratio of the percentage of a given isomer in a lipid class to the percentage of that isomer produced in the incubation. For the *t*-18:1 substrates, the discrimination index is the ratio of the percentage of the isomer in a lipid class to the percentage of the unconverted substrate remaining after the incubation was calculated. Values below 1.0 indicate discrimination against the isomer and values above 1.0 indicate discrimination for, or accumulation of, the isomer.

Figure 1 shows the pattern of incorporation of *c,c*-18:2 products into lipid classes. For comparison, the relative amounts of *c,c*-18:2 isomers not incorporated (i.e., the FFA fraction) also are shown. It appears from that figure that the PL, mainly composed of PC and PE, is enriched in the *c,c*-isomers for each acid assayed, except for the *c,c*-products formed from desaturation of *t*11- and *t*13-18:1. This indicates that these unusual octadecadienoic acids (and the common *c*9,*c*12-18:2) are good substrates for the acyl-CoA and phospholipid transferases, in agreement with reports in the literature (25). For the products from *t*11, the FFA remained most radioactive, indicating that *c*9,*c*11-18:2 is a relatively poor substrate for the acyltransferases. The reason for this may be found in the conjugated double bond structure distinguishing this molecule from the other isomeric products in which the double bonds are separated by one or more methylene groups.

CE were enriched only by *c*4,*c*9-18:2, in agreement with the observation of Sgoutas that fatty acyl CoA esters are esterified with cholesterol in vitro in the order: *cis*-monounsaturated > saturated = *trans*-monounsaturated > polyunsaturated (21).

TAG were enriched in the propylene-interrupted *c,c*-acids, *c*4,*c*9- and *c*9,*c*14-18:2. As the double bonds moved together, the relative amounts of the *c,c*-isomers incorporated into TAG decreased. For the FFA, the opposite pattern was found. The conjugated *c*9,*c*11-isomer was found at highest levels in the FFA, whereas the amounts of free *c,c*-18:2 acids decreased as the double bonds moved apart. In interpreting these results, it must be remembered that the incorporation patterns shown in Figures 1, 2 and 3 are the results of complex, multi-step reactions, in which the substrate is competing with the products generated and with microsomal fatty acids for a variety of acyltransferases.

The pattern of insertion of *c,t*-18:2 products into lipid classes is shown in Figure 2. The percentage conversion of *t*5-18:1 to *t*5,*c*9-18:1 was too low to allow accurate measurement of the incorporation into lipid classes, and consequently, this isomer was not included in this study. Only the *c*9,*t*12-, the *c*9,*t*14- and to a lesser degree, the *c*9,*t*13-isomers were enriched in the PL. Thus, as a group, the *c,t*-isomers are relatively poor substrates for incorporation into PL. On the other hand, the CE fraction showed enrichment in each isomer except *c*9,*t*12-18:2. This suggests that the *c,t*-18:2 isomers are treated by the acyl CoA-cholesterol-O-acyltransferase as if they were monounsaturated

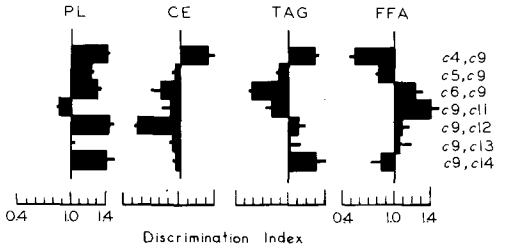

FIG. 1. Incorporation of *c,c*-18:2 products into PL, TAG, CE and FFA. The results are expressed as ratios of the % of the label in a lipid class present as *c,c*-18:2 to the % of labeled *c,c*-18:2 in the total labeled acids in the incubation. Mean ± SD for each isomer is shown (n=6). Bars to the left indicate discrimination against those isomers, and bars to the right indicate discrimination for those isomers.

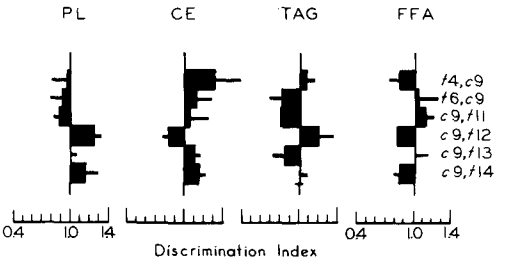

FIG. 2. Incorporation of *c,t*-18:2 products into PL, TAG, CE and FFA. The results are expressed as ratios of the % of the label in a lipid class present as *c,t*-18:2 to the % of labeled *c,t*-18:2 in the total labeled acids in the incubation. Mean ± SD for each isomer is shown (n=6). Bars to the left indicate discrimination against those isomers, and bars to the right indicate discrimination for those isomers.

fatty acids with *cis* double bonds at the 9-position (21). The *c*9,*t*12-isomer was enriched in the TAG fraction and in the PL. The *t*4,*c*9-isomer was enriched in the TAG.

Incorporation of the *t*-18:1 substrates themselves, shown in Figure 3, indicated they were relatively poor substrates for incorporation into PL. The *t*4-18:1 was a poor substrate for incorporation into TAG and CE, too, and was consequently enriched in the FFA. Patterns of incorporation showed only minor deviations from the mean for the other isomers.

When *t*5-18:1 was incubated with microsomes prepared from rats fed a diet supplemented with corn oil, the distribution of substrate and products of the lipid classes showed a pattern (not shown here) that was virtually identical to the pattern for that isomer incubated with microsomes from EFA-deficient rats. This indicates that the incorporation patterns depicted in Figures 1, 2 and 3 probably may not be significantly changed by the higher levels of linoleic acid and polyunsaturated members of the linoleic acid family in the microsomes.

Position Specificity in the Incorporation into Phospholipids

The distribution of total labeled acids between PC and PE for each *t*-18:1 isomer, plus its metabolic products, is given in Table 3. In each case, the incorporation into PC exceeded that into PE by about a factor of 2, perhaps because the amount of PC exceeds the amount of PE in the microsomes. The PC and PE fractions were separated from the crude lipid extract and each was treated with phospholipase A_2. The isomeric distribution of the fatty acids esterified to the 1- and 2-positions of PC and PE, respectively, was determined following $AgNO_3$-TLC, and the radioactivities of the fractions were counted. The patterns of incorporation of *c,c*-18:2 products are shown in Table 4, *c,t*-18:2 products in Table 5 and the *t*-18:1 substrates in Table 6. The results are tabulated as ratios of the percentage of the isomer found in a given position to the percentage conversion for the isomer.

Table 4 shows the insertion of *c,c*-products into PC and PE. Except for the *c*9,*c*11- and the *c*9,*c*12-isomers, the incorporation of these products into the 2-position of PC was strongly favored. As already indicated, the *c*9,*c*11-isomer was a poor substrate for incorporation into PL, probably due to its conjugated double bonds (8). It did not show preference for the 1- or 2-positions of PC, and it showed only moderate preference for the 2-position of PE. The *c*9,*c*12-isomer (linoleic acid) rather surprisingly showed enrichment in the 1-position of PC and only moderate enrichment in the 2-position of PE. From literature data on the insertion of linoleic acid into PL in vitro (26) enrichment in the 2-position would have been expected, and the reason for preference for the 1-position of PC is obscure at present, perhaps related to the mechanism of isomerization by which it was produced. Linoleic acid formed from 12-18:1 thus is not found in the usual location of linoleic acid in PL, and enters another metabolic pool.

The lower portion of Table 4 (part B) gives the results of incorporation of the *c*5,*c*9- and *c*6,*c*9-isomers into PC using liver microsomes from rats fed a corn-oil-supplemented diet. The data agreed with the data obtained for the *c,c*-products of 5*t*- and 6*t*-18:1 acids incubated with microsomes from EFA-deficient rats, indicating that the pattern observed in these experiments was not altered by the presence of linoleic acid and other ω6 acids in the concentrations found in microsomes from non-EFA-deficient rats. The results shown in Table 4 are

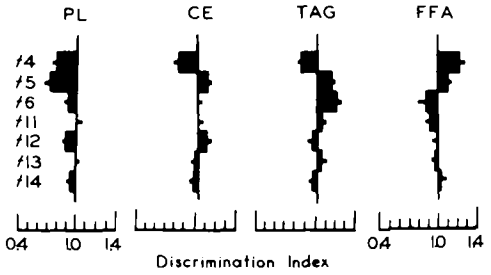

FIG. 3. Incorporation of *t*-18:1 substrates into PL, TAG, CE and FFA. The results are expressed as ratios of the % of the label in a lipid class present as *t*-18:1 substrate to the % of the substrate remaining in the incubated mixture. Mean ± SD for each isomer is shown (n=6). Bars to the left indicate discrimination against those isomers, and bars to the right indicate discrimination for those isomers.

TABLE 3

Distribution of Radioactive *t*-18:1 Substrates Plus Their Products between PC and PE[a]

t-18:1 Isomer	Total radioactivity (%)	
	PC	PE
Δ4	16.0 ± 1.4	8.1 ± 0.2
Δ5	18.1 ± 1.6	7.5 ± 1.2
Δ6	23.6 ± 4.3	9.1 ± 1.7
Δ11	8.3 ± 0.3	3.8 ± 0.3
Δ12	17.3 ± 0.1	11.4 ± 0.1
Δ13	17.6 ± 0.9	4.8 ± 0.1
Δ14	9.2 ± 0.1	2.6 ± 0.1

[a]Values are means and SD (n=6).

TABLE 4

Incorporation of *c,c*-18:2 Products into 1- and 2-Positions of PC and PE[a]

c,c-18:2 Isomer	PC		PE	
	1	2	1	2
A EFA-deficient rats				
*c*4,*c*9	0.33 ± .07	2.01 ± .03	0.37 ± .04	1.76 ± .04
*c*5,*c*9	0.39 ± .03	2.02 ± .02	0.51 ± .05	1.97 ± .02
*c*6,*c*9	0.38 ± .03	2.33 ± .05	0.41 ± .03	2.08 ± .17
*c*9,*c*11	0.69 ± .09	0.84 ± .02	0.32 ± .06	0.85 ± .09
*c*9,*c*12	1.13 ± .28	0.78 ± .01	0.82 ± .05	1.22 ± .18
*c*9,*c*13	0.37 ± .15	1.89 ± .10	0.75 ± .11	1.72 ± .08
*c*9,*c*14	0.58 ± .07	3.92 ± .17	0.91 ± .27	3.05 ± .48
B EFA-supplemented rats				
*c*5,*c*9	0.37 ± .07	2.09 ± .03	–	–
*c*6,*c*9	0.33 ± .05	2.58 ± .22	–	–

[a]The results are expressed as ratios of the percentage of the label within the 1- and 2-positions present as *c,c*-18:2 isomer to the percentage of that isomer in products and substrates after incubation. Data are means and SD (n=6).

TABLE 5

Incorporation of *c,t*-18:2 Products into 1- and 2-Positions of PC and PE[a]

c,t-18:2 Isomer	PC		PE	
	1	2	1	2
A EFA-deficient rats				
*t*4,*c*9	0.39 ± .38	0.85 ± .16	0.20 ± .11	0.95 ± .16
*t*6,*c*9	0.18 ± .01	1.43 ± .09	0.16 ± .02	1.74 ± .22
*c*9,*t*11	0.45 ± .08	0.65 ± .06	0.35 ± .01	0.66 ± .22
*c*9,*t*12	0.69 ± .22	1.40 ± .03	0.57 ± .11	1.96 ± .18
*c*9,*t*13	0.18 ± .05	2.19 ± .02	0.23 ± .04	1.71 ± .09
*c*9,*t*14	0.17 ± .02	1.71 ± .10	–	1.24 ± .35
B EFA-supplemented rats				
*t*5,*c*9	0.45 ± .02	0.30 ± .05	–	–
*t*6,*c*9	0.33 ± .04	1.84 ± .14	–	–

[a]Results are expressed as ratios of the percentage of the label in the 1- and 2-positions present as *c,t*-18:2 to the percentage of that isomer present in the labeled acids after incubation. Data are mean and SD (n=6).

generally in agreement with results reported by Lands et al. (27) and Okuyama et al. (22) that the transfer to position 2 of the PL is favorable for fatty acids with double bonds at positions 5, 9 and 12.

Incorporation of *c,t*-18:2 isomers into PC and PE is expected to be guided mainly by the *c*9-double bond present in the molecules, and hence enrichment in the 2-position is expected. The results in Table 5 clearly demonstrate that incorporation into the 2-position was indeed favored for these substrates, with the exception of the conjugated *c*9,*t*11-isomer. The conversion rate for *t*5-18:1 to *t*5,*c*9-18:2 was too low to allow accurate determination of the specificities of this isomer and it is not included in Table 5. The *t*6,*c*9-18:2 isomer generated from *t*6-18:1 employing non-EFA-deficient microsomes (part B, Table 5) showed incorporation patterns consistent with the results obtained with microsomes from EFA-deficient rats.

Table 6 shows insertion of the *t*-18:1 substrates themselves into PC and PE. The isomers were enriched in the 1-position, as expected, except for the *t*11 and *t*12-isomers, which showed no preferences for the 1- or 2-positions. Okuyama et al. studied the insertion of *t*-monoenoic acids into PL in vitro (22), and they found them to be generally good substrates for the acyltransferases with insertion

TABLE 6

Incorporation of *t*-18:1 Substrates into 1- and 2-Positions of PC and PE[a]

t-18:1 Isomer	PC		PE	
	1	2	1	2
A EFA-deficient rats				
*t*4	1.56 ± .10	0.31 ± .07	1.56 ± .02	0.47 ± .04
*t*5	1.53 ± .02	0.22 ± .02	1.44 ± .02	0.27 ± .02
*t*6	1.48 ± .01	0.35 ± .07	1.48 ± .02	0.36 ± .02
*t*11	1.14 ± .03	1.08 ± .01	1.25 ± .02	1.08 ± .05
*t*12	1.01 ± .07	1.00 ± .01	1.07 ± .01	0.88 ± .04
*t*13	1.30 ± .04	0.57 ± .02	1.22 ± .03	0.71 ± .01
*t*14	1.14 ± .01	0.56 ± .03	1.11 ± .03	0.71 ± .10
B EFA-supplemented rats				
*t*5	1.49 ± .04	0.28 ± .02	–	–
*t*6	1.34 ± .02	0.43 ± .10	–	–

[a]Results are expressed as ratios of the percentage of the label in the 1- and 2-positions present as *t*-18:1 substrate to the percentage of substrate in the total labeled acids. Data are means and SD (n=6).

into the 1-position favored. Furthermore, they demonstrated sensitivity to configurational differences in the acids governing the transfer rates to the 1-position. A more detailed comparison with the data of Okuyama et al. is not possible because the experimental conditions were not the same.

The incorporation of *t*5- and *t*6-isomers into PC in incubation systems with liver microsomes from rats fed corn oil were not significantly different from the incorporations with microsomes prepared from EFA-deficient rats.

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Phospholipid Synthesis in Human Embryo Fibroblasts Infected with Herpes Simplex Virus Type 2

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ABSTRACT

The effect of herpes simplex virus type 2 infection on the synthesis of phospholipids in human embryo fibroblasts was determined at temperatures permissive (35 C) or nonpermissive (42 C) for virus replication. Incorporation of [^{32}P]i was decreased by herpes simplex virus type 2 infection after 6 hr, which corresponds to the time of initiation of progeny virus production. No differences were observed in the relative incorporation of [^{32}P]i into phospholipid classes. In another series of experiments, cells were labeled with [^{3}H]ethanolamine before infection and with [^{14}C]ethanolamine after infection. The incorporation of [^{14}C]ethanolamine was also decreased after 6 hr of infection. When choline was substituted for ethanolamine, a similar, although less pronounced, decrease in incorporation was seen in infected cells compared to mock-infected cells. During abortive infection at 42 C, incorporation of [^{3}H]thymidine into cellular DNA was stimulated, but the incorporation of phospholipid precursors was decreased. Total phospholipid composition and phospholipid acyl group composition were not changed appreciably during abortive or productive infection, regardless of whether the cells were labeled before or after infection. In conclusion, these data indicated that, during herpes simplex virus type 2 infection, the incorporation of lipid prescursors into phospholipid was decreased. The stimulation of cellular DNA synthesis previously observed during abortive infection at 42 C was not paralleled by a detectable stimulation of total phospholipid synthesis. Neither productive nor abortive infection resulted in significant phospholipid compositional changes in the host cell; however, both resulted in a marked inhibition of phospholipid synthesis.

INTRODUCTION

Although many studies have thoroughly detailed the decrease of host cell protein and nucleic acid synthesis caused by herpes viruses (1), there have been few studies of the changes in phospholipid metabolism caused by herpes virus infection. Asher et al. (2) found that herpes virus infection of BSC_1 cells did not cause a change in the rate of incorporation of choline into lipids and did not change the relative incorporation into phosphatidylcholine (PC) or sphingomyelin. On the other hand, Ben-Porat and Kaplan (3) reported that pseudorabies virus infection of rabbit kidney cells causes a slight increase in the incorporation of [^{32}P]i, [^{3}H]myo-inositol and [^{3}H]choline into the total phospholipid pool (1.18-, 1.29- and 1.18-fold, respectively). These authors also found that, when infected cells were labeled with [^{3}H]choline or [^{32}P]i between 3 and 10 hr postinfection, they contained 2-3 times more label in sphingomyelin than in uninfected cells; however, when the cells were labeled before infection, there was no difference in the amount of label in sphingomyelin (4). This suggested that the infection by herpes viruses had little effect on the synthesis of the glycerolipids; rather, infection stimulated sphingomyelin synthesis. Since these few reports suggest that phosphoglyceride synthesis is not affected by viral infection at a time when cellular protein synthesis was decreased, we decided to investigate this problem further by studying herpes simplex virus type 2 (HSV-2) infected human embryo fibroblasts (HEF). Further, we did these studies under conditions that lead to productive (37 C) and abortive infections (42 C). Nondefective HSV-2 infection, at supraoptimal temperature (42 C), results in the synthesis of viral-specific polypeptides (5) but does not result in progeny virus production or cell death (6). However, the HSV-2 infected cultures incubated at supraoptimal temperatures are stimulated to incorporate increased amounts of [^{3}H]thymidine into cellular DNA (5,7,8) and assume some of the properties of transformed cells (6,9).

We have previously described the pathways by which HEF synthesize lipids (10). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are synthesized from their base precursors and PE is methylated to yield PC. To determine the effects of productive or abortive HSV-2 infection on these key metabolic pathways, we have monitored the labeled phospholipids of cells incubated with [^{3}H]-labeled precursors before infection and with the [^{14}C]-labeled

Abbreviations: HSV-2, herpes simplex virus, type 2; HEF, human embryo fibroblasts; MEM, minimum essential medium; TS, Tris-buffered saline; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; S, sphingomyelin.

precursors after infection. By these methods, the rate of degradation of phospholipids can be compared to the rate of incorporation of phospholipid precursors. In addition, we have used [^{32}P]i to contrast the synthesis of phospholipids in HSV-2 infected and mock-infected cells.

EXPERIMENTAL PROCEDURES

Virus and Cell Culture

Secondary cultures of HEF were purchased from Flow Laboratories, Rockville, MD, and used between passages 12 and 20 as previously described (8). Growth medium consisted of Eagle's minimum essential medium (MEM) with Hank's base salts (Flow Laboratories, Rockville, MD) supplemented with 10% fetal calf serum, 10% tryptose phosphate broth, 0.22% $NaHCO_3$, 2 mM L-glutamine, 100 U of penicillin/mL and 100 μg of streptomycin/mL of medium.

HSV-2 strain ANG was obtained from L. Falk, University of Chicago, Chicago, IL. The stocks of virus were prepared in HEF as previously described (8). The virus was typed by analysis of restriction enzyme patterns of purified DNA by Bernard Roizman (University of Chicago) and by type-specific immunofluorescence tests by Richard Courtney (University of Tennessee). Viral infectivity was measured by plaque assay in HEF. Cell cultures and virus stocks were tested and found negative for mycoplasmal contamination.

Radioisotopic Labeling of Cells

HEF were incubated in 60 mm plastic cell culture dishes for 48 hr during logarithmic growth phase with 10 μCi/mL of [^{3}H]ethanolamine, 3.8 Ci/mmol, followed by incubation in MEM without fetal calf serum for 24 hr to deplete unincorporated labeled ethanolamine. The medium was then removed and the monolayers were rinsed with Tris-buffered saline (TS), 25 mM Tris, 0.85% saline, pH 7.4. Cells were then infected at a multiplicity of infection of 2 plaque-forming units per cell or mock-infected with an equal volume of TS. After 1 hr of virus absorption at 35 C, the cells were rinsed with TS and re-fed with MEM containing 1 μCi/mL [^{14}C]ethanolamine, 44 mCi/mmol. In some experiments, radioactive choline was substituted for ethanolamine ([^{3}H]choline, 10 μCi/mL, 15 Ci/mmol; [^{14}C]choline, 1 μCi/mL, 60 mCi/mmol). When [^{32}P]i was used to label cells, it was added at 1 μCi/mL in MEM. Radioisotopes were purchased from Amersham/Searle, Arlington Heights, IL. Although the same number of cells was planted in each experiment, the absolute amount of precursor incorporation varied due to cell growth rate among experiments and did not allow averaging data from several experiments. The data are therefore presented as the mean of values from 1 of 5 similar experiments.

Abortive HSV-2 Infection

Conditions of abortive infection were as previously described (5). Briefly, the cells were incubated for 24 hr preinfection with 20 μg of 5-flourouracil (Sigma Chemical Co., St. Louis, MO) and 1 μg of thymidine/mL of growth medium at 35 C. The conditions of infection were identical to those already described except that the cells were incubated at 42 C after 1 hr of virus attachment at 35 C.

Cell Harvest and Lipid Analysis

Cells were harvested by scraping the cells from the plate directly into methanol and extracted by the method of Bligh and Dyer (11). Aliquots of the $CHCl_3$ extract were counted to determine total radioactive precursor incorporation into lipid. Phospholipids were analyzed by 2-dimensional thin layer chromatography (TLC) on Silica Gel H plates. The first solvent system consisted of chloroform/methanol/water (87:31:5, v/v) and the second solvent system was butanol/acetic acid/water (80:26:26, v/v). The lipids were visualized by staining with I_2 vapor, and following evaporation of the I_2 the silica gel containing the lipid was scraped into a scintillation vial and counted using a toluene/Triton X-100/water (2:1:0.2, v/v) scintillation cocktail. These techniques yielded results in which the distribution of radiolabel among compounds in duplicate samples varied by less than 10%. Phospholipid phosphorus was determined by the method of Chalvardjian and Rudnicki (12).

Phospholipids were separated from neutral lipids by chromatography on columns of Unisil, Clarkson Chemical Company, Williamsport, PA (ca. 10 cm in a Pasteur pipet plugged with glass wool) (13). Neutral lipids were eluted with 10 mL of chloroform and phospholipids were eluted with 10 mL of methanol.

Methyl esters of fatty acids in the phospholipid fraction were prepared by transmethylation with methanol/sulfuric acid (14), and were identified by gas liquid chromatography (GLC) as described by Waite et al. (15).

RESULTS

Time Course of Infection

In order to correlate virus replication with changes in cellular phospholipid synthesis,

cultures were prepared as in the labeling experiments, infected with 2 infectious virions/cell and the cells plus extracellular fluids were monitored for total viral yield by plaque assay in HEF (Fig. 1). Infectious virus was synthesized within 3 hr after infection, and there was a logarithmic increase in virus production from 3 to 24 hr after infection. Typical viral cytopathology was first evident at 12-18 hr postinfection and there was extensive morphological changes and multinucleate cell formation at 24 hr postinfection. The yield of virus at 24 hr postinfection was ca. 100 virions/cell. Approximately 50% of the virus produced under these conditions of infection was released from the cells into the extracellular fluids (data not shown).

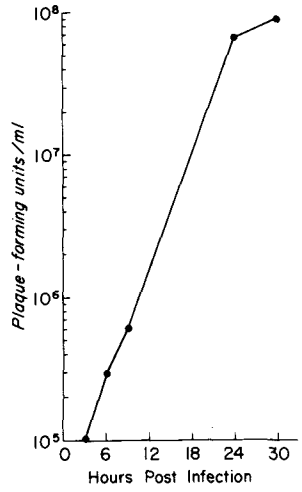

FIG. 1. Total HSV-2 synthesis in HEF infected at a multiplicity of 2 PFU/cell. At the times indicated, cells were scraped from the culture surface, sonicated, and the infectious virus contained in the cells plus culture medium was assayed by plaque count in HEF cells (●—●).

Effects of Productive Infection on the Incorporation of Lipid Precursors

To determine differences in the de novo synthesis of phospholipid in HSV-2 infected and mock-infected cells, HEF were infected as already described or mock-infected and the cells were incubated in medium containing 1 μCi/mL [^{32}P]i. At various times after infection, the cells were scraped from the plate, collected by centrifugation, and the lipids extracted and analyzed. In both infected and uninfected cells, ca. 3.0% of the total label was cell-associated, indicating that infected cells transported [^{32}P]i into the cells at the same rate as uninfected cells. However, after 3-4 hr postinfection, the infected cells incorporated less [^{32}P]i into phospholipids than uninfected cells (Fig. 2). Cells infected with 10 virions/cell incorporated slightly less [^{32}P]i than the cells infected with 2 virions/cell (Fig. 2). This decreased incorporation was evident when the results were presented as [^{32}P]i cpm/culture as in Figure 2 or as [^{32}P]i/mol phospholipid phosphorus which was lower in the infected cultures compared to the mock-infected cultures at later times after infection. The specific activities at 24 hr for m.o.i. of 0, 2 and 10 were 1482.9 ± 96.0, 867.6 ± 42.7 and 821.6 ± 92.0 cpm [^{32}P]i/nmol of phospholipid, respectively. Together, these data indicate that the decreased incorporation of [^{32}P]i is not due to the loss of cell mass during infection but rather a decrease of phospholipid synthesis. Incorporation of [^{32}P]i into all classes of phospholipids was decreased by HSV-2 infection; however, there was no change in the relative incorporation into phospholipid classes due to virus infection (Fig. 3). Incorporation of [^{32}P]i into phosphatidylinositol (PI) plus phosphatidylserine (PS) fraction was rapid in both infected and uninfected cells, accounting for about 60% of the

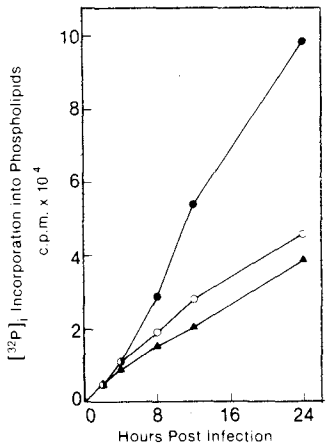

FIG. 2. Incorporation of [^{32}P]i into phospholipids of infected (moi = 2, ○—○; moi = 10, ▲—▲) and mock-infected cells (●—●). The HEF cells were infected with HSV-2 (moi 0, 2 or 10) and incubated with [^{32}P]i 1 μCi/mL for the times indicated. The cells were harvested and analyzed as described in Experimental Procedures. Results are expressed as the average of duplicates from 1 of 3 similar experiments. Standard deviation was less than 5%.

label at 2 hr. This decreased with time, approaching 25% at 12 hr. The rapid incorporation into PI + PS probably is due to the increase in PI turnover resulting from serum stimulation when the cells are re-fed following infection or mock-infection. Concomitant with the decrease in the percentage of PI + PS was an increase in

PE; PC remained relatively constant throughout the experiment.

A double isotopic labeling technique was used to differentiate synthesis of phospholipids before or after viral infection. HEF incorporated ethanolamine into PE and PC by the sequential methylation of PE (10). Thus, 2 important pathways in phospholipid synthesis could be measured. HSV-2 infection caused slightly increased degradation of [^{3}H]ethanolamine-labeled phospholipids (Fig. 4) which was consistently observed in all experiments. The incorporation of [^{14}C]ethanolamine into phospholipids by infected cells was markedly decreased between 12 and 24 hr postinfection (Fig. 4). The degradation of [^{3}H]labeled phospholipids and the de novo synthesis of [^{14}C]-labeled phospholipids resulted in a decrease in the ratio of [^{3}H] to [^{14}C] after the replacement of [^{3}H]ethanolamine by [^{14}C]ethanolamine. The decrease in preincorporated label at 3-4 hr postinfection was consistently observed in both infected and mock-infected cells and probably is due to a stimulated turnover and reincorporation of phospholipid headgroups resulting from serum addition.

When cells were prelabeled with choline, the degradation of labeled phospholipids after infection was slower than when ethanolamine was used as the precursor. Infection by HSV-2 decreased the incorporation of [^{14}C]choline added after infection. The greatest difference in incorporation into mock-infected cells and infected cells was after the first 6 hr of infection; thus, the decreased incorporation of precursors appears to parallel the start of infectious virus production (cf. Figs. 1 and 5). Although these results are similar to those obtained with [^{14}C]ethanolamine, the decrease in [^{14}C]choline incorporation resulting from HSV-2 infection appears to be less pronounced. This difference between the synthesis of PE and PC by infected cells was not found when [^{32}P]i incorporation was measured (Fig. 2). At present, the reason for the quantitative difference noted using [^{32}P]i and [^{14}C] base is unresolved. However, it does not appear to be a transport phenomenon since, as found using [^{32}P]i, the transport of choline and ethanolamine into the cells was not a limiting factor for incorporation based on the observation that the pools of unincorporated label in infected and mock-infected cells were similar (data not shown).

Effects of Abortive Infection on Phospholipid Precursor Incorporation

Cultures of HEF were prepared as in the

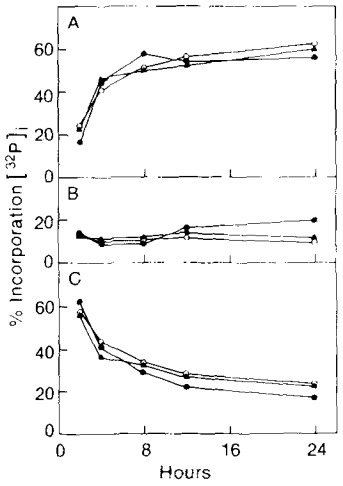

FIG. 3. Phospholipid distribution of [^{32}P]i in cells labeled at the time of infection with [^{32}P]i as in Fig. 2. At the times indicated, the cells were harvested and analyzed as described in Experimental Procedures. Mock-infected (●—●); infected, moi = 2 (▲--▲); infected, moi = 10 (○—○). Results are expressed as percent of total phospholipid [^{32}P]i in PE, A; PC, B; PS + PI, C.

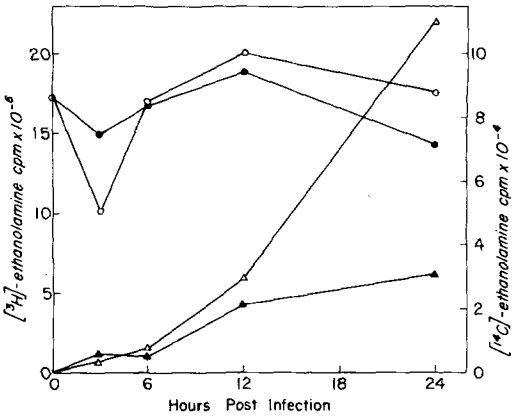

FIG. 4. Degradation of ethanolamine-containing phospholipid synthesized before HSV-2 infection and de novo synthesis after productive infection at 35 C. HEF cells were labeled 48-24 hr preinfection with [^{3}H]ethanolamine (10 μCi/mL, 3.8 Ci/mmol) then incubated from 24 to 0 hr preinfection in nonradioactive media to deplete the endogenous pool of [^{3}H]ethanolamine. The cells were then infected with 2 PFU/cell of HSV-2. During virus infection at 35 C, the cells were labeled with [^{14}C]ethanolamine (1 μCi/mL, 44 mCi/mmol). At the indicated times, the cells were trypsinized and pelleted by low-speed centrifugation and the precursor incorporation into phospholipid was measured (see Experimental Procedures). [^{3}H]Ethanolamine, infected ●—●, mock-infected ○—○; [^{14}C]ethanolamine, infected ▲—▲, mock infected △—△. Results are the average of duplicate determinations from 1 of 5 similar experiments.

previous experiments except that the cells were incubated with 5-fluorouracil and thymidine for 24 hr before infection to arrest cells in DNA synthesis and incubated at 42 C after infection. Under these conditions, cellular DNA synthesis was stimulated by virus infection, but virus-specific DNA synthesis was decreased. When compared to mock-infection, abortive infection decreased the incorporation of phospholipid precursors (phosphate, ethanolamine and chline) (data not shown) and was similar to the decrease caused by productive infection (see Figs. 2, 4 and 5).

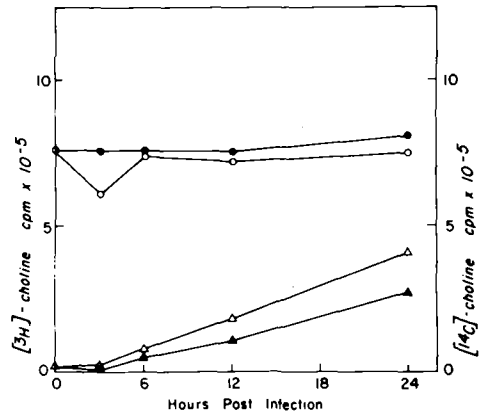

FIG. 5. Degradation of choline-containing phospholipids synthesized before infection, and de novo synthesis after infection. The experiments were performed as described in the legend to Fig. 4, except that [^{3}H]choline (10 μCi/mL, 15 Ci/mmol) was used before infection and [^{14}C]choline (1 μCi/mL, 60 mCi/mmol) was used after infection. [^{3}H]Choline, infected ●—●, mock-infected ○—○; [^{14}C]choline, infected ▲—▲, mock-infected △—△. Results are the average of duplicate determinations from 1 of 5 similar experiments.

Phospholipid Composition of Productively and Abortively Infected Cells

In both infected and mock-infected cells, ethanolamine was incorporated predominantly into PE with little methylation to form PC (Table 1). There was a small accumulation of phosphatidyl-N-monomethylethanolamine, but the methylation pathway does not appear to be a significant source of PC. Choline was incorporated predominantly into PC with some incorporation into sphingomyelin (Table 1). All the compositional data presented were derived from cultures harvested at 24 hr postinfection; in other experiments not presented, we found that harvest time did not change the labeling pattern as presented in Table 1. The slight shift in the incorporation of [^{3}H]choline into PC rather than sphingomyelin was not statistically significant, and no compositional differences were observed in cells labeled before or after infection.

Further experiments were designed to determine whether the total phospholipid distribution changed as a result of productive or abortive infection. The cells were grown in the presence of [^{32}P]i continuously 24 hr before and after infection, and the phospholipids and fatty acids were analyzed at 24 hr postinfection. At both 35 C (productive infection) and at 42 C (abortive infection) (Table 2), the differences in phospholipid composition were slight. Total phospholipid synthesis as measured by [^{32}P]i incorporation into phospholipids was depressed by both productive and abortive infections.

TABLE 1

Incorporation of Labeled Precursors into Phospholipids of Human Embryo Fibroblasts Productively Infected with Herpes Simplex Virus Type 2 at 35 C[a]

Phospholipid	Precursor: Ethanolamine, Control	Precursor: Ethanolamine, Infected	Precursor: Choline, Control	Precursor: Choline, Infected
Phosphatidylethanolamine	89.8 ± 3.3[b]	89.0 ± 2.3	0.3 ± 0.1	0.4 ± 0.1
Phosphatidyl-N-monomethylethanolamine	8.4 ± 2.9	6.7 ± 1.9	0.0	0.0
Phosphatidyl-N-N-dimethylethanolamine	1.3 ± 0.5	0.8 ± 0.3	0.0	0.0
Phosphatidylserine + phosphatidylinositol	2.1 ± 0.3	3.3 ± 0.8	0.5 ± 0.2	0.4 ± 0.3
Phosphatidylcholine	2.6 ± 0.6	2.7 ± 1.6	73.0 ± 3.8	78.1 ± 2.9
Sphingomyelin	0.4 ± 0.1	0.6 ± 0.1	18.8 ± 2.2	14.6 ± 2.1

[a]HEF cells were grown in the presence of [^{3}H]ethanolamine or [^{3}H]choline as described in the legend to Fig. 4. At 24 hr postinfection, the cells were harvested and the lipids were extracted and analyzed by 2-dimensional TLC (see text for details).

[b]Results are presented as the percentage of total cpm in each phospholipid ± standard deviation. Results presented are the average of duplicate determinations from 3 experiments.

TABLE 2

Phospholipid Composition of Human Embryo Fibroblasts Infected with Herpes Simplex Virus Type 2[a]

Phospholipid	Incubation temperature			
	35 C		42 C	
	Control	Infected	Control	Infected
Phosphatidylethanolamine	24.5[b] ± 1.7	20.8 ± 2.05	26.7 ± 2.4	23.8 ± 7.1
Phosphatidyl-N-monomethylethanolamine	1.0 ± 0.4	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Phosphatidyl-N-N-dimethylethanolamine	0.2 ± 0.1	0.5 ± 0.5	0.2 ± 0.1	0.2 ± 0.1
Phosphatidylcholine	49.4 ± 4.0	55.3 ± 1.8	45.7 ± 0.1	49.1 ± 5.8
Phosphatidylserine + phosphatidylinositol	14.5 ± 1.2	14.0 ± 0.6	12.8 ± 3.0	13.8 ± 3.0
Sphingomyelin	7.2 ± 2.3	5.5 ± 0.7	9.8 ± 0.1	8.1 ± 0.4
Cardiolipin	1.8 ± 0.1	1.6 ± 0.6	2.1 ± 0.8	2.1 ± 1.0

[a]HEF cells were incubated for 24 hr preinfection with 20 μg of 5-fluorouracil and 1 μg of thymidine/mL. Cultures were then infected with HSV-2 (moi, 2.0) or mock-infected and incubated at 35 C or at 42 C. The cells were labeled continuously before and after infection with [^{32}P]i. At 24 hr postinfection, the cultures were harvested and the lipids were extracted and analyzed by 2-dimensional TLC (see text).

[b]Results are presented as percentage of total cpm in each compound. Each value represents the mean of 3 experiments ± standard deviation.

Phospholipid Acyl Group Composition of Productively and Abortively Infected Cells

To determine if HSV-2 caused a change in phospholipid acyl composition, cells were cultured and infected as described previously and the cultures were harvested at 24 hr post-infection. Phospholipids were separated from the lipid extract by column chromatography and the fatty acids were analyzed by GLC. The fatty acid composition was not significantly different in either productively or abortively infected cells compared to the mock-infected cells incubated at the same temperature (Table 3). In contrast with virally transformed cells (16), in general, or HSV-2 transformed cells (13), there was not a significant change in either the percentage of saturated vs percentage unsaturated or in the ratio of oleate to arachidonate in the HSV-2 infected cells.

DISCUSSION

Our results show that HSV-2 infection of HEF cells reduced the incorporation of phosphate, ethanolamine and choline into phospholipids during the time of maximal viral synthesis. Similar results have been reported by Vance and Burke (17), who observed a 50% decrease in the total activity of CDP-choline: 1,2-diglyceride choline phosphotransferase in Semliki Forest virus infected baby hamster kidney cells. However, Rauscher virus infection of BALB/c mice has been found to cause an increased activity of choline phosphotransferase in liver microsomes (18). Poliovirus also has been shown to stimulate the synthesis of phospholipids (19), even though the virus contained no lipids. Asher et al. (2) have shown that during the first 18 hr, herpes virus infected BSC_1 cells incorporate choline at a rate comparable to control cells. Ben-Porat and Kaplan (3) reported an increase in the incorporation of phospholipid precursors into pseudorabies virus infected cells compared to mock-infected cells between 2 and 9 hr post-infection. Thus, virus infection may have different effects on the various pathways of phospholipid metabolism. However, since there is little relationship between experimental designs used, no clear pattern of metabolic alteration can be assigned to any virus group.

The decrease in lipid metabolism that we have observed occurred concomitantly with the initiation of progeny virus synthesis and may be secondary to the decrease of cellular protein synthesis caused by herpes virus infection (1). The rate of degradation of ethanolamine phospholipids, but not choline phospholipids, was increased in infected cells compared to mock-infected control cells. The increased degradation of ethanolamine phosphoglycerides may be due to the increased release of lysosomal phospholipases which have been shown to hydrolyze PE at a rate twice that of PC (20). These data suggest that the decreased phospholipid synthesis postinfection may be due to a virally induced decrease in enzyme synthesis, and/or an increase in the degradation of phospholipid. The incorporation of [^{32}P]i into phospholipids was also decreased by HSV-2 infections even though there was not an appreciable decrease in the total amount of membranous phospholipid. These data differ from those presented by Asher et al. (2) and Ben-Porat and Kaplan (3), who reported no suppression of choline incorporation into

TABLE 3

Fatty Acid Composition of Human Embryo Fibroblasts Infected with Herpes Simplex Virus Type 2[a]

Cells (temperature of incubation)	Fatty acids in phospholipids (%)											
	16:0[b]	16:1	18:0	18:1	18:2	20:3	20:4	20:5	22:4	22:5	22:6	% Saturated	18:1/20:4
Control (35 C)	24.0 ± 3.2[c]	3.9 ± 1.2	19.6 ± 1.2	27.8 ± 1.8	1.5 ± 0.7	2.2 ± 1.2	10.2 ± 2.6	0.7 ± 0.5	2.1 ± 0.3	2.4 ± 0.3	2.2 ± 1.4	43.6 ± 3.7	3.0 ± 1.1
Infected (35 C)	24.0 ± 2.8	5.3 ± 0.3	19.4 ± 0.2	27.4 ± 2.1	2.3 ± 0.4	2.4 ± 1.7	11.9 ± 2.4	1.0 ± 0.5	2.8 ± 0.6	3.1 ± 0.6	3.0 ± 0.3	43.5 ± 4.5	2.4 ± 0.2
Control (42 C)	23.4 ± 2.7	3.6 ± 0.5	20.6 ± 3.3	26.2 ± 1.1	1.7 ± 0.4	2.0 ± 1.2	9.0 ± 1.7	1.8 ± 0.9	3.0 ± 0.9	2.8 ± 0.7	2.9 ± 0.8	44.0 ± 5.8	3.0 ± 0.8
Infected (42 C)	27.8 ± 6.2	3.7 ± 0.7	20.7 ± 3.4	24.8 ± 2.9	1.7 ± 0.7	1.1 ± 1.2	7.1 ± 2.0	1.7 ± 1.2	1.8 ± 1.3	2.0 ± 0.9	2.2 ± 1.1	48.4 ± 9.2	3.2 ± 0.7

[a]Phospholipids were separated from the lipid extract, obtained as described in the legend to Table 2, by chromatography on 10-cm columns of Unisil. The fatty acids in the phospholipid fraction were then hydrolyzed, methylated and analyzed by GLC as detailed in the text.

[b]Fatty acid methyl esters are denoted by chain length followed by number of double bonds. Identification was made by chromatographing standard fatty acid methyl esters in parallel to the samples.

[c]Results are presented as the mass percentage of total fatty acids (mean of 4-5 experiments ± standard deviation).

herpes simplex virus infected monkey kidney cells and pseudo-rabies virus infected rabbit kidney cells. We observed a lag time of about 12 hr before suppression of incorporation was evident. Thus, the lack of suppression observed by others may be related to the time course of infection, the difference in virus type, host cell or the decreased suppression of choline incorporation compared to ethanolamine incorporation. The differences in virus type may be especially relevant since it has recently been shown that different strains decrease cellular macromolecule synthesis with different efficiencies (21). We did not find differences in the total phospholipid composition of HSV-2 infected HEF cells whether they were productively or abortively infected. Thus, our data agree with Ben-Porat and Kaplan (4), in that no difference in sphingomyelin content was seen between the total membrane fractions of infected and uninfected cells.

Although abortive infection has been shown to cause a marked increase in host cell DNA synthesis and may lead to cell transformation (8.9), we were unable to detect a concomitant increase in phospholipid synthesis. This may be due to the low frequency of HEF transformation by HSV-2 (9). Marcon and Kucera (7) have shown that the increased DNA synthesis observed during abortive infection results from high levels of synthesis in ca. 3 to 8% of the cells in the culture. The stimulated cells may have an increased rate of lipid synthesis, but the increased synthesis may be masked by the large percentage of unstimulated cells in the culture.

In summary, HSV-2 infection suppresses phospholipid synthesis in HEF cells and the suppression is probably secondary to the virally induced inhibition of host cell protein synthesis. The suppression does not appear to be due to virus replication per se since suppression of phospholipid synthesis occurs at temperatures nonpermissive for viral replication. Infection does not abolish the ability of the host to respond to serum stimulation by a rapid turnover of PI; however, the amount of PI synthesis as well as the synthesis of the phospholipids is markedly decreased.

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Sterols and Their Biosynthesis in Some Freshwater Bivalves

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ABSTRACT

A number of free sterols and sterol esters of three freshwater mussels was separated and identified. A slow rate of biosynthesis *de novo* of sterols was demonstrated in *Anodonta cygnea*. Injected cholesterol was found to undergo esterification, oxidation, Δ^{22}-dehydrogenation and C-24 alkylation. Methyl-[^{14}C]methionine was proved to be incorporated in C-24 alkylsterols. Abnormally large amounts of cholesterol injected in *A. cygnea* were metabolized toward restoration of the normal composition of sterols. This was achieved by intensified metabolism of cholesterol, mainly by conjugation, oxidation and Δ^{22}-dehydrogenation.

INTRODUCTION

The sterol composition of marine bivalves has been investigated in more detail than that of any other class of marine invertebrates. In contrast, there are only two reports on the sterol composition of freshwater mussels, which have different living conditions and diets from marine species. The sterol compositions of *Cristaria plicata* and *Carbicula japonica* were reported in 1971 (1). Their main sterol is cholesterol (I), accompanied by small quantities of 22-dehydrocholesterol (II), 24-methylcholest-5,22-dien-3β-ol (III), 24-methylenecholesterol (IV) and C_{29}-sterols (Fig. 1). On the basis of their results, the authors concluded that 60-70% cholesterol is characteristic of sterols of freshwater mussels, whereas it comprises only 30-40% of the total sterols in marine species. The proportions of 47.4% cholesterol (2) found in *Anodonta cygnea* and 58.7% in *Mytilus edulis* (3) were in disagreement with this statement.

Investigations on sterol biosynthesis and interconversions in bivalves have given the most contradictory results of any group of invertebrates, and this has led to conflicting views concerning their ability to produce sterols *de novo* (4). The earliest studies (5,6) obtained evidence for the labeling of cholesterol from acetate. In *A. cygnea*, Voogt (2) established the incorporation of acetate in C_{27}, C_{28} and C_{29}-sterols, confirming sterol alkylation in this mussel. In other representatives of the same order (Eulamellibranchia), a higher incorporation of [2-^{14}C]acetate than that of [1-^{14}C]-acetate, and no incorporation of mevalonate, was demonstrated (2), although according to other results, mevalonate was incorporated into sterols of mussels (7,8).

Sterol alkylation in bivalves and other marine invertebrates has also been a subject of controversy. Alkylation of cholesterol to 24-methylenecholesterol was observed in *Saxidomus giganteus* (9) as well as transformation of desmosterol into 24-methylenecholesterol, cholesterol and Δ^{22}-dehydrocholesterol (10). These results, taken in conjunction with the other reports, indicate that bivalve mollusks may be the first recorded animals capable of 24-alkylation. Experiments with some other marine invertebrates (11,12), including the oyster, *Ostrea griphea* (7), indicated that methionine had not taken part in sterol alkylation. These data led to a search for another alkylating agent. In *A. cygnea* and other Eulamellibranchia (2), the utilization of acetate as a C-24-alkylating agent was proposed.

In a recent communication, we reported the

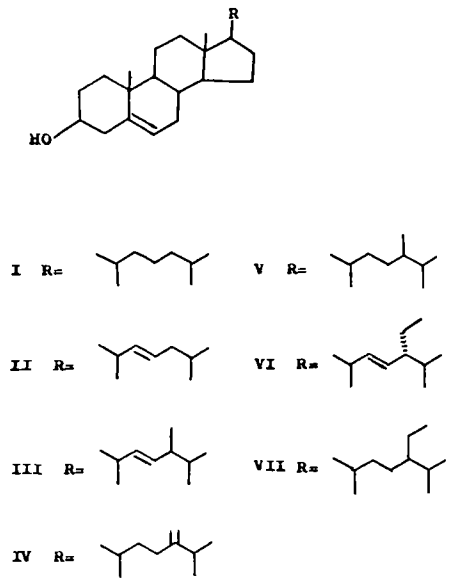

FIG. 1. Structures of sterols.

identification of 15 sterols in *Mytilus galloprovincialis* (13), a mussel occurring widespread in the Black Sea. In this paper, we report the results of similar studies on freshwater mussel sterol composition. We also expected to obtain some information about the influence of endogenous and exogenous factors on sterol composition as well as the capability of these organisms for synthesizing and transforming sterols. The experimental animals included three freshwater bivalves common in Bulgaria: *Anodonta cygnea*, *Pseudoanodonta complanata* and *Unio timidus*.

MATERIALS AND METHODS

Analytical GLC of sterols (as acetates) was performed on a Perkin-Elmer F-17 GC instrument equipped with a column containing 3% OV-17 on Chromosorb Q at 280 C. Cholesterol acetate (Merck) was used as a standard.

GC/MS analysis of the sterol mixtures was performed on MM 70-70 instrument with a 3% OV-101 column at 70 eV.

Measurement of ^{14}C radioactivity was performed using an LKB-1210 liquid scintillation counter capable of calculating the absolute radioactivity in dpm. The counter was equipped with an external standardization system which automatically corrected the quenching of the sample; background was 20 cpm.

All samples were counted to a minimum of 10,000 cpm, which warrants a counting error well below 3%.

The following labeled compounds were used in tracer experiments: [4-^{14}C] cholesterol, sp act 59 mCi/mmol, manufactured by "Izotop" (USSR), [2-^{14}C] Na acetate, sp act 4.81 mCi/mmol, manufactured by the Institute of Isotopes of the Hungarian Academy of Sciences and L-methyl-[^{14}C] methionine, sp act 1.06 mCi/mmol, manufactured by the National Atomic Energy Commission Institute of Isotopes (Hungary).

Isolation of Free Sterols (FS) and Sterols Esters (SE)

A. cygnea was collected in a freshwater pool near the city of Sofia. *U. timidus* and *P. complanata* were collected in the Danube river near the city of Rousse. The animals were fixed in methanol, minced, and extracted three times with methanol. The combined extracts were concentrated, diluted with water, and extracted three times with chloroform. The total lipids were chormatographed on a silica gel (Merck) column (1:30). Subsequent elution with petroleum ether/ethyl ether mixtures resulted in isolation of sterol esters (SE) and free sterols (FS). The SE were hydrolyzed by refluxing for 2 hr with 2 N KOH/MeOH. The FS were purified by prep TLC (Silica Gel G) and analyzed by GLC (as acetates). The amounts of the isolated free sterols and those of the sterol esters were ca. 30 mg and 14 mg per mussel.

Separation of Sterols

The following additional procedures were used for the separation of some sterols: (a) continuous TLC on Silica Gel G/$AgNO_3$ (14). Sterol acetates were separated by prep TLC on Silica Gel G containing 16% $AgNO_3$. After 48 hr continuous development with petroleum ether (bp 55-60 C)/chloroform (9:1), four zones were separated. GLC analysis indicated that zones II, III and IV contained one single sterol each. In this way, 28C$\Delta^{5,22}$(III), 27C-$\Delta^{5,22}$(II) and 29C$\Delta^{5,22}$(VI)-sterols were isolated in a pure state. Zone I was a mixture of 27CΔ^5 (I), 28CΔ^5(V) and 29CΔ^5(VII)-sterols.

(b) Separation of sterols from stanols. Sterol acetates (50 mg) were oxidized with 90 mg *m*-chloroperbenzoic acid in chloroform and the stanol acetates were separated from the epoxides of sterol acetates by prep TLC (15) and analyzed by GLC.

Tracer Experiments

Each of the water solutions (1 mL) of [2-^{14}C] sodium acetate (0.5 mCi) and L-methyl-[^{14}C] methionine (0.1 mCi) and water suspensions of [4-^{14}C] cholesterol (0.1 mCi in 1 mL) and 30 mg cholesterol (2 mL), was distributed within a pair of animals and injected in the adductor muscle. Similarly, a water suspension (3 mL) of 120 mg cholesterol mixed with [4-^{14}C] cholesterol (0.1 mCi) was distributed among three other animals. The injections were always made in the muscle of the animal in order to exclude any participation of intestinal bacteria in sterol transformations. No radioactive material was found to flow out of the injected animal tissue. After adequate incubation in pure water at 15 C, the mussels were killed and the sterols investigated as already described. The isolation and purification of free sterols was achieved by digitonin precipitation (experiments with labeled acetate) or prep TLC separation of sterols, acetylation, prep TLC and finally argentated prep TLC of sterol acetates. Sterol metabolites contained in the water phase after extraction of lipids with $CHCl_3$ were hydrolyzed by a 2-hr boiling in 1 N NaOH and the sterols were extracted with ether. The purity of each fraction was checked by analytical GLC before measuring the radioactivity and by the established constancy of radioactivity after recrystallization of sterols II, III and VI.

RESULTS AND DISCUSSION

The sterols found in the investigated mussels were identified as: Δ^{22}-cholesterol, RT = 0.92. MS: m/z 384(22%), 369(4%), 366(5%), 300 (20%), 273(16%), 272(10%), 271(15%), 255 (35%), 231(5%), 213(13%). Cholesterol, RT = 1. MS: m/z 386(30%), 371(17%), 368(18%), 353(17%), 301(17%), 275(34%), 273(11%), 255(15%), 231(12%), 213(21%). 5α-Cholestanol, RT = 1. MS (mixed with cholesterol): m/z 388, 373, 257, 233, 215. 24-Methylcholesta-5,22-dien-3β-ol, RT = 1.12. MS: m/z 398(17%), 383(3%), 380(4%), 365(3%), 300 (14%), 272(11%), 271(16%), 255(33%), 231 (7%), 213(15%). 24-Methylenecholesterol, RT = 1.22 (mixed with 24-methylcholest-5-en-3β-ol). MS: m/z 398, 314. Poriferasterol, RT = 1.34. MS: m/z 412(12%), 397(3%), 394 (4%), 300(12%), 272(13%), 271(14%), 255 (21%), 231(7%), 213(13%). 24-Ethylcholest-5-en-3β-ol, RT = 1.51. MS: m/z 414(18%), 399(10%), 396(10%), 381(8%), 329(9%), 303 (15%), 273(10%), 255(15%), 231(9%), 213 (15%). 5β-Cholestanol: isolated after elimination of unsaturated sterols through *m*-chloroperbenzoic acid oxidation of the total sterol mixture. RT = 0.82, corresponding to that of an authentic sample.

RT, MS and mobility on argentated silica gel are identical to those of authentic samples.

Stanols comprise less than 1% of the total sterol mixture of these mussels. In *A. cygnea*, collected in autumn in Bulgaria, the main stanol was 5β-cholestanol (coprostanol), accompanied by small amounts of 28C and 29C stanols. In *A. cygnea*, collected at the same location in summer, a mixture of 5α-cholestanol and 5β-cholestanol was present. In *U. timidus*, collected in the Danube river, only 5α-cholestanol was found.

Δ^{22}-Sterols were determined as *trans*-22-dehydrosterols on the basis of GLC. The precipitation of isolated sterols with digitonin confirmed the β-orientation of the 3-OH group.

The composition of FS and SE in the investigated animals is shown in Table 1.

C-24 configurations of 24-alkyl sterols are rarely determined because of the difficulties of isolation in a pure state. It is 24α- for terrestrial plants and 24β- for most of the algae. This difference can be used as an indicator in the investigations of the food chains. The isolation of some 24-alkyl sterols in a pure state enabled us to establish their C-24 configuration by comparison of mp of free sterols and their acetates with literature data. Sterol VI (mp 152-153 C, acetate mp 147-8 C) was identified

TABLE 1

Sterol Content of Freshwater Mussels (in GLC area %)

Species	*Anodonta cygnea*							*Unio timidus*			*Pseudoanodonta complanata*	
Date	July	October		October (starvation)		July (Netherlands)		August			August	
Sterols / Sterol form[a]	FS	FS	SE	FS	SE	Total sterols		FS	SE		FS	SE
27CΔ[5] (I)	42	54	54	62	45	47.4		52	56		48	49
27CΔ[5,22] (II)	2	3	3	3	2	4.2		2	2		4	3
28CΔ[5,22] (III)	24	16	17	15	21	18.5		16	16		12	13
28CΔ[5], 29CΔ[5,22] (V+VI)	25	21	20	16	21	8.5 + 8.5		27	22		26	23
29CΔ[5] (VII)	7	6	6	4	11	10.2		3	4		10	12

[a] FS–free sterols; SE–sterol esters.

as poriferasterol (16) (lit mp 155-156 C, acetate mp 146-148 C), whereas these data for stigmasterol are mp 170-171 C and acetate mp 141 C (17). These constants for sterol III (mp 143-144 C, acetate mp 136-137 C) corresponded to data previously obtained for crinosterol (17), but are lower than those found more recently (mp 148 C, acetate mp 157-158 C) (18), and could be regarded as an indication of the presence of impurities. GLC and GC/MS investigations rejected this possiblity, confirming the purity of the isolated sterol. Most likely, in this case we had a mixture of C-24 epimers of this sterol. Recently, Khalil et al. (19) found such a mixture in scallop sterols.

The results summarized in Table 1, in agreement with previous results (1), gave some evidence that the sterol composition of the particular freshwater bivalves examined is much simpler than that of the marine bivalves. Probably this simpler sterol composition is a peculiarity connected with the requirements of the membrane functions in these animals living in lower water salinities.

Recently, we reported that sterol esters isolated from microalgae (20), sponges (13) and jellyfish (21) contained mainly sterols corresponding to the earlier biosynthetic stages, whereas the free sterols from the same organisms contained mainly representatives of the later biosynthetic stages (C-24-alkylated and Δ^{22}-sterols). In the three freshwater mussels, almost no difference in the sterol composition of FS and SE was found (Table 1). Similar results were obtained with the Black Sea mussel *M. galloprovincialis* (13) and the gastropod *Rapana thomasiana* (22), as well as with a squid (23) (i.e., with representatives of the main classes of Mollusca). Later, the same principle was demonstrated with decapods (24). These results showed that the difference between FS and SE, observed in lower organisms, was not valid for mollusks and probably not for other evolutionary higher invertebrates.

The sterol composition of *A. cygnea*, collected in the Netherlands, is similar to that of the *A. cygnea* collected in Bulgaria (Table 1). These data, as well as the data for sterol composition of the *A. cygnea* collected in summer and in autumn (Table 1), indicated that although the biological individuality of this mussel is an important factor determining its sterol composition, seasonal changes in life conditions are significant factors, too. This prompted us to examine how much change in exogenous sterol input, e.g., the decrease of phytoplankton in autumn, could influence the sterol composition in this mussel, and how much sterol biosynthesis *de novo* could contribute to it. The latter is connected with the concept that the bivalves are neither able to synthesize sterols *de novo* nor to transform them, so that sterol composition is entirely determined by that of their food (3).

As the biogenetic experiments were carried out using starvation conditions, the changes in sterol composition during a two-week period of starvation were studied beforehand. The results showed an increase of cholesterol concentration (Table 1), as well as a two-fold increase of sterol esters. The latter effect of starvation is obviously of more common occurrence, as it has been observed with fungi (25) and with Ostrea (26).

Our feeding experiments showed (Table 2) that after 14 days of incubation, [2-^{14}C] acetate was incorporated into the sterols, and the rate of incorporation was very low. Therefore, the biosynthesis *de novo* does operate in this animal, but it provides an insignificant part of total sterols. This process was also slow in Voogt's experiments (2), and perhaps it explains the established lack of incorporation in some of the previous investigations.

Our feeding experiments with [4-^{14}C]-cholesterol gave evidence about the capability of the mussels to transform this sterol. The total radioactivity of sterols remained almost the same after 7 and 14 days of incubation and that pointed to their slow metabolism in *A. cygnea*. The presence of radioactivity in sterol esters proved the ability of *A. cygnea* to esterify sterols, too, so far demonstrated in mussels only in *M. edulis* (27). There was a decrease of cholesterol activity in SE, while at the same time, the activity of 27C$\Delta^{5,22}$ (II), 28C$\Delta^{5,22}$ (III) and 29C$\Delta^{5,22}$ (VI) sterols derived from it increased faster than in the free sterols. The decrease of SE activity from the 7th to the 14th day was paralleled by an increase of that in FS. Other processes taking place included Δ^{22}-dehydrogenation and C-24 alkylation to 28C and 29C sterols.

Appearance of activity in $\Delta^{5,22}$-sterols (II, III and IV) indicated the presence of a Δ^{22}-dehydrogenase in *A. cygnea*, which had not been established so far in freshwater mussels. Changes in 22-dehydrocholesterol activity with time showed that the dehydrogenation was a comparatively fast process and the resulting sterol further metabolized.

Current opinion (28) is that marine invertebrates are incapable of alkylating sterols and that the sterols originate entirely from the diet. A number of scientists have shown alkylation in marine invertebrates, including the bivalves, but none of them has established the nature of the alkylating agent (2,9,10). Our experiments with

TABLE 2

Activity of Sterols with Time after Incubation of *A. cygnea* with Labeled Precursors (in dpm/mg)

Precursors	[4-^{14}C]Cholesterol				[$^{14}CH_3$]Methionine				[2-^{14}C]Acetate	
Sterol form	FS		SE		FS		SE		FS	
Time / Sterols	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d
Total	283,895	318,783	24,271	14,383	70	201	146	85	0	125
27CΔ^{5}, 28CΔ^{5}, 29CΔ^{5} (I,V,VII)	420,539	453,401	48,961	21,143	83	182	X[a]	90	X	X
29CΔ^{5,22} (VI)	2,414	5,222	328	1,468	38	197	X	254	X	X
28CΔ^{5,22} (III)	4,476	6,084	168	553	6	7	X	0	X	X
27CΔ^{5,22} (II)	13,813	8,963	196	373	0	0	X	0	X	X

[a]X—insufficient amount of sterol for determination.

L-methionine-methyl-^{14}C demonstrated its incorporation into sterols, without randomization, as indicated by the lack of activity in 22-dehydrocholesterol (Table 2). Activity after 14 days' incubation was considerably higher than that after 7 days. Therefore, the process of alkylation is a slow one and possibly the failure in the incorporation of methionine (7,11,12) is due to insufficient time of incubation. At the same time, activity appeared in the sterol esters sooner than in the free sterols and gradually decreased, in agreement with the assumption that alkylation takes place with esterified sterols (20). It might be concluded that, irrespective of whether acetate can take part in the C-24 alkylation in *A. cygnea*, this process also can go through the normal pathway with methionine.

An interesting result of this experiment was the evidence for cholesterol alkylation in *A. cygnea*—a process generally accepted as being performed via desmosterol. So far, this intermediate has been found in marine mussels (29), but not in freshwater mollusks (1,2), and our attempts to detect it in *A. cygnea* also led to negative results. The situation with some decapods is analogous (30).

The results of our experiments with an abnormally high input of cholesterol demonstrated to what extent the sterol composition depends on diet sterol input. After an incubation of 7 and 14 days with cholesterol sufficient to increase the content in the mussel from 54 to 70%, the composition of FS in *A. cygnea* was the same as in the control, but in the SE there was an increase of cholesterol (8% after 7 days and 17% after 14 days). This indicated a tendency of this organism to maintain its sterol composition at the level determined by its physiological requirements of the moment. The nature of processes leading to normalization of sterol composition after an abnormally high sterol input in *A. cygnea* can be deduced from the following tracer experiments.

Injection of large amount of labeled cholesterol (increase of cholesterol content to 80%) in *A. cygnea* led to a temporary increase of the cholesterol content equal to 24, 22 and 14% for 6 hr, 26 hr and 7 days of incubation. Activity in the water medium remained negligible after 26 hr of incubation, but appeared after the 7th day. Considerable activity was established in the water contained in the body of the mussel. Alkaline hydrolysis of this water afforded cholesterol (evidenced by GLC and by formation of epoxide), with activity equal to that of cholesterol in FS—an indication of excretion of cholesterol as water-soluble conjugates. In this way, conjugation, without additional transfor-

mation, was shown to be one of the mechanisms for the elimination of the surplus cholesterol. These results are summarized in Table 3.

The low activity of sterol esters, increasing strongly with time, as well as the increase of cholesterol content in SE from 4 up to 11%, was an indication that SE, possibly due to their small importance for membrane functions, should be another way of sterol surplus inactivation. On the other hand, the total activity of SE was low, which is an indication that the acceleration of metabolic processes other than esterification plays the decisive role in the normalization of cholesterol content.

Another interesting observation was the high activity of alkylated sterols isolated from SE after a short time of incubation (6 hr). This observation, supported by the fast appearance of radioactivity with methionine (described earlier in this communication), is an indication that C-24 alkylation of cholesterol may take place in its esterified form.

An interesting fact is that cholesterol was readily incorporated into an oxidized sterol with unknown structure, which demonstrated the presence of sterol-oxidizing enzymes in *A. cygnea*.

Summarizing the results obtained (Tables 2 and 3), it may be concluded that *A. cygnea* is able to biosynthesize sterols *de novo*, although very slowly, and to alkylate them at C-24 with methionine. It is also able to esterify and alkylate cholesterol, to oxidize it, and to introduce a C-22 double bond, but all of these processes are rather slow. Any abnormally large sterol input into *A. cygnea* is modified during the restoration of the normal level by acceleration of sterol metabolism, the major processes being oxidation and transformation to water-soluble conjugates.

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TABLE 3

Activity of Sterols with Time after Incubation of *A. cygnea* with a Large Amount of Cholesterol (in dpm/mg)

Sterol form		FS			SE		
Time / Sterols		6 hr	26 hr	7 days	6 hr	26 hr	7 days
Total		332,404	242,421	146,974	1,286	2,862	28,313
$27C\Delta^5$, $28C\Delta^5$, $29C\Delta^5$	(I,V,VII)	355,935	348,115	308,194	2,226	9,418	34,440
$29C\Delta^{5,22}$	(VI)	970	1,329	6,216	1,380	662	470
$28C\Delta^{5,22}$	(III)	1,072	1,213	1,182	922	620	570
$27C\Delta^{5,22}$	(II)	7,539	15,871	26,375	87	804	207
Oxidized sterol		23,120	44,440	60,420	X[a]	X	X

[a]X—insufficient amount of sterol for determination.

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Biohydrogenation of Cholesterol as an Index of Bacterial 7α-Dehydroxylase Activity

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ABSTRACT

Fecal steroid compositions of 82 human subjects of various ages and diets and gastrointestinal status were examined by gas liquid chromatography. Progressive increases in bacterial activities on both bile acids and neutral sterols were observed with the advance of age in infants and children. The patterns in the 4-year-olds approached those observed in adults. Bacterial activites on fecal steroids were found to be decreased in adult subjects with acute shigellosis and in those challenged by castor oil. In contrast, no significant changes in fecal steroid profiles were observed in the subjects with traveller's diarrhea assoicated with toxigenic *Escherichia coli*. The effects of diarrhea on fecal steroids of infants under 1½ years were less consistent than those of adults. However, a close relationship was observed between the degree of 7α-dehydroxylation of cholic acid (expressed as the ratio of deoxycholic to the sum of deoxycholic and cholic acids) and the percentage of cholesterol in the feces ($r = -0.921$, $p < 0.001$). The correlation between the production of lithocholic acid and the percentage cholesterol was also good ($r = -0.739$, $p < 0.001$). Analysis of neutral steroids may be a good index of intraluminal bile acid metabolism.

INTRODUCTION

It is known that intestinal sterols may be metabolized by microorganisms in the human intestinal tract. The primary bile acids (BA), cholic and chenodeoxycholic acid, are subjected to deconjugation, dehydroxylation, and dehydrogenation by intestinal microorganisms (1). Two of the major metabolites of BA in the feces of healthy adults are deoxycholic acid (DOC) and lithocholic acid (LC). They are produced by bacterial 7α-dehydroxylation of cholic (C) and chenodeoxycholic acids (CDC), respectively. Neutral sterols are also altered in the intestinal tract by microorganisms (2). Cholesterol and plant sterols are converted to 5β-steroids and to a lesser extent to 5α-steroids by saturation of the Δ^5-double bond of the common intermediate 4-cholesten-3-one. Subsequent reduction of the 3-keto group yields predominantly 3β-hydroxyl sterols such as coprostanol (CO) which is the principal neutral steroid in the feces of normal adults. A number of authors have reported the correlation between the formation of coprostanol and the conversion of primary to secondary BA in rats (3) and in humans (4). However, quantitative evaluation of the correlation between these fecal steroids on a large number of samples is still lacking. In this paper, we report the results of a systematic study of both BA and neutral sterol profiles of fecal samples from both adults and children with or without gastrointestinal problems. The results indicate that gas liquid chromatography (GLC) may be used to examine the action of microorganisms on the steroids in the gastrointestinal tract.

METHODS AND MATERIALS

Subjects

Eighty-two human subjects recruited from various research protocols in the Section of Nutrition and Gastroenterology were investigated in this study. They were grouped as: group 1—31 healthy adult male controls aged 18 to 45 years with no history of gastrointestinal problems or liver disease. All were on regular mixed diets; group 2—5 healthy infant controls (3 males and 2 females) aged 4 to 18 months with no history of diarrhea, steatorrhea or liver diseases. They were fed commercial infant formulas or were partially weaned and were receiving solid foods; group 3—4 healthy male children aged 44 to 65 months who were on regular mixed diets when studied; group 4—4 adult male subjects with acute shigellosis after being challenged by *Shigella flexneri* 2a (M42-43) (5). These volunteers were healthy inmates at the Maryland House of Correction, Jessup, MD, who were participating in an evaluation of the efficacy of an oral shigella vaccine. The samples from these subjects were collected during infection but before antibiotic

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treatment; group 5–10 adult subjects (7 males, 3 females) with traveller's diarrhea (TD) associated with toxigenic *Escherichia coli* before antibiotic treatment (6,7); group 6–15 infants and children (10 males, 5 females) aged 12 days to 18 months with acute or chronic diarrhea of nonspecific etiology (ANSD or CNSD) but with no evidence of malnutrition or liver disease. The duration of diarrhea was < 1 week for ANSD and > 2 weeks for CNSD patients. The samples were collected before any antibiotic treatment; group 7–8 infants (7 males and 1 female) aged 2 to 16 months with acute diarrhea associated with Shigella, Salmonella or toxigenic *E. coli* but with no evidence of malnutrition or liver disease. No antibiotics were given before stools were collected; group 8–5 healthy adult male subjects with diarrhea after challenged by a single dose (30 mL) of castor oil, U.S.P. (Phillips Roxane Laboratories, Inc., Columbus, OH).

Collection of Specimens

All fecal specimens were collected in plastic containers and were either frozen at -20 C immediately after collection, or were diluted by adding known amounts of an ethanol/methanol (95:5) mixture and kept at 0 C until analyzed. The samples were collected from various research protocols over a time span of 6 years but were analyzed within 2 weeks after the completion of each study. We found no differences in the results of steroid analysis between duplicate samples preserved by either method, nor were any differences found in fecal steroids between sexes within the same group of subjects.

Steroid Analysis

Bile acids were analyzed as methyl ester-trifluoroacetate derivatives by GLC according to the technique described by Kuksis (8) and Yousef et al. (9). Neutral steroids were analyzed as methyl ester-trimethylsilyl ether derivatives by the combined thin layer/GLC techniques described by Miettinen et al. (10) with slight modification (5). All GLC analyses were done in a Packard Model 420 gas chromatograph with dual flame ionization detectors.

For BA analyses, the following chromatographic conditions were used: a 6 ft × 2 mm U-shaped glass column was packed with 3% OV-210 on 80/100 mesh Chromosorb W-AW-DMCS-HMDS (Applied Science Laboratories, Inc., State College, PA). The operating temperatures were: column, 230 C; detector, 250 C; and injection port, 260 C. The carrier gas was nitrogen at a flow rate of 30 mL/min with inlet pressure of 20-40 psig. For the analysis of neutral sterols (NS), 3% OV-225 on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, Inc.) was used (5). Other chromatographic conditions were similar to those used in BA analysis. 5α-Cholestane was used as an internal standard for quantitation of both BA and NS. Detailed procedures of thin layer chromatography (TLC) for the separation of BA have been described elsewhere (11,12).

BA and NS standards were obtained from Steraloids (Pawling, NY), Applied Science Laboratories (State College, PA) and Supelco (Bellefonte, PA). All solvents were of reagent grade and obtained from Aldrich (Milwaukee, WI) and Mallinckrodt (St. Louis, MO). Glass plates (20 × 20 cm), precoated with Silica Gel G to a thickness of 250 μm, were purchased from Brinkmann (Westbury, NY).

7α-dehydroxylation of cholic acid was expressed as

$$\frac{\%\,DOC}{\%\,DOC + \%\,C} \times 100,$$

and

7α-dehydroxylation of chenodeoxycholic was expressed as

$$\frac{\%\,LC}{\%\,LC + \%\,CDC} \times 100$$ (see Table 1).

Total steroid concentrations were expressed as mg/g of feces (wet wt). Daily steroid excretion was expressed as mg/kg of body wt/day (see Table 1).

Statistical Analysis

Statistical evaluation among various groups was done by Student's t-test. No significant difference was recorded when $p > 0.05$. All values of steroid composition were expressed as mean ± standard error of mean (SEM) % of total BA or NS in the feces so that the effect of dilution could be avoided (Table 1). Correlation between the degree of 7α-dehydroxylation of BA and the percentage of cholesterol in the feces was determined by linear regression methods.

RESULTS

Effect of Age on Fecal Steroids

Progressive changes in both BA and NS profiles were observed with maturation in normal infants and children. In the infants under 1½ years (group 2), the degrees of 7α-dehydroxylation of C and CDC were 18.4 ± 7.3 and 25.4 ± 6.7. In 4-year-olds (group 3), the corresponding values were 81.6 ± 11.1 and 86.5 ± 9.4; in adults (group 1), the values were 95.0 ± 0.6 and 93.7 ± 0.7 (Table 1). The findings indicated that bacterial 7α-dehydroxyl-

TABLE 1

Comparison of the Degree of Bacterial 7α-Dehydroxylation and Biohydrogenation of Cholesterol in Fecal Samples from 82 Human Subjects

Group no.	No. of subjects	Age	Sex	GI Status	$\frac{DOC}{C+DOC}$ (× 100)	$\frac{LC}{CDC+LC}$ (× 100)	%CH	$\frac{CO}{CH+CO}$ (× 100)	Total concentrations (mg/g) BA	Total concentrations (mg/g) NS	Total excretions (mg/kg/day) BA	Total excretions (mg/kg/day) NS
1	31	Adult	M	Control	95.0 ± 0.6[a]	93.7 ± 0.7[a]	26.5 ± 2.3[a]	71.0 ± 2.3[a]	2.15 ± 0.29[c]	5.50 ± 0.69[c]	(n=24) 3.4 ± 0.6	(n=24) 10.4 ± 1.7
2	5	< 1½ yr	3 M 2 F	Control	18.4 ± 7.3	25.4 ± 6.7	94.7 ± 3.7	3.8 ± 3.5	0.57 ± 0.18	1.13 ± 0.22	3.8 ± 2.0	15.4 ± 4.5
3	4	4 yr	M	Control	81.6 ± 11.1[b]	86.5 ± 9.4[b]	27.0 ± 1.5[a]	69.8 ± 1.9[a]	0.30 ± 0.12[f]	2.33 ± 0.59[c]	2.0 ± 0.7	17.9 ± 3.0
4	4	Adult	M	Acute Shigellosis	43.7 ± 8.7[d]	77.3 ± 6.9[d]	86.2 ± 8.7[d]	12.5 ± 8.5[d]	0.75 ± 0.33	2.10 ± 0.70	4.9 ± 1.0	6.8 ± 1.7
5	10	Adult	7 M 3 F	TD	87.6 ± 4.9	88.3 ± 4.6	37.4 ± 7.6	60.0 ± 7.3	1.48 ± 0.24	2.55 ± 0.49[f]	(n=6) 7.4 ± 1.4[f]	(n=6) 10.1 ± 2.7
6	15	< 1½ yr	10 M 5 F	ANSD/CNSD	10.8 ± 4.8	53.5 ± 7.3[c]	92.6 ± 3.8	6.6 ± 3.8	0.10 ± 0.02[a]	0.74 ± 0.14	(n=11) 1.3 ± 0.2	(n=11) 15.5 ± 4.8
7	8	< 1½ yr	7 M 1 F	ASD	6.1 ± 1.8	51.5 ± 7.1[c]	99.3 ± 0.2	0.4 ± 0.1	0.18 ± 0.04[c]	1.56 ± 0.39	(n=5) 2.5 ± 1.1	(n=5) 12.7 ± 2.9
8	5	Adult	M	Castor oil	71.6 ± 8.9[e]	76.1 ± 9.4[e]	45.5 ± 5.4[e]	47.5 ± 4.3[d]	0.72 ± 0.17	1.39 ± 0.32[f]	(n=4) 2.2 ± 0.5	(n=4) 7.2 ± 3.6

All values were expressed as mean ± SEM.

[a]$p < 0.001$; [b]$p < 0.005$; [c]$p < 0.05$ as compared to group 2; [d]$p < 0.001$; [e]$p < 0.005$; [f]$p < 0.05$ as compared to the paired controls in group 1.

M = male, F = female, DOC = deoxycholic, C = cholic.

LC = lithocholic, CDC = chenodeoxycholic, CH = cholesterol.

TD = travellers' diarrhea associated with toxigenic *E. coli*.

ANSD = acute nonspecific diarrhea, CNSD = chronic nonspecific diarrhea.

ASD = acute diarrhea of specific etiology (e.g., Shigella, Salmonella).

N = number of subjects, unless otherwise indicated (as in total excretions).

ation of primary BA increased with age and that the patterns in the 4-year-olds approached those observed in adults. In the NS fraction, cholesterol decreased with age: 94.7 ± 3.7%, 27.0 ± 1.5% and 26.5 ± 2.3% of the total NS in the feces for infants, children, and adults, respectively.

Effect of Diarrhea on Fecal Steroids

In adult subjects with acute shigellosis (group 4), bacterial action on fecal BA and NS was reduced. The degrees of 7α-dehydroxylation of C and CDC (43.7 ± 8.7 and 77.3 ± 6.9, respectively, see Table 1) were signifcantly lower than those of nondiarrheal controls (group 1 and pair controls, $p < 0.001$). Consistent with the BA findings, percentage cholesterol was increased in acute shigellosis (86.2 ± 8.7% as compared to 26.5 ± 2.3% for nondiarrheal controls, $p < 0.001$). On the other hand, bacterial activities on fecal BA and NS in adults with TD associated with toxigenic *E. coli* (group 5) were not reduced. The degrees of 7α-dehydroxylation of C and CDC were 87.6 ± 4.9 and 88.3 ± 4.6, respectively. Consistent with the BA findings, the percentage of cholesterol was not significantly changed during diarrhea (37.4 ± 7.6%). It is notable that bacterial activities on fecal steroids for adult subjects challenged by castor oil were also reduced during diarrheal episode. The degrees of 7α-dehydroxylation of C and CDC were decreased: 71.6 ± 8.9 ($p < 0.005$ as compared to group 1 and their own controls before challenge) and 76.1 ± 9.4 ($p < 0.005$), respectively. In addition, the percentage of cholesterol in the feces was increased: 45.5 ± 5.4% ($p < 0.005$ as compared to group 1 and their own controls before challenge) as a result of diarrhea. These data are consistent with the observation that intestinal transit times (ITT) were shortened during the diarrheal episode associated with castor oil (unpublished data from this laboratory).

The effects of diarrhea on fecal steroids of infants under 1½ years were less consistent than those of adults. The degrees of 7α-dehydroxylation of C in the feces of these infants were lower than those in nondiarrheal controls (10.8 ± 4.8 for group 6; 6.1 ± 1.8 for group 7 as compared to 18.4 ± 7.3 in group 2 controls) but the differences were not significant statistically. This is consistent with the data from the analysis of NS fractions in the same samples. The percentage of cholesterol in the feces was not changed in the infants with diarrhea of various etiologies (92.6 ± 3.8 for group 6; 99.3 ± 0.2 for group 7 as compared to 94.7 ± 3.7 for group 2 controls). However, the degrees of 7α-dehydroxylation of CDC were significantly increased during diarrheal episodes (53.5 ± 7.3 for group 6; 51.5 ± 7.1 for group 7 as compared to 25.4 ± 6.7 for group 2 controls, $p < 0.05$ in both cases).

Correlation between Biohydrogenation of Cholesterol and 7α-Dehydroxylation of Cholic and Chenodeoxycholic Acids

Table 2 gives a comparison of correlation coefficients of BA and NS in fecal specimens of all 82 subjects. It is apparent that there were good correlations between percentage DOC or percentage LC of total BA and percentage CO or percentage CH of total NS in the feces with correlation coefficients varying from -0.697 to -0.831 ($p < 0.001$ in all cases). The degrees of 7α-dehydroxylation of CDC also correlated well with % CH and the ratio of CO/(CO + CH) in the same feces ($r = -0.739$ and 0.737, respectively, $p < 0.001$). However, the best correlation was achieved between the degrees of 7α-dehydroxylation of C and percentage CH and the ratio of CO/(CO + CH) in the same set of samples ($r = -0.921$ and 0.919, respectively, $p < 0.001$) (see Fig. 1).

DISCUSSION

This study clearly shows the role of intestinal microorganisms on the profiles of fecal steroids in humans, and conversely, fecal steroid composition was clearly shown to be a useful index of intestinal microecology, although further definition in a wider range of pathophysiological conditions is required.

Effect of Age on Fecal Steroids

It is generally believed that the fetus is free of microorganisms before birth. The intestinal

TABLE 2

Comparison of Correlation Coefficients of Bile Acids and Neutral Sterols in the Fecal Specimen[a]

Y	X	r
% CO	% DOC	0.807
% CO	% LC	0.700
% CH	% DOC	-0.831
% CH	% LC	-0.697
% CH	DOC/(C + DOC)	-0.921
% CH	LC/(CDC + LC)	-0.739
CO/(CH + CO)	DOC/(C + DOC)	0.919
CO/(CH + CO)	LC/(CDC + LC)	0.737

[a]CO = coprostanol (See footnotes under Table 1 for other abbreviations).

Number of paired data = 82. $p < 0.001$ for all cases.

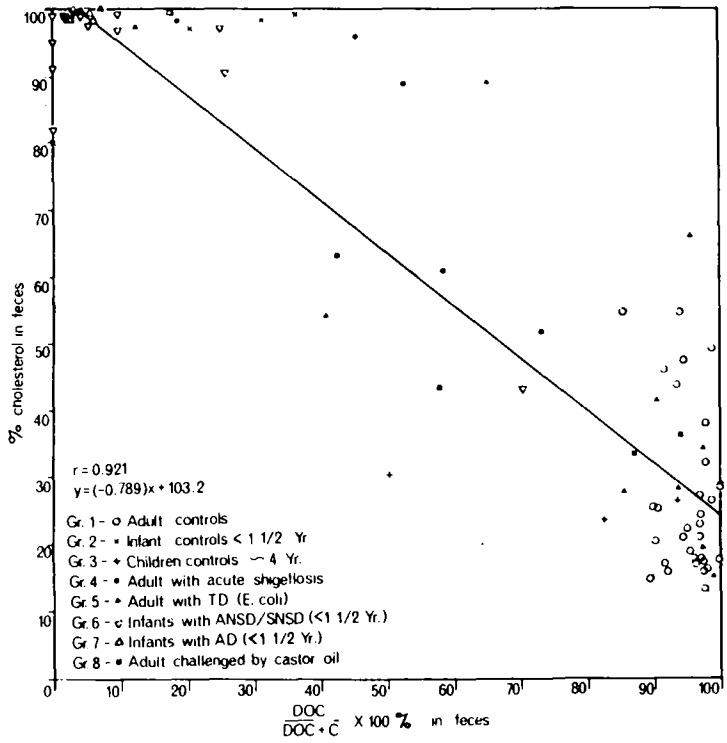

FIG. 1. Correlation between the percentage cholesterol and the production of deoxycholic acid (DOC) from cholic acid (C) in the fecal samples from 82 human subjects of all ages regardless of the type of diet and gastrointestinal status. Correlation coefficient $r = -0.921$, $p < 0.001$.

bacterial flora develop rapidly through contamination from the environment and undergo significant qualitative and quantitative changes with maturation (13,14). In a previous study from this laboratory (15), we have shown that bacterial 7α-dehydroxylation of C and CDC acids and the production of bacterial metabolites (such as CO) of cholesterol increased with age and approached adult levels in children of 4 years or older. The data from this study further illustrated this. It is noted that none of our 31 adult control subjects (group 1) had degrees of 7α-dehydroxylation of C or CDC less than 85% whereas only one out of all 28 infants under 1½ years (groups 2, 6 and 7) had values greater than 45%. This particular patient, age 6 months, had acute diarrhea of nonspecific etiology (ANSD) and the degrees of 7α-dehydroxylation of C and CDC were 70.1% and 54.3%, respectively, in his stool specimens. The patterns in the NS fractions were similar. None of the 31 adult controls (group 1) had greater than 55% CH in their feces whereas only one out of all 28 infants under 1½ years (groups 2, 6 and 7) had less than 80% CH. Again, this is the same ANSD patient with the elevated degrees of 7α-dehydroxylation of C and CDC. The percentage CH was 43.1% in his stools. Nevertheless, there was a clean and obvious separation for the degrees of bacterial modification on fecal steroids between normal infants (group 2) and adults (group 1) (see Fig. 1). It should be noted also that a child of 6 years with severe combined immune deficiency (SCID) who had been kept in strict reverse isolation from birth had a fecal steroid profile similar to that of the control infants under 1½ years (16). It should be further recognized that the difference in steroid profiles observed in infants, children and adults could be attributed, at least in part, to dietary effects. For example, healthy adult volunteers on a fiber-free liquid formula diet show a significant reduction in fecal bacterial flora (17), an increase in transit time (18), a reduction in the interaction of intestinal steroids with the colonic flora (18), and a reduction in excretion of both acidic and neutral steroids (18). The effects of dietary fiber on intestinal sterols have been discussed in detail by Huang et al. (19).

Effect of Diarrhea on Fecal Steroids

As indicated in our previous papers on the effects of diarrhea on fecal steroids of adult subjects (5-7), 2 distinct fecal steroid patterns appear to be associated with 2 basic human mechanisms of diarrhea production. One, typified by enterotoxin-producing *E. coli*, is mediated via enterotoxin-stimulated secretion in the small bowel without invasion by the bacteria. This is associated with a fecal steroid profile generally similar to those of nondiarrhea controls. The other, exemplified in shigellosis, requires invasion of the intestinal mucosa, particularly in the large bowel and this is characterized by a reduction in bacterial metabolites of BA and NS in the feces. This is also consistent with our observation that ITT was shortened in acute shigellosis but not in the experimentally induced TD (7). In this respect, it is important to note that bacterial activities on fecal steroids were also decreased in castor-oil-induced diarrhea, and this is associated with the shortened ITT observed in these subjects (unpublished data from this laboratory). It is also notable that bacterial activities on fecal steroids were not significantly reduced in cholera despite the shortened ITT observed (unpublished data from this laboratory). Thus, the effect of diarrhea on fecal steroid patterns cannot be explained entirely by ITT in this case. The effect of diarrhea on fecal steroid profiles of young infants was not readily seen, however, probably due to a lack of bacterial metabolites of intestinal sterols in their stool specimens even in nondiarrheal state.

Correlation between Biohydrogenation of Cholesterol and 7α-Dehydroxylation of Cholic and Chenodeoxycholic Acids

Recent studies have shown that the conversion of primary BA to secondary metabolites is associated with the production of coprostanol from cholesterol in the feces, and that dietary modifications may affect the transformation of these steroids through the alteration of intestinal flora (3,4). The present study confirmed these observations and further provided a quantitative evaluation of the correlation between the degree of 7α-dehydroxylation of C and CDC acids and that of biohydrogenation of cholesterol based on the data from the analyses of fecal steroids of 82 human subjects of all ages regardless of the types of diet and gastrointestinal status. It seems obvious that a close relationship was observed between the degree of 7α-dehydroxylation of C acid and the percentages of CH of total NS in the fecal samples. The correlation between the production of LC from CDC acid and the biohydrogenation of CH was also good, but with greater variability. The reason for this discrepancy is not known. Factors such as hepatic synthesis of LC by an alternate pathway (20), binding of BA by dietary fiber (19,21) and bacteria (22), intestinal absorption, solubility (23), and the differences in pool size and synthesis rate of C and CDC acids (24) should be considered. Studies in this area are in progress in our laboratory.

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Ketone Bodies, Glucose and Glutamine as Lipogenic Precursors in Human Diploid Fibroblasts

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ABSTRACT

Incorporation of [^{14}C] from acetoacetate, D(-)- and L(+)-3-hydroxybutyrate, glucose, glutamine, acetate and palmitate in cellular lipids were studied in cultures in human diploid fibroblasts (HDF). The results showed that acetoacetate was 2-10 times more effective as a lipogenic precursor than was either D- or L-3-hydroxybutyrate. Its extent of incorporation into lipids was 2- to 8-fold more than the other precursors examined under conditions when the overall rates of nonsaponifiable and saponifiable lipogenesis as measured by $^{3}H_2O$ incorporation were essentially unchanged. Acetoacetate supported both saponifiable and nonsaponifiable lipid syntheses with half-saturation values (K_m app.) of 185 μM and 30 μM, respectively. Glucose stimulated acetoacetate incorporation into lipids whereas, conversely, acetoacetate inhibited [^{14}C] glucose incorporation into lipids. The presence of low density lipoproteins (LDL) cholesterol (@40 μg cholesterol/mL) inhibited the incorporation of [^{14}C] from acetoacetate 56% into nonsaponifiable lipids; the inhibition was consistently higher (75%) when [^{14}C] glucose or glutamine were the precrusors. The loss of 3-hydroxy-3-methyl-glutaryl CoA (HMG CoA) reductase activity upon addition of LDL-cholesterol was greater than the suppression of [^{14}C] incorporation from acetoacetate or glucose into nonsaponifiable lipids. In the presence of glucose, [^{14}C] acetoacetate was incorporated into 3-βOH sterols (digitonin precipitable) 7.7 ± 1.1 times more effectively than was [^{14}C] glucose. The results suggest that HDF would be a suitable model to investigate the effects of various precrusors of HMG CoA on the rate of cholesterol biosynthesis.

INTRODUCTION

The role of ketone bodies as precursors of complex lipids has received increasing attention following the initial studies by Edmond (1). It was shown that DL-3-hydroxybutyrate was the preferred substrate when compared to acetoacetate, acetate or mevalonate, for sterol and fatty acid synthesis in the brain, spinal cord and skin of the developing rat. Since this work, lipogenesis from ketone bodies has been investigated using cerebral cortex slices (2), whole brain homogenates (3), intrascapular (4,5) and intracranial (6) injection of precursors as well as in vitro with primary cultures of oligodendrocytes (7), among others. While in the whole animal, D- or L-3-hydroxybutyrate was incorporated into lipids to a greater extent than was acetoacetate (4), in the case of in vitro studies, the reverse was true (3,7).

The purpose of this study was to demonstrate ketone-body-supported lipid synthesis in cultures of human diploid fibroblasts (HDF) and to investigate its regulation by low density lipoprotein-cholesterol complex (LDL). Our interest in this model system stems from previous work regarding cholesterol biosynthesis (8,9), and from the fact that diabetes mellitus is accompanied both by elevations in blood ketone body concentrations and by atherosclerotic cardiovascular disease which is a major contributor to the mortality and morbidity of this disorder. The results indicate that acetoacetate is a preferred substrate for lipogenesis compared to D- or L-3-hydroxybutyrate, glucose, or glutamine in HDF. The supression of nonsaponifiable lipid synthesis by LDL is not as complete when acetoacetate is the precursor as it is when glucose or glutamine are the substrates. In addition, [^{14}C]-acetoacetate is incorporated into 3-βOH sterols some 7 times more effectively than is [^{14}C]-glucose.

MATERIALS AND METHODS

Chemicals

The [3-^{14}C] acetoacetate was purchased as the ethyl ester from New England Nuclear Corp., Boston, MA, and hydrolyzed by adding a 6-fold excess of water followed by 100 mM NaOH to bring the pH between 10 and 11. This was incubated at 37 C for 90 min, then diluted to 50 mL with water and the pH adjusted to 7.0. This was then applied to a 1.4 cm x 40 cm Dowex 1-8X-(Cl^-) column and eluted with a linear gradient (100 mL total) of 0-50 mM HCl. The [3-^{14}C] acetoacetate elutes as a well defined peak at slightly over 20 mM HCl. The [U-^{14}C] glutamine, [U-^{14}C] glucose, [D,L 3-^{14}C]-3-hydroxybutyrate and D(-)[3-^{14}C] 3-hydroxybutyrate were also purchased from New England Nuclear Corp. The L(+)[3-^{14}C]-hydroxybutyrate was prepared by treating the racemic mixture with D-3-hydroxybutyrate

dehydrogenase (EC 1.1.1.30) purchased, as were all other chemicals, from Sigma Chemical Co., St. Louis, MO, and then separating the [3-^{14}C] acetoacetate from the L(+)[3-^{14}C]-3-hydroxybutyrate on a Dowex 1-8X(Cl^-) column as just described. The desired specific activity was obtained by adding nonradioactive L(+)-3-hydroxybutyrate prepared by the method of McCann (10).

Tissue Culture

The cultures of HDF were obtained from normal individuals, grown and maintained as previously described (11). The conditions of their use appear in the legends of the figures.

Lipid Extraction and Saponification

Cell pellets were obtained by treating the Petri dishes with trypsin, scraping with a Teflon policeman, transferring with saline to a tube, and centrifuging at ca. 250 G for 10 min at room temperature. The pellets were then washed 3 times with saline. Lipids were extracted from these pellets and saponified following the procedure described for tissue samples (1,4). The radioactivity in the saponifiable and nonsaponifiable fractions was determined by liquid scintillation counting.

Digitonides

The 3-βOH sterols were precipitated as the digitonides from the nonsaponifiable fraction (12).

Calculations

For calculating the C_2 unit incorporation into lipids, the following methods were adopted.

(a) Palmitate. It has been shown by Stanley and Tubbs (13) using liver mitochondria that the oxidation of long-chain free fatty acids yields acetyl CoA as the only compound obtained in a precursor-product relationship. This also was presumed to occur in human diploid fibroblasts. In the event that this assumption is not correct, as is the case for the liver peroxisomal β-oxidation of fatty acids (14), the result would be that fewer than 7 acetyl units would be generated after the initial cleavage of the ^{14}C-containing acetyl unit. Thus, the C_2 unit would have a higher specific activity than if palmitate yielded 8 acetyl units. The overall result would then be that the value listed for palmitate incorporation is a maximum.

(b) Glucose. For any label to appear in lipids from this substrate, it must have been oxidized to the level of acetyl CoA and therefore the specific activity of acetyl units derived from this substrate is based on glucose yielding 2 C_2 units.

(c) Acetoacetate. This substrate can be either cleaved by 3-ketothiolase into 2 acetyl CoA units and used for lipogenesis or, after CoA-acylation, it could be used directly. In either case, the specific activity of an acetyl group is one-half that of the acetoacetate itself.

(d) Glutamine. It has previously been shown that significant amounts of glutamine are metabolized by human diploid fibroblasts to pyruvate and lactate (15), and thus, glutamine may yield one acetyl unit that would have, therefore, the same specific activity as the glutamine.

Determination of K_m Apparent for Lipogenesis

Five [^{14}C] acetoacetate concentrations ranging from 0.05-1.0 mM were used to obtain rates that were graphed in double-reciprocal form. Correlation coefficients of these plots for linear regression were 0.992, 0.993 and 0.992 for incorporation of [^{14}C] into saponifiable, nonsaponifiable and total lipids, respectively.

Lipoproteins

The low density human β-lipoprotein (LDL) preparations, as well as lipoprotein-deficient human plasma, were obtained following the procedure of Brown et al. (16).

HMG CoA Reductase (ED 1.1.1.34)

The activity of this enzyme was determined essentially by the method of Goodwin and Margolis (17).

RESULTS

The incorporation of [3-^{14}C] acetoacetate, [3-^{14}C] D-, or [3-^{14}C]-L-3-hydroxybutyrate and [U-^{14}C] glucose into saponifiable and nonsaponifiable lipids by HDF in log and stationary phase was linear up to 12 hr (results not shown). Acetoacetate was incorporated into fatty acids and sterols 2- to 5-fold better than was either D- or L-3-hydroxybutyrate and, as such, was the ketone body of choice for this study (data not shown). The data in Table 1 show the effect of precursors of acetyl groups on the isotope incorporation from [3-^{14}C] acetoacetate or [U-^{14}C] glucose into sterol and fatty acid fractions by log phase HDF. It should be noted that the rates of isotope incorporation in Table 1 are lower than those reported in Tables 3 and 4 because the media contained normal plasma with LDL present. No significant effect of D-3-hydroxybutyrate on [3-^{14}C] acetoacetate incorporation was observed; however, L-3-hydroxy-

TABLE 1

Substrates of Lipogenesis in Human Diploid Fibroblasts (nmol acetyl groups incorporated in lipids/12 hr/mg protein)

Precursor	Addition	Lipids saponifiable	% Control	Lipids nonsaponifiable	% Control	Total lipids % Control
[3-^{14}C] Acetoacetate (100 μM)	None	1.00	100	0.70	100	100
[3-^{14}C] Acetoacetate (100 μM)	D-3-hydroxybutyrate (1.0 mM)	1.17	117	0.65	93	107
[3-^{14}C] Acetoacetate (100 μM)	L-3-hydroxybutyrate (1.0 mM)	0.58	58	0.48	69	62
[3-^{14}C] Acetoacetate (100 μM)	Glucose (1.0 mM)	2.65	265	1.25	178	229
[U-^{14}C] Glucose (1.0 mM)	None	2.70	100	0.78	100	100
[U-^{14}C] Glucose (1.0 mM	Acetoacetate (100 μM)	1.78	66	0.55	71	67
[U-^{14}C] Glucose (1.0 mM)	Acetoacetate (500 μM)	1.30	48	0.45	58	50
[U-^{14}C] Glucose (1.0 mM)	Acetoacetate (1000 μM)	1.00	37	0.50	64	43

	Lipogenesis, K_m app.		
	Saponifiable Lipids	Nonsaponifiable lipids	Total lipids
[3-^{14}C] Acetoacetate	185 μM	30 μM	130 μM

Cultures of HDF in early log phase were grown in ITU media† containing normal human plasma. At time 0 min, growth media were replaced with fresh media containing the indicated ^{14}C-substrates and other additions as noted. The results were the average of 3, 100 mm Petri dish (P-100) cultures, in duplicate. The data for K_m app. were obtained under the same experimental conditions with varying concentrations of [^{14}C] acetoacetate in 4.0 mL vol. The [3-^{14}C] acetoacetate was added at 1 μCi/P-100 and the [U-^{14}C] glucose at 2 μCi/P-100.

†This media is identical to the HTU media (11) with the exception that inosine replaces hypoxanthine.

butyrate was inhibitory. The addition of 1 mM glucose caused a 2-fold stimulation of the incorporation of [3-^{14}C] acetoacetate into total lipids.

As was shown in Table 1, the addition of acetoacetate in increasing concentrations resulted in a progressive inhibition of [U-^{14}C]-glucose incorporation into lipids. The inhibition by acetoacetate was greater for the appearance of ^{14}C label from [U-^{14}C] glucose into sterols than into fatty acids. This is just the opposite of the previously mentioned stimulation of [3-^{14}C] acetoacetate incorporation by glucose into these same 2 classes of lipids.

The K_m app. values for the formation of fatty acids, sterols and total lipids from [3-^{14}C]-acetoacetate in log phase HDF are also listed. The K_m app. for acetoacetate incorporation into sterols is ca. 17% of that for incorporation into the fatty acid fraction.

The incorporation of ^3H_2O into saponifiable and nonsaponifiable lipids by HDF is shown in Table 2. The appearance of [^{3}H] in the saponifiable fraction varied by 20% between experiments with glucose and acetoacetate, whereas it varied by less than 4% into the nonsaponifiable fraction. In contrast, the incorporation of [^{14}C] into these 2 fractions varied over a range of 100%. Also shown is a comparison between [^{14}C] glucose incorporation at 1.0 mM (17 mg%) and 5.5 mM (100 mg%). There was no increase in label incorporation into the nonsaponifiable lipids at the higher concentration whereas label appearance in the saponifiable fraction increased by 60%.

The nonsaponifiable fraction synthesized from [^{14}C] acetoacetate was chromatographed on thin layer silicic acid plates after mixing with authentic cholesterol. The radioautographs developed from plates run both in benzene/ethyl acetate (2:1, v/v) and hexane/ethyl ether/glacial acetic acid (80:20:4, v/v) showed that this sterol fraction consisted primarily of a single spot (>75% of ^{14}C). This spot overlapped but it was not totally coincident with that of the cholesterol standard, visualized by anisaldehyde (not shown).

The formation of the digitonides from the nonsaponifiable fraction is also shown in Table 2. In the presence of unlabeled glucose, the incorporation of [^{14}C] from [3-^{14}C] acetoacetate into 3-βOH sterols is substantially greater (7.7 ± 2 times) than when [U-^{14}C] glucose is the precursor. The 3-βOH sterols comprise 68 ± 11% (SEM) of the nonsaponifiable fraction when acetoacetate is the precursor and 31 ± 5% (SEM) when glucose is the labeled substrate (data not shown).

As expected, the production of saponifiable

TABLE 2

Lipogenesis and 3-βOH-Sterol Formation[a]

Precursor	Addition	^{3}H-Labeled (nmol $^3H_2$ incorporated/12 hr/mg)		^{14}C-Labeled (nmol acetyl units incorporated/12 hr/mg protein)	
		Saponifiable	Nonsaponifiable	Saponifiable	Nonsaponifiable
[U-^{14}C] Glucose (1.0 mM)	None	9.0	14.5	2.0	4.1
[U-^{14}C] Glucose (5.5 mM)	None	–	–	3.2	4.1
[3-^{14}C] Acetoacetate (0.5 mM)	None	9.0	15.0	4.2	7.8
[3-^{14}C] Acetoacetate (0.5 mM)	Glucose (1.0 mM)	11.2	14.6	4.1	9.1

[^{14}C] Precursor	Digitonide formation[b] (nmol acetyl units precipitable as digitonide/12 hr/mg)
[3-^{14}C] Acetoacetate	9.6 ± 1.5 (n = 4)
[3-^{14}C] Acetoacetate + cold glucose	15.5 ± 3.6 (n = 9)
[U-^{14}C] Glucose	2.0 ± 0.4 (n = 9)

[a]The incorporation of ^3H_2O and [^{14}C] substrates into lipids was performed as in Table 3 with LDL-cholesterol-deficient media (final cholesterol content = 1.2 μgm/mL) except that ^3H_2O was added to the media @1.0 mCi/mL in a final vol of 4.0 mL.

[b]The digitonides are expressed as means ± SEM. These results were obtained by averaging a larger group of experiments than in a.

lipids from acetoacetate was not affected to any substantial degree by the addition of either LDL, or cholesterol by itself, to the LDL-free media (Table 3). In contrast, the nonsaponifiable lipogenesis from acetoacetate was inhibited ca. 50%. However, in the case of [U-^{14}C]-glucose and [U-^{14}C] glutamine, the inclusion of LDL caused a greater inhibition, 75% or more of the incorporation of ^{14}C into the sterol fraction. Surprisingly, addition of LDL resulted in an inhibition (≤20-40%) of glucose and glutamine incorporation into saponifiable lipids. It has already been mentioned that the inclusion of glucose to the media containing [3-^{14}C]-acetoacetate stimulated lipogenesis (Table 1), and additionally, it had the effect of relieving the LDL-inhibition of acetoacetate incorporation into the sterol fraction. Conversely, the inclusion of acetoacetate to the media containing [U-^{14}C] glucose inhibits the [^{14}C] incorporation into lipids (cf. Table 1) and reduces the LDL-mediated inhibition of lipogenesis. The degree of inhibition of lipogenesis effected by LDL with acetoacetate and glucose as precursors is generally the same for log or stationary phase HDF. The addition of LDL caused a near total loss of HMG CoA reductase activity, the putative rate-limiting enzyme in cholesterol synthesis (Table 3).

The comparative rates of isotope incorporation from various [^{14}C] sources of acetyl CoA into saponifiable and nonsaponifiable lipids are shown in Table 4. These results show that, in HDF, acetoacetate is at least as effective a lipid precursor as the other precursors tested.

DISCUSSION

The data in the present work establish acetoacetate as a major lipogenic precursor in HDF (cf. Tables 1-4).

The addition of glucose resulted in a >2-fold stimulation of [3-^{14}C] acetoacetate-supported lipogenesis (Table 1). This is due presumably to the provision, by glucose, of reducing equivalents required for lipogenesis. Similar stimulation by glucose was reported using cerebral cortex slices from 1-week-old rats (2); however, others using brain homogenates from rats of the same age did not observe this glucose effect (3). It is not clear why the latter study failed to demonstrate this stimulation of lipogenesis. An obvious difference between homogenates (3), slices (2) or HDF (present work) is the greater degree of cellular integrity in the latter 2 systems.

The inhibitory effect of acetoacetate on lipogenesis from glucose is consistent with the concept of competitive acetyl group donors. This inhibition has also been observed using cerebral cortex slices where acetoacetate decreased label incorporation from glucose, but not the total lipid synthesis (2).

The overall rate of nonsaponifiable lipogenesis does not appear to differ whether acetoacetate is present along with glucose or not (Table 2). Under these conditions, it is evident that acetoacetate produces substantially more 3-βOH sterols (>7X) than does glucose. These results are consistent with the concept that, during increased blood [acetoacetate] and [glucose], i.e., diabetes mellitus, there may be a significant contribution of acetoacetate to cholesterogenesis. Only a marginal increment in saponifiable lipogenesis, and none for nonsaponifiable lipid synthesis occurred when the glucose concentration was raised from 1.0 mM (17 mg%) to 5.5 mM (100 mg%) (Table 2). These data, therefore, indicate that, even when blood [glucose] is decreased in the presence of ketosis (e.g., ketotic hypoglycemia), acetoacetate-supported steroid biosynthesis may proceed unimpaired.

Using acetoacetate as substrate, the values for half-saturation (K_m app.) of the lipogenic process (cf. Table 3) are within the normal physiological range for this compound, i.e., 200-400 μM in the newborn human infant (18), and is in accord with a concept of a particular anabolic function for this ketone body.

Although the identities of the major nonsaponifiable lipids arising from acetoacetate have not bee determined, its migration in 2 separate chromatographic systems was very close to that of cholesterol. This, and the observation that the majority of the [^{14}C]-acetoacetate incorporation is into 3-βOH sterols, would support our hypothesis that ketone bodies may be important in cholesterogenesis and the involvement of the steroid in diabetic atherosclerotic vascular disease.

The synthesis of sterols by log phase HDF from acetoacetate is inhibited by LDL (56% inhibition at 40 μg cholesterol/mL). This is less than the inhibition when glucose or glutamine are the substrates for nonsaponifiable lipid formation (75% inhibition of both at 40 μg cholesterol/mL). The addition of glucose results in a marginal reduction of the LDL inhibition of nonsaponifiable lipogenesis from acetoacetate or glutamine (Table 3). The differential effect of LDL on label incorporation from [^{14}C] acetoacetate, as compared to other precursors, into nonsaponifiable lipids might be explained by the multiplicity of products for which the synthesis is influenced by the activity of HMG CoA reductase in HDF,

TABLE 3

Effect of LDL on Lipogenesis in Human Diploid Fibroblasts (nmol acetyl groups incorporated into lipids/12 hr/mg protein)

	Addition	Growth phase	Lipids saponifiable	% Control	Lipids nonsaponifiable	% Control	% Loss of HMG CoA reductase activity
[3-^{14}C] Acetoacetate (500 μM)	None	Log	2.06	100	4.84	100	0
[3-^{14}C] Acetoacetate (500 μM)	LDL	Log	1.73	84	2.13	44	87
[3-^{14}C] Acetoacetate (500 μM)	Cholesterol	Log	2.39	116	3.82	79	
[3-^{14}C] Acetoacetate (500 μM)	Glucose (1.0 mM)	Log	3.35	100	9.86	100	0
[3-^{14}C] Acetoacetate (500 μM)	Glucose (1.0 mM + LDL)	Log	3.45	103	5.52	56	86
[U-^{14}C] Glucose (1.0 mM)	None	Log	2.80	100	8.00	100	0
[U-^{14}C] Glucose (1.0 mM)	LDL	Log	1.57	56	2.00	25	92
[U-^{14}C] Glucose (1.0 mM)	Cholesterol	Log	2.24	80	2.80	35	
[U-^{14}C] Glucose (1.0 mM)	Acetoacetate (500 μM)	Log	1.80	100	3.90	100	
[U-^{14}C] Glucose (1.0 mM)	Acetoacetate (500 μM + LDL)	Log	2.12	118	2.03	52	
[U-^{14}C] Glutamine (1.0 mM)	None	Log	0.30	100	1.00	100	
[U-^{14}CpGlutamine (1.0 mM)	LDL	Log	0.15	50	0.25	24	
[U-^{14}C] Glutamine (1.0 mM)	Glucose (1.0 mM)	Log	0.77	100	1.95	100	
[U-^{14}C] Glutamine (1.0 mM)	Glucose (1.0 mM + LDL)	Log	0.45	58	0.72	37	
[U-^{14}C] Glutamine (1.0 mM)	Acetoacetate (500 μM)	Log	0.47	100	1.00	100	
[U-^{14}C] Glutamine (1.0 mM)	Acetoacetate (500 μM + LDL)	Log	0.23	50	0.27	27	
[3-^{14}C] Acetoacetate	None	Confluent	1.93	100	4.70	100	0
[3-^{14}C] Acetoacetate	LDL	Confluent	1.79	93	2.35	50	57
[U-^{14}C] Glucose	None	Confluent	1.32	100	2.85	100	0
[U-^{14}C] Glucose	LDL	Confluent	0.63	48	0.80	28	78

Cells were grown in either early log phase or to early confluency, then the ITU media was replaced by the same media in which the normal human plasma was substituted with LDL-free plasma. The cells were acclimated for 24-30 hr; after this time, media were again replaced with fresh media of the indicated composition. When LDL was added, the final cholesterol content was 40 μg/mL. Free cholesterol was also added at this concentration. At 12 hr, the duplicate cultures were harvested and processed as described in Methods. In the expression of the relative HMG CoA reductase activities, the experimental values are expressed as percent of acivity lost as compared to the appropriate control, indicated by 0% loss. The [U-^{14}C] glutamine was added at 2.4 μCi/P-100, whereas the other ^{14}C-substrates were as in Table 1.

TABLE 4

Comparative Rates of Lipogenesis
(nmol of acetyl units incorporated into lipids/12 hr/mg protein)

Precursor	Saponifiable lipids	Nonsaponifiable lipids
[2-^{14}C] Acetate (300 μM)	3.89	4.06
[3-^{14}C] Acetoacetate (500 μM)	11.30	12.45
[U-^{14}C] Glucose (1.0 mM)	1.67	1.55
[U-^{14}C] Glutamine (1.0 mM)	6.6	6.6
[1-^{14}C] Palmitate (100 μM)	–	13.4

Experimental conditions for growth of HDF in early log phase were as described in Table 2, except that, after acclimation, the final media contained LDL-free plasma and the indicated additions. The data presented for all substrates were obtained in the same experiment; the results were in duplicate as before. The reason that no entry appears in the saponifiable column for [^{14}C] palmitate is that, when it was present, the newly synthesized fatty acids were not identified separately from the [^{14}C] palmitate, which was directly incorporated into the saponifiable lipid fraction. The [1-^{14}C] palmitate was added at 5 μCi/P-100; the [2-^{14}C] acetate at 2 μCi/P-100 and the rest of the [^{14}C] precursors were added as in Tables 1 and 2.

i.e., cholesterol, dolichol, polyisoprenes and isopentyl t-RNA (19). The presence of glucose also eliminates (viz, acetoacetate) or reduces (viz, glutamine) the degree of inhibition of saponifiable lipogenesis in log-phase HDF by LDL. The mechanism whereby LDL inhibits saponifiable lipid synthesis is unclear, although it may be related to a provision of phospho- and other lipids for the cell by the LDL; therefore, a smaller proportion of saponifiable lipids would be formed from the ^{14}C-precursor.

The HMG CoA reductase measured under these conditions was almost totally inhibited by LDL. This inhibition is of similar time-course and concentration effectiveness shown by others (16). The inhibition of nonsaponifiable lipid synthesis was in all cases less than the inhibition of HMG CoA reductase. This further supports the concept that LDL-derived cholesterol regulation of HMG CoA reductase is multifaceted in nature. Human HDF appear to be a suitable model to investigate these instructions (19).

The ability and relative efficiency of D- and L-3-hydroxybutyrate, acetate, palmitate, glucose and glutamine to act as substrates for saponifiable and nonsaponifiable lipid synthesis in HDF have also been studied (Table 4). It appears that glutamine is an effective precursor of complex lipids in these cells. Since glutamine is at least as important as glucose for energy metabolism in HDF (11,20) and is present at a high concentration in tissue culture media, its ability to also contribute significantly to lipid anabolism is of particular importance.

Finally, the formation of sterols and other lipids from ketone bodies assumes particular importance in diabetes mellitus in which hyperglycemia exists and the blood levels of acetoacetate frequently exceed the K_m app. values reported in this study. Such a condition is of added significance in view of the observation that synthesis of nonsaponifiable lipids is not regulated by LDL as effectively when acetoacetate is the substrate as it is with glucose or glutamine.

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Effect of the Environment and Fasting on the Lipid and Fatty Acid Composition of *Diplodom patagonicus*

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ABSTRACT

Some effects of food, habitat and temperature on the lipid composition of a freshwater mollusk, *Diplodom patagonicus*, were studied. Animals kept and fasted up to 60 days in an aquarium at 9 C and 20 C showed a decrease of the total lipid content that corresponded to a decrease of triacylglycerols and diacylglycerol ethers. This decrease evoked an increase of polar-to-nonpolar lipid ratio. However, no significant change in the total fatty acid composition was shown. Moreover, a decrease of temperature from 20 C to 9 C decreased the incorporation of labeled linoleic and α-linolenic acid into the lipids, but did not modify the unsaturated:saturated acid ratio of the mollusk lipids during this period. A change of habitat from lake to estuary changed very significantly the fatty acid composition of the animal. The ω6 acids, linoleic and arachidonic, typical of *D. patagonicus* living in the lake, were partially replaced by ω3 acids. That this change was due to a change of food composition was indicated by the fatty acids of corresponding sediments. Therefore, the fatty acid composition of *D. patagonicus* is highly sensitive to food composition and varies little with temperature and seasonal changes.

INTRODUCTION

The lipid constituents and the fatty acid composition of the freshwater bivalve mollusk *Diplodom patagonicus*, collected in Lake Nahuel Huapi, Argentina, showed a composition that is extremely rich in fatty acids of the linoleic family (1). This composition, high in linoleic and arachidonic acids and very poor in members of the α-linolenic acid series, is peculiar because it not only differs from marine bivalves, but also from other freshwater animals (2).

The lipid composition changed with the season but the fatty acid composition was constant and apparently independent of the temperature of the natural environment. These results prompted us to investigate the effect of food and habitat changes on the lipid composition of this mollusk, as well as the effect of temperature, under controlled laboratory conditions.

MATERIALS AND METHODS

Materials

D. patagonicus specimens were collected in Lake Nahuel Huapi as described previously (1). The temperature of the water was 8 C. They were transported to our laboratory in vessels containing sand and water from the lake. In the laboratory, they were kept in a glass aquarium with aerated tap water at 15 C for 1 day before the experiments were started.

D. variabilis samples were collected at Río Santiago, off the coast of the river Río de la Plata, Buenos Aires, Argentina. This place is rather far from the mouth of the river and oceanic influences are limited. The salinity is 0.04-0.05% NaCl.

Sediment samples from Nahuel Huapi and Río Santiago were collected from the bottom of the lake and estuary in the same area.

[1-^{14}C] Linoleic (61 mCi/mmol) and [1-^{14}C] α-linolenic acids (58 mCi/mmol), 98% radiochemically pure, were provided by the Radiochemical Centre, Amersham, England. They were neutralized with ammonia and used as ammonium salts. Unlabeled linoleic and α-linolenic acids (99% pure) were provided by the Hormel Institute, Austin, MN.

Methods

To study the effect of temperature and fasting on the fatty acid composition, 16 *D. patagonicus* specimens were kept in aquaria with aerated, fresh water and without any food during the times specified below. These aquaria were placed in chambers at 20 ± 1 C and 9 ± 1 C. From each aquarium, 2 groups of 2 animals each were taken at the 17th and 60th days. The animals were killed, the soft tissue dissected and the lipids extracted and analyzed as described previously (1).

To study the compositional changes evoked

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by the environment, a group of animals collected in October from Lake Nahuel Huapi was transported and kept in the Río de la Plata estuary until December (the temperature of the water varied from 14 to 18 C). They were immersed 2 m deep, in a cage lying at the bottom of the estuary. At that time, 4 groups of 2 animals each were processed separately to obtain the total lipids.

Eight groups of 2 animals each, maintained for 60 days at 20 C and 9 C, were used for the experiments with radioactive tracers. Five μmol (5 μCi) of [1-^{14}C]ammonium linoleate or [1-^{14}C]ammonium α-linolenate complexed with albumin was added to 250-mL flasks containing each group. The animals were kept for 2 hr at the specified temperatures. After that time, they were killed, washed with sufficient distilled water, and the lipids extracted and analyzed.

Extraction and Analysis of Lipids and Fatty Acids

Total lipids of soft tissues were extracted with $CHCl_3/CH_3OH$ (2:1, v/v) and weighed as described previously (1). They were separated into components by thin layer chromatography (TLC) on Silica Gel G, using plates of 20 x 40 cm. The phospholipids were separated first with $CHCl_3$/methanol/acetic acid/water (65:25:4:4, v/v) in the lower part. The plate was then dried and the neutral lipids were separated in the second half with petroleum ether/ethyl ether/acetic acid (80:20:1, v/v). Spots were revealed by charring and were quantitated by densitometry (3). Aliquots of total lipids were saponified, and the recovered fatty acids were extracted and esterified. The methyl esters of fatty acids were analyzed by gas liquid chromatography (GLC) as already described (1).

In the experiments with radioactive tracers, the lipids were again separated by TLC and the radioactivity was measured on the plates with a scanner (Berthold, Germany).

RESULTS AND DISCUSSION

Fasting Effect

It has already been shown that, in the natural habitat, the lipid composition of *D. patagonicus* is little modified throughout the year (1). Triacylglycerols, however, decreased in winter. The lipid content of the Diplodom species is decreased by fasting. In an experiment in which *D. patagonicus* was fasted in the aquarium at room temperature (15-20 C) for 55 days, a decrease of the lipid content from 1.26 to 1.06% (wet wt of soft tissue) was found. However, 17 days of fasting in the aquarium maintained at 9 C did not modify significantly the composition of either the lipids of the mollusk or the fatty acids. Even an increase of temperature to 20 C, which could be expected to enhance the metabolism and consumption of energy stores of these animals, did not modify these results (Fig. 1). However, after 60 days of fasting, it was shown that both groups of mollusks, whether maintained at 9 C or 20 C, significantly decreased the proportion of triacylglycerols and diacylglycerol ethers. Moreover, at both temperatures, the decrease was the same. The triacylglycerols decreased from 24.1 ± 0.9% of total lipid to half of this value and the diacylglycerol ethers from 11.1 ± 0.8% to one-twelfth of this figure. The proportion of free fatty acids and phosphatidylcholine plus ceramide amino ethyl phosphonate increased correspondingly (Fig. 1). In consequence, the ratio of polar to nonpolar lipids increased from 0.8 to 1.5.

Table 1 shows that the fatty acid composition of *D. patagonicus* was not, for practical purposes, changed during 17, and even 60 days, of fasting. Therefore, long periods of fasting are apparently required to compel the mollusk to use its storage triacylglycerol for energy, but this metabolism would not select specific acids.

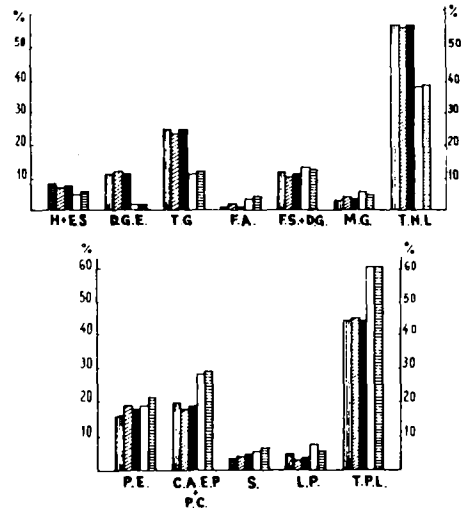

FIG. 1. Effect of fasting and environmental temperature on lipid composition of *D. patagonicus*. Composition: original (▥); after 17 days of fasting at 20 C (▨); after 17 days of fasting at 9 C (■); after 60 days of fasting at 20 C (□); after 60 days of fasting at 9 C (▤). H: hydrocarbons, ES: esterified sterols, DGE: diacylglycerol ethers, TG: triacylglycerols, FA: fatty acids, FS: free sterols, DG: diacylglycerols, MG: monoacylglycerols, TNL: total neutral lipids, PE: phosphatidylethanolamine, CAEP: ceramide aminoethyl phosphonate, PC: phosphatidylcholine, S: sphingomyelin, LP: lysophospholipids, TPL: total phospholipids.

TABLE 1

Effect of Fasting and Environmental Temperature on Fatty Acid Composition of *D. patagonicus*[a]

Fatty acids	Before fasting 13 C[b]	After 17 days of fasting 20 C	After 17 days of fasting 9 C[c]	After 60 days of fasting 20 C[c]	After 60 days of fasting 9 C[c]
14:0	3.6 ± 0.5	2.8	3.2 ± 0.3	2.4 ± 0.2	3.5 ± 0.6
15:0 + X_1	1.9 ± 0.3	2.4	2.2 ± 0.3	1.5 ± 0.3	1.5 ± 0.5
16:0	17.6 ± 1.3	15.3	13.1 ± 1.5	16.2 ± 1.1	16.4 ± 1.2
16:1	3.8 ± 0.9	4.2	4.9 ± 0.4	3.0 ± 0.5	3.0 ± 0.6
17:0 + X_2	2.4 ± 0.6	2.2	1.4 ± 0.5	1.2 ± 0.3	1.7 ± 0.6
18:0	8.6 ± 1.2	8.4	7.1 ± 1.0	8.2 ± 0.6	7.8 ± 0.6
18:1ω9	5.3 ± 0.4	5.3	5.1 ± 0.7	4.2 ± 0.5	3.8 ± 0,2
18:2ω6	8.8 ± 1.4	10.6	11.2 ± 1.2	7.4 ± 0.9	8.8 ± 0.5
18:3ω6	t	t	0.3 ± 0.1	t	t
20:1 + 18:3ω3[d]	10.8 ± 2.1	12.3	14.3 ± 1.9	17.0 ± 1.1	12.4 ± 0.3
18:4ω3	1.2 ± 0.5	1.5	1.0 ± 0.6	t	0.7 ± 0.2
20:3	0.5 ± 0.2	1.6	1.0 ± 0.6	t	0.7 ± 0.2
20:4ω6[e] + 22:1 NMI 22:2[f] + 20:4ω3	17.2 ± 2.1	15.5	15.6 ± 1.9	19.5 ± 1.8	21.7 ± 1.2
20:5ω3	6.3 ± 0.5	7.4	7.9 ± 0.5	9.8 ± 0.4	7.0 ± 0.5
X_3	2.7 ± 1.3	3.3	3.9 ± 0.8	1.7 ± 1.3	2.5 ± 0.4
22:4ω6	1.6 ± 0.3	1.7	2.3 ± 0.3	2.1 ± 0.2	1.9 ± 0.2
22:4ω3	2.7 ± 0.2	2.2	2.8 ± 0.3	3.2 ± 0.2	3.1 ± 0.1
22:5ω3	2.7 ± 0.5	1.3	1.4 ± 0.1	1.3 ± 0.3	1.7 ± 0.2
22:6ω3	0.7 ± 0.2	0.8	0.5 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
	1.6 ± 0.2	1.2	0.8 ± 0.1	0.5 ± 0.1	1.1 ± 0.2

[a]Measured on EGSS-X at 180 C.

[b]Results expressed as the mean of 5 samples ± extreme deviation of the mean.

[c]Results expressed as the mean of 2 samples ± extreme deviation of the mean.

[d]20:1 ca. 90% of total.

[e]20:4ω6 ca. 80% of total.

[f]22:2 ca. 80% of total.

Temperature Effect

There is good evidence that, in many animals, the adaptation to a lower temperature produces an increase of the unsaturation index of the fatty acids, largely through an increase of polyenoic acids of 20 and 22 carbons, and a decrease of palmitic and stearic acids (4-7). This result has been interpreted as one of the mechanisms used by poikilothermic animals to maintain the fluidity of membranes at an appropriate level.

However, we found that seasonal changes modifying the lipid distribution of *D. patagonicus* did not significantly alter the fatty acid composition in spite of lake temperature variations between 6 C and 13.5 C (1). These results were attributed to an invariant food consumption based on a rather constant food composition. This apparent independence of fatty acid composition of *D. patagonicus* from habitat temperature was also noted in *Mesodesma mactroides* (8), *Chlamys tehuelcha* (9) and *Mytilus platensis* (10). In those marine mollusks, preferential food composition was suggested as determining the final composition of the fatty acids of the animal. These results are apparently confirmed in the present experiments in which the mollusks were maintained at 9 C and 20 C in conditions that eliminated any food effect (Table 1 and Fig. 1). After 60 days of fasting, mollusks maintained at 9 C and 20 C did not modify their relative fatty acid compositions and only partially changed their lipid distributions.

However, the temperature of the habitat modifies the intake and incorporation of fatty acids added to the medium. When the ammonium salts of ^{14}C-labeled linoleic or α-linolenic acids bound to albumin were added to the flasks containing the mollusks, it was found that after a 2-hr period, significant radioactivity was incorporated into the lipids of the animal (Fig. 2). The amount incorporated depended on the temperature. Animals adapted to 20 C showed an incorporation higher than at 9 C. After a period of adaptation of 60 days at the aforementioned temperatures, ca. 25 and 32% of the label of 18:2 and 18:3 acids were incorporated, respectively, at 20 C, whereas the incorporation was only ca. 11 and 15%, respectively, at 9 C. Therefore, these results show again that the temperature of the habitat

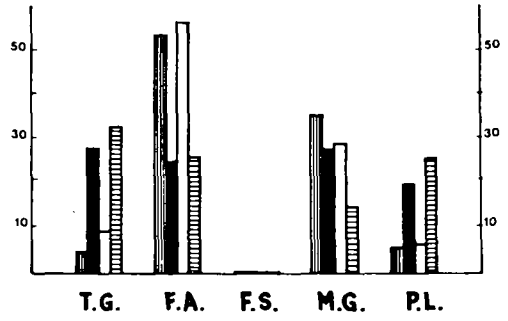

FIG. 2. *D. patagonicus* uptake of fatty acids and incorporation into lipids. Distribution of radioactivity. Incubation with 18:2ω6 at 9 C (▥); incubation with 18:2ω6 at 20 C (■); incubation with 18:3ω3 at 9 C (□); incubation with 18:3ω3 at 20 C (▤). TG: triacylglycerols, FA: fatty acids, FS: free sterols, MG: monoacylglycerols, PL: phospholipids.

determined the amount of fatty acid incorporated but did not modify the saturated/unsaturated acid ratio of ca. 0.76 and 0.74, respectively, for these temperatures.

Figure 2 shows that, in mollusks adapted to 9 C for 60 days, the labeled acids were largely "incorporated" as free acids, whereas at 20 C less radioactivity was found in free acids and more was incorporated into triacylglycerols and phospholipids. This could be explained by temperature activation of lipid-metabolizing reactions.

All these experiments would suggest that the fatty acid composition of the mollusk is largely dependent on the food composition. If so, the change of environmental temperature over the range studied would modify the amount of fatty acids incorporated without changing the fatty acid unsaturation.

Effect of Natural Habitat Change

The effect of natural habitat on the fatty acid composition of *D. patagonicus* was studied by transferring the mollusk from Lake Nahuel Huapi to the Río de la Plata River. It signified a change from a snow-melt water lake in the Andes mountains, poor in nutrients, to a warm river 1,600 km away and on the Atlantic coast, rich in nutrients and plankton. The zone into which the mollusks were placed may be considered an estuary, in spite of limited oceanic influences and low salinity. The Nahuel Huapi specimens were collected and transferred in October. They were analyzed in December and compared to fresh specimens collected in Nahuel Huapi in the same December to eliminate seasonal factors.

Results are presented in Figures 3 and 4 and show small changes in the lipid distribution,

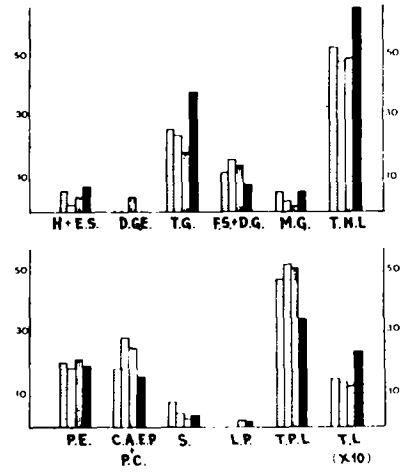

FIG. 3. Effect of habitat change on lipid composition. *D. patagonicus* from lake, collected in October (▤); *D. patagonicus* from lake, collected in October and maintained in estuary until December (□); *D. patagonicus* from lake, collected in December (▥); *D. variabilis* from estuary, collected in December (■). The other references are the same as those in Fig. 1.

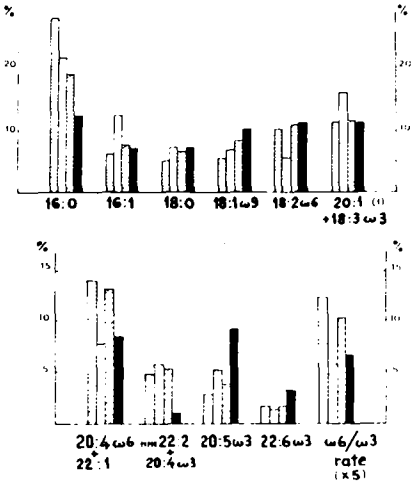

FIG. 4. Effect of habitat change on fatty acid composition. The references are the same as those in Fig. 3. (1): In the second bar, ca. 35% corresponds to the α-18:3 acid. In the other bars, it is 10%.

whereas there are large changes recognizable in the fatty acid composition (Fig. 4). The small changes of the lipid distribution were, however, in general similar in both groups of mollusks, either that remaining in the lake or that transferred to the Río de la Plata. Some of these changes, such as the proportion of triacylglycerols, were higher in the mollusks from the lake than in the animals carried to the Río de la

Plata and may be attributed to a seasonal effect.

Large changes in fatty acid composition were especially obvious in the fatty acids of the linoleic and α-linolenic acid series. Linoleic and arachidonic acids, very significant components of *D. patagonicus* from the lake, were reduced to ca. 1/2 the original value when the animals were carried to the Río de la Plata, whereas ω3 acids such as 20:5 and 18:3 were increased (Fig. 4).

These changes can be correlated with the fatty acid composition of the lake and estuary sediments, respectively (Table 2). In Lake Nahuel Huapi, the sediment was rich in linoleic acid, with values about 12% in winter (samples taken in August and July, Table 2). However, the content of α-linolenic acid was less than 4%, and 20:5ω3 only reached about 2%. In the Río de la Plata area, where *D. patagonicus* had been kept, the opposite distribution was found. Linoleic acid was only about 5%, whereas the α-linolenic was about 14% (Table 2).

The fatty acid composition of *D. patagonicus* in the original habitat would show considerable terrestrial influence as indicated by a very significant predominance of ω6 acids. In the Río de la Plata, a marine influence would be shown by the increase of ω3 acids. However, both acids from the substrate, linoleic (ω6) and α-linolenic (ω3), are of vegetable origin, and would be converted to 20:4ω6 and 20:5ω3, respectively, by the mollusk (Fig. 4). Moreover, it is significant that the composition of the transferred *D. patagonicus* approaches the fatty acid pattern of the *D. variabilis*, a related species natural to the Río de la Plata (Fig. 4). However, in spite of the decrease of arachidonate and an increase of eicosapentaenoate, the compositions of both *D. patagonicus* and *D. variabilis* living in the Río de la Plata differ significantly from marine bivalves. In marine bivalves, the arachidonic acid does not usually exceed 4% whereas the 20:5ω3 is higher than 10% (7-9).

The adaptation of *D. patagonicus* to the new habitat and the influence of the new food composition on the mollusk may be easily shown by the ratio of ω6/ω3 acids. In *D. patagonicus* from Lake Nahuel Huapi, this ratio lies between 2.5 and 2.0 whereas, in the mollusk adapted to the Río de la Plata estuary, it was decreased to 1.1. In the autochthonous related species, it was 1.2. It may be concluded that the stability (1) of the typical fatty acid composition of *D. patagonicus* living in Lake Nahuel Huapi, particularly rich in ω6 and poor in ω3 acids, would be the consequence of a food of rather stable composition, rich in linoleic acid, and of little sensitivity of the animal to temperature and seasonal changes.

TABLE 2

Percentage Fatty Acid Composition of Sediment from Lake Nahuel Huapi and Río de la Plata Estuary[a]

Fatty acids[b]	Lake Nahuel Huapi	Río de la Plata estuary
12:0	1.5 ± 0.5	t
14:0	2.4 ± 1.2	5.2 ± 0.1
15:0 + X[c]	t	7.1 ± 0.2
16:0	20.8 ± 0.7	24.4 ± 0.4
16:1	24.4 ± 0.9	22.4 ± 1.9
18:0	5.7 ± 0.2	5.5 ± 1.3
18:1ω9	25.2 ± 0.4	12.2 ± 0.7
18:2ω6	12.2 ± 0.4	5.3 ± 0.0
18:3ω3	3.7 ± 0.1	14.0 ± 0.1
18:4	t	0.5 ± 0.5
20:4ω6	2.1 ± 0.4	1.2 ± 0.2
20:5ω3	2.0 ± 0.5	2.2 ± 0.4

[a]Lipid in sediment (% wet wt): Nahuel Huapi, 0.010 ± 0.003; Río de la Plata (Río Santiago), 0.028 ± 0.001.

[b]Results are the mean of the analysis of 2 samples ± extreme deviation of the mean.

[c]Peak X corresponds to a saturated fatty acid. The composition was calculated from a gas liquid chromatogram.

The effect of habitat is not only shown by the increase of ω3 acids but also by that of the 16:1 (Fig. 4). The increase of this acid is a typical effect of the habitat in the *D. patagonicus* since it was not found in *D. patagonicus* remaining the the lake, and even in *D. variabilis*. By contrast, a decrease of palmitic acid was shown in both *D. patagonicus* samples, and could be related to seasonal changes (1).

In *D. patagonicus*, a very constant and high concentration of 22:2 was also found, in spite of season (1) and habitat changes (Fig. 4). However, the acid is a minor component of the *D. variabilis*. It has been identified as a non-methylene-interrupted acid (1). Non-methylene-interrupted acids, 20:2 and 22:2, have been recognized in some mollusks (11). However, the origin of these acids is still open to discussion, since they could be synthesized endogenously by the animal (12) or have an exogenous formation.

In *D. patagonicus*, the extremely constant concentration of 22:2 acid, in spite of season and habitat variations, as well as the low concentration found in *D. variabilis*, would favor the hypothesis of a direct biosynthesis in *D. patagonicus*.

Similarly, the concentration of eicosenoic acid is also constant, in spite of a change of habitat (Fig. 4), or after fasting (Table 1). This result would suggest, coincidently with a recent

report by Ackman et al. (13), that its origin is probably endogenous, and that it would be synthesized by elongation of 18:1 acid. Unfortunately, we have not as yet been able to determine experimentally the position of the double bond.

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METHODS

Effect of Ca^{++} on Triphosphoinositide Extraction in Fusing Myoblasts

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ABSTRACT

Ca^{++}-dependent degradation of triphosphoinositide has been postulated to regulate levels of membrane-bound Ca^{++} and to generate a 1,2-diacylglycerol fusogen in cell fusion. Triphosphoinositide metabolism was therefore studied during Ca^{++}-induced fusion of cultured chick embryo myoblasts. Using a frequently cited extraction procedure, it was found that apparent Ca^{++}-dependent triphosphoinositide degradation was actually due to inhibition of extraction. A new procedure using the ion-pairing reagent tetrabutylammonium sulfate was developed which was unaffected by Ca^{++} and gave 2- to 20-fold greater extraction of triphosphoinositide than existing procedures. With this procedure, no changes in triphosphoinositide metabolism were found during myoblast fusion.

The quantitatively minor plasma membrane lipid TPI has been reported to be rapidly degraded in response to stimuli which increase intracellular Ca^{++} (1-5). The Ca^{++}-dependent degradation of TPI to 1,2-diacylglycerol and inositol-1,4,5-triphosphate might then amplify the effect of a *trans*-membrane Ca^{++} signal by causing the release of TPI-bound Ca^{++} from the plasma membrane inner leaflet (2,6). In addition, it has been postulated that the generation from TPI of the known fusogen 1,2-diacylglycerol may be involved in Ca^{++}-dependent membrane fusion processes such as secretion (7,8) and cell-cell fusion (9).

In order to determine if the latter proposed mechanism might be involved in a naturally occurring cell fusion process, TPI metabolism was examined in rapidly fusing chick myoblasts. During the course of this study, it was discovered that changes in extracellular and/or intracellular Ca^{++} concentration can markedly alter the extractability of TPI. This could lead to erroneous conclusions regarding the role of TPI turnover in various Ca^{++}-dependent processes. Accordingly, various procedures were examined for their ability to extract TPI in the presence or absence of Ca^{++}. In addition, a new extraction procedure which uses TBAS was developed. This procedure extracted significantly greater amounts of TPI than previously described procedures and was unaffected by the presence of Ca^{++}. Using this procedure, no changes in TPI metabolism were found to occur during myoblast fusion.

METHODS

Culturing of Myoblasts

Myoblast cell cultures were prepared from breast muscle of 12-day white leghorn chick embryos as described previously (10), except that 2.5 mg/mL collagenase (Worthington Type III) was used in place of trypsin. Cells plated at 1.5×10^6/60 mm dish were grown in Ca^{++}-free DMEM containing 10% fetal bovine serum (treated with Chelex-100 resin) and 1% 11-day chick embryo extract. The final concentration of Ca^{++} in the medium was adjusted to 0.1 mM, which permits the normal differentiation of mononucleated myoblasts but prevents myotube formation (11). After 30-40 hr, the cells were fed with fresh medium containing 10^{-5} M cytosine arabinoside to kill any proliferating cells. Rapid fusion of three-day-old myoblasts was initiated by addition of Ca^{++} to a final concentration of 2 mM.

Thin Layer Chromatography

Unlabeled TPI standard was added to extracts of ^{32}P-labeled phospholipids, which were then analyzed by one-dimensional chromatography on 0.2 mm Mylar-backed Silica Gel-60 plates (E. Merck) developed sequentially in 2 solvent systems. The plate was developed twice

Abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TBAS, tetrabutylammonium sulfate; TPI, triphosphoinisitide.

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with chloroform/methanol/ammonium hydroxide/water (60:35:2.5:2.5, v/v/v/v) to move the monophosphorylated phospholipids into the upper two-thirds of the plate. After the second development, phospholipid standards in a separate lane were located by spraying with Phospray (Supelco). The second solvent system (n-propanol/4.3 N ammonium hydroxide, 65:35, v/v) was then allowed to run until the front reached the phosphatidylinositol standard spot (ca. one-third up the plate) to resolve TPI. TPI was located by spraying with Phospray. Plates were dried with a hair dryer at ambient temperature for 15 min between developments. Radioactive phospholipids were located by autoradiography (X-Omat RP film from Kodak), scraped into vials and counted in a scintillation counter using 3 mL ACS (Amersham) and 1.5 mL H_2O to form a gel.

RESULTS

A commonly used procedure for TPI extraction uses KCl in the aqueous phase to promote partitioning of the polar TPI into the organic phase (12). With this procedure, the addition of Ca^{++} (2 mM) to initiate myoblast fusion caused an apparent 87% decrease in ^{32}P-labeled TPI (Table 1). Since this decrease occurred within 15 sec of Ca^{++} addition (data not shown), it seemed that Ca^{++} might interfere with TPI extraction, even in the presence of 2 M KCl. When 2 mM EDTA was present in the extraction mixture, the Ca^{++}-dependent decrease in labeled TPI was partially prevented (Table 1, experiment 1). When another commonly used TPI extraction solution containing HCl was used, no significant Ca^{++}-induced difference in TPI metabolism was detected. Fur-

TABLE 1

Effects of Ca^{++} and Extraction Procedures on Triphosphoinositide Extraction[a]

		[^{32}P]-Triphosphoinositide cpm/dish	
	Ca^{++}	Exp 1[b]	Exp 2[c]
C:M:KCl[d]	–	5400 ± 2300	–
	+	700 ± 180	–
C:M:EDTA[e]	–	4950 ± 620	890 ± 390
	+	2340 ± 90	170 ± 30
C:M:HCl[f]	–	20000 ± 700	3700 ± 200
	+	23500 ± 500	3900 ± 340
C:M:TBAS[g]	–	52600 ± 2400	18000 ± 1900
	+	47500 ± 1000	23000 ± 1700

[a]Three-day myoblasts were washed once in HEPES-buffered DMEM and then incubated for 30 min in 1.5 mL HEPES-buffered DMEM containing 50 μCi/mL [^{32}P]-PO_4^{3-}. The labeled medium was removed and the indicated extracting solution was immediately added to the dish. After scraping the dish with a rubber policeman, the solution was transferred to a test tube for completion of the extraction procedure. Label in TPI was determined after thin-layer chromatography as described in Methods. Triphosphoinositide values represent the mean cpm/dish ± 1/2 the range of duplicate 60 mm dishes.

[b]Where indicated, the cells were incubated with 2 mM Ca^{++} for the entire 30-min labeling period.

[c]Where indicated, Ca^{++} (2 mM) was added 5 min before harvesting the cells.

[d]The cells were scraped in 4 mL chloroform/methanol/water (1:2:0.8, v/v/v). After transfer to a test tube, 1 mL each of chloroform and 2 M KCl were added sequentially to complete the extraction. After centrifugation, the aqueous upper phase was discarded and the chloroform layer was washed 3X with theoretical upper phase.

[e]The cells were scraped in 4 mL chloroform/methanol/2 mM EDTA (1:2:0.8, v/v/v). The extraction was completed by sequential addition of 1 mL chloroform and 1 mL H_2O. After centrifugation, the aqueous phase was discarded and the chloroform layer was washed 3X with theoretical upper phase.

[f]The cells were scraped in 3 mL chloroform/methanol/HCl (conc.) (200:200:1, v/v/v). After transferring to a test tube, the mixture was evaporated under N_2. Chloroform (1 mL) was added and washed with 1 mL 0.1 N HCl and then 3X with 1 mL of theoretical upper phase made with chloroform/0.1 N HCl (1:1, v/v).

[g]The cells were scraped in 3 mL chloroform/methanol (1:1, v/v) containing 10 mM tetrabutylammonium sulfate (TBAS). After transfer to a test tube, the extracts were evaporated under N_2. Chloroform (1 mL) was added and washed once with 1 mL 0.1 N HCl and 3X with 1 mL of theoretical upper phase made with chloroform/0.1 N HCl (1:1, v/v).

thermore, the HCl method extracted ca. 4-fold more TPI from control (low Ca^{++}) myoblasts than either the KCl or EDTA method. These results suggested that Ca^{++} inhibited the extraction of the highly charged TPI and raised the possibility that additional TPI remained unextracted with the HCl method. The development of a more efficient extraction procedure was therefore undertaken. A method was developed in which the amphiphilic ion-pairing reagent TBAS was used to displace Ca^{++} from the TPI and facilitate its extraction by formation of a lipophilic ion pair. As the data in Table 1 show, the TBAS method extracted 2- to 5-fold more TP than the HCl method. Using the TBAS method, no significant effect of Ca^{++} on TPI metabolism was detected. Thus, Ca^{++} does not appear to stimulate myoblast fusion by activating a TPI-specific hydrolase which would result in increased diacylglycerol levels.

DISCUSSION

Several laboratories have reported alterations in TPI metabolism in tissues treated with agents whose effects are mediated by changes in intracellular Ca^{++}. These agents include acetylcholine and other muscarinic agonists in rabbit iris muscle (3,4), the polypeptide hormones ACTH and vasopressin in rat brain (5), and the divalent cation ionophore A23187 in guinea pig brain synaptosomes (8). These findings have led to the postulate that Ca^{++}-dependent TPI turnover is a common response to agents which increase intracellular Ca^{++}. Such turnover, it is postulated, would have the result of releasing TPI-bound Ca^{++} from the plasma membrane inner leaflet, thus further increasing the Ca^{++} concentration and amplifying subsequent Ca^{++}-dependent responses. The present finding that Ca^{++} inhibits extraction of myoblast TPI under certain extraction conditions suggests the possibility that, in some instances, the apparent Ca^{++}-dependent degradation of TPI in other cell types may have resulted from decreased TPI extraction rather than from increased turnover. The results presented here demonstrate that it is essential to carry out appropriate controls to establish the Ca^{++}-independence of the TPI extraction method employed, particularly in situations where levels of TPI-bound Ca^{++} may vary. Where possible, evidence for alterations in TPI turnover should include quantitation of the TPI hydrolysis products (e.g., 1,2-diacylglycerol, inositol phosphates, diphosphoinositide or phosphatidylinositol).

Extraction mixtures containing the ion-pairing reagent TBAS extracted 2 to 20 times more TPI than the polar lipid extraction methods described previously (the KCl and HCl methods). In the absence of a quantitative method to determine the total cellular TPI, the absolute efficiency of TPI extraction in the presence of TBAS could not be determined. It is clear, however, that our results raise the possibility that previous measurements of TPI levels in various tissues may have resulted in erroneously low values. Use of the more efficient TBAS method should provide a more accurate estimate of total TPI in these tissues.

When those methods which are not affected by Ca^{++} (HCl and TBAS) were used, no Ca^{++}-induced alterations in labeled TPI were observed during Ca^{++}-dependent myoblast fusion. Furthermore, no changes in the levels of possible degradation products of TPI such as diacylglycerol and inositol phosphates or an increase in diphosphoinositide or phosphatidylinositol, could be detected (data not shown). Using an HCl extraction method, Allan and Michell (9) found degradation of TPI under conditions in which erythrocytes can be induced to fuse. The present findings suggest that the erythrocyte model fusion system differs mechanistically from naturally occurring fusion or that TPI degradation in the erythrocyte system may not be related directly to fusion.

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Separation of Pairs of C-24 Epimeric Sterols by Glass Capillary Gas Liquid Chromatography

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ABSTRACT

Pairs of C-24 epimeric sterols have been very difficult to separate by physical methods. We report here the partial or complete separation of the trimethylsilyl ethers of nine pairs of C-24 epimeric sterols by gas liquid chromatography on a glass capillary column coated with SP-2340. The trimethylsilyl ethers of the epimeric pairs of sterols with saturated side chains and a pair with two double bonds in the side chain were completely separated from each other by GLC. The epimeric pairs with a double bond at C-22 showed partial separation. The 24β-epimer with a saturated side chain eluted before the corresponding 24α-epimer. This order was reversed for pairs of C-24 epimeric sterol trimethylsilyl ethers containing a double bond in the side chain at C-22.

INTRODUCTION

The assignment of the stereochemical configuration at C-24 of sterols has always been a challenging aspect of structural identification of sterols isolated from foods and other natural products. Until the early 1970s, specific rotations were used in assigning stereochemical configuration at C-24. Unfortunately, the differences in specific rotations were often very small, making these assignments tenuous, especially when mixtures of the C-24 epimers were present.

The discovery that proton magnetic resonance (PMR) spectroscopy could be used to distinguish the C-24 epimeric sterols and stearanes was a major advance in this field of research (1-6). For example, work done with PMR confirmed the correctness of the original conclusion (7) that the principal sterol from *Chlorella ellipsoidea* was 22,23-dihydrobrassicasterol (24β-methyl) and not campesterol (24α-methyl) (1). In a similar way, it was verified that C-24 epimeric sterols, such as brassicasterol and campesterol, can occur together in rapeseed oil (1). By use of PMR spectroscopy, it was found that 24-ethylcholesterol from a series of bryophytes and primitive and advanced tracheophytes usually has the 24α-configuration, whereas the 24-methylcholesterol from the same sources consisted of an epimeric mixture (8,9). PMR analyses permitted the first characterization of campesta-5,22E-dien-3β-ol (22-dehydrocampesterol) from the marine diatom, *Phaeodactylum tricornutum* (10), and the assignment of configuration at C-24 of sterols from the Atlantic scallop, *Placopecten magellanicus*, with quantitative estimation of the α- and β-epimers (11). PRM analyses permitted the first identification of both brassicasterol and 22-dehydrocampesterol in the oyster, *Crassostrea virginica* (12). Thus, PMR spectroscopy was the only tool available for determining both sterol configurations at C-24 and for semiquantitation of a mixture of epimers. The principal disadvantages of the PMR spectroscopic method for analyses of C-24 epimeric sterols is the limited availability of NMR instruments of 100 MHz or higher, and the requirement for quantities of sterol sometimes not easily obtainable from complex biological samples.

Since the early work of Beerthuis and Recort (13), gas liquid chromatography (GLC) has been recognized as an extremely powerful tool for separating and identifying sterols. In a study of GLC analysis of 92 sterols, each could be distinguished from the other, except for pairs of C-24 epimeric sterols (14). However, the first successful separation of the trimethylsilyl (TMS) ethers of the diastereoisomers of α-tocopherol by GLC on a glass capillary column (15) strongly suggested that C-24 epimeric sterols could be separated by this method. We have examined the TMS derivatives of nine pairs of C-24 epimeric sterols by capillary GLC and now report the physical separation of these epimeric compounds.

MATERIALS AND METHODS

Sterols

Samples of brassicasterol and 22,23-dihydrobrassicasterol were from earlier syntheses (16,17). The dihydrobrassicasterol and porif-

Trivial and Equivalent Names of Sterols[a]

Brassicasterol =	Ergosta-5,22E-dien-3β-ol
22-Dehydrocampesterol =	Campesta-5,22E-dien-3β-ol
22,23-Dihydrobrassicasterol =	Ergost-5-en-3β-ol
Campesterol =	Campest-5-en-3β-ol
Ergostanol =	Ergostan-3β-ol
Campestanol =	Campestan-3β-ol
Δ^7-Ergostenol =	Ergost-7-en-3β-ol
Δ^7-Campestenol =	Campest-7-en-3β-ol
Poriferasterol =	Poriferasta-5,22E-dien-3β-ol
Stigmasterol =	Stigmasta-5,22E-dien-3β-ol
Clionasterol =	Poriferast-5-en-3β-ol
Sitosterol =	Stigmast-5-en-3β-ol
Chondrillasterol	Poriferasta-7,22E-dien-3β-ol
Spinasterol =	Stigmasta-7,22E-dien-3β-ol
Δ^7-Poriferastenol =	Poriferast-7-en-3β-ol
Δ^7-Stigmastenol = schottenol =	Stigmast-7-en-3β-ol
25-Dehydrochondrillasterol =	Poriferasta-7,22E,25-trien-3β-ol
25-Dehydrospinasterol =	Stigmasta-7,22E,25-trien-3β-ol

[a]For the sterols, we have used ergostane, campestane, stigmastane, and poriferastane as stem names and this obviates use of α and β and R and S when referring to 24-alkyl sterols. However, according to IUPAC/IUB 1967 Revised Tentative Rules for Steroid Nomenclature (Biochem. J. 113, 5[1969]), brassicasterol, campesterol, campestanol, Δ^7-campestenol, chondrillasterol, poriferasterol, sitosterol, Δ^7-stigmastenol, and 25-dehydrospinasterol have the 24R configuration. 22-Dehydrocampesterol, 22,23-dihydrobrassicasterol, ergostanol, Δ^7-ergostenol, spinasterol, stigmasterol, 22,23-dihydroporiferasterol, Δ^7-poriferastenol, and 25-dehydrochondrillasterol have the 24S configuration.

erasterol were also obtained from *C. ellipsoidea* (7). Campesterol was isolated from a soybean sterol mixture by several crystallizations from acetone. Stigmasterol was a gift from Upjohn (Kalamazoo, MI). Campestanol and ergostanol were obtained by catalytic hydrogenation of campesterol and 22,23-dihydrobrassicasterol, respectively. The Δ^7-campestanol was obtained from hydrogenation of 7-dehydrocampesterol in the presence of Raney nickel. Sitosterol and 22,23-dihydroporiferasterol were prepared from stigmasterol and poriferasterol, respectively, by a published method (18). Chondrillasterol, Δ^7-poriferastenol and Δ^7-ergostenol were isolated from *Chlorella emersonni* (19). Spinasterol and Δ^7-stigmastenol were isolated from alfalfa leaves. 25-Dehydrochondrillasterol and 25-dehydrospinasterol were gifts from W. Sucrow (University of Paderborn, Paderborn, West Germany). 22-Dehydrocampesterol was isolated from a marine diatom *P. tricornutum* (10).

Derivatization

The sterol TMS ethers were prepared by adding 100 μL of pyridine and 100 μL of Regisil (Regis Chemical, Morton Grove, IL) to 1 mg or less of sterol, allowing it to stand for 30 min, adding about 1 mL of petroleum ether and evaporating to dryness under nitrogen. The TMS ethers were redissolved in isooctane to give solutions containing approximately 2 μg/μL.

Gas Liquid Chromatography

The TMS ether of each sterol was analyzed separately by capillary GLC and then as an epimeric mixture. The GLC analyses were made on a 115 m × 0.25 mm glass capillary column coated with SP-2340, a cyanosilicone phase of chromatographic properties similar to those of polar polyester phase. The column, purchased from Quadrex Corp., New Haven, CT, provided ca. 290,000 theoretical plates (n) and 230,000 effective theoretical plates (N) for cholesterol TMS ether. The capacity ratio or partition ratio (k) for cholesterol TMS ether was ca. 6 at 195 C oven temperature (20).

The chromatograph was a Hewlett Packard Model 5840, equipped with a flame ionization detector, an automatic liquid sampler (Model 7671A), and an all-glass J&W splitter (J&W Scientific, Inc., Orangevale, CA). The chromatographic conditions were: attn. 0; split ratio, 1/50 to 1/100; sample size, ca. 1.7 μL of a solution containing ca. 2 μg/μL; injection port temperature, 280 C; column temperature, 185 or 195 C; detector temperature, 300 C; carrier gas, H_2 at 20 to 34 psig; average linear velocity, 19 cm/sec. Helium, rather than hydrogen, was tried as the carrier gas but it resulted in a doubling of retention time and a concomitant decrease in peak size and symmetry. The oven was maintained under a positive pressure of nitrogen and the split flow was vented out the window to minimize the danger from the use of hydrogen as the carrier gas. Nitrogen was

introduced at the end of the column as the auxillary gas at a flow rate of 1.4 times that of the hydrogen flow to the detector. Peak areas were determined by integrating 0.02-min slices under the curve and summing the relevant portions to obtain peak areas.

RESULTS

The separations of the TMS ethers of nine pairs of C-24 epimeric sterols are shown in Figures 1 and 2. Cholesterol TMS ether was included in each sample as an internal standard but it does not appear on the short section of the chromatogram shown. The TMS ethers of epimeric pairs of sterols with saturated side chain are completely separated, as in the case of campesterol and 22,23-dihydrobrassicasterol, whereas those pairs with a double bond at C-22 are less well separated (brassicasterol and 22-dehydrocampesterol). In the chromatography of these 9 epimeric pairs of sterols, it was consistently observed that the 24β-epimers of sterols with saturated side chains eluted before the corresponding 24α-epimers (Table 1, and Figs. 1 and 2). The order was reversed for epimeric pairs of sterols, such as brassicasterol-22-dehydrocampesterol, poriferasterol-stigmasterol, and chondrillasterol-spinasterol, that contain a double bond in the side chain at C-22 (Figs. 1 and 2).

Comparison of the results for the separation of epimers in Figures 1 and 2 shows that the oven temperature of 185 C gave slightly better separations than the oven temperature of 195 C at the expense of longer retention times.

Quantitative measurements for chromatographed samples of prepared mixtures of known concentrations of the difficult-to-separate brassicasterol and 22-dehydrocampesterol were in close agreement with actual concentrations (Fig. 3). Precision was good and coefficients of variation ranged from 0.9 to 6.2%. Evaluation of the precision of the relative retention times for several sterol TMS ethers indicated that the coefficient of variation was extremely small, ranging from 0.037 to 0.147% (Table 2).

In this study and in the earlier study of the separation of diastereoisomers of tocopherol

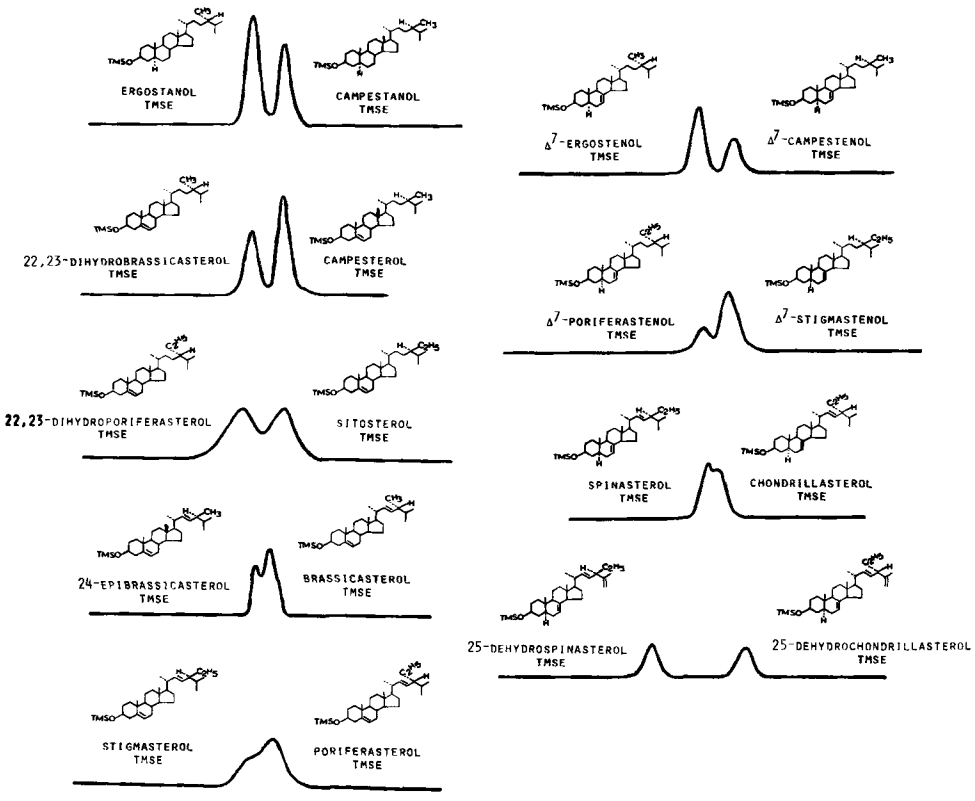

FIG. 1. Gas chromatographic separation of pairs of C-24 epimeric sterol trimethylsilyl ethers on SP-2340 at 195 C (elution time from left to right).

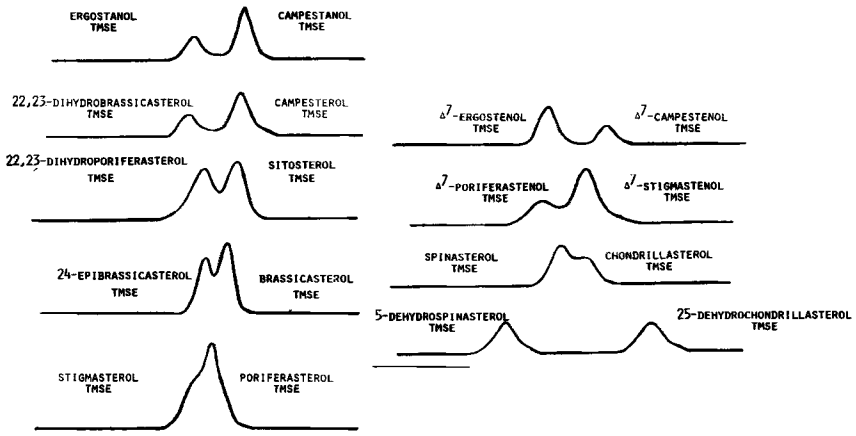

FIG. 2. Gas chromatographic separation of pairs of C-24 epimeric sterol trimethylsilyl ethers on SP-2340 at 185 C (elution time from left to right).

TABLE 1

Relative Retention Times (RRT) of Trimethylsilyl Ethers of Pairs of C-24 Epimeric Sterols[a,b]

Sterol	Orientation at C-24	RRTs Column 185 C	RRTs Temperature 195 C
Campestanol	α	1.324	1.309
Ergostanol	β	1.304	1.291
Campesterol	α	1.336	1.317
22,23-Dihydrobrassicasterol	β	1.315	1.299
Sitosterol	α	1.629	1.584
22,23-Dihydroporiferasterol	β	1.616	1.571
22-Dehydrocampesterol	α	1.214	1.112
Brassicasterol	β	1.133	1.119
Stigmasterol	α	1.405	1.370
Poriferasterol	β	1.414	1.377
Δ^7-Campestenol	α	1.674	1.646
Δ^7-Ergostenol	β	1.653	1.624
Δ^7-Stigmastenol	α	2.045	1.976
Δ^7-Poriferasterol	β	2.023	1.959
Spinasterol	α	1.753	1.704
Chondrillasterol	β	1.766	1.712
25-Dehydrospinasterol	α	2.057	1.992
25-Dehydrochondrillasterol	β	2.116	2.046

[a]Relative to cholesterol TMS ether.

[b]Refer to text for chromatographic conditions.

(15) on glass capillary columns coated with SP-2340, separation efficiency and peak shape were extremely sensitive to sample size; results were acceptable only with very small samples. Choice of sample size was a compromise between the minimal quantity needed for accurate area measurements and the maximal quantity that gave good separations of epimeric pairs.

DISCUSSION

This is the first convenient physical separation of TMS ethers of pairs of C-24 epimeric sterols. Recently, it has been shown that (24R)- and (24S)-methyl-5α-cholestanes as well as (24R)- and (24S)-methyl-5α-cholestanol acetates could be separated by glass capillary gas liquid chromatography (21). The time required

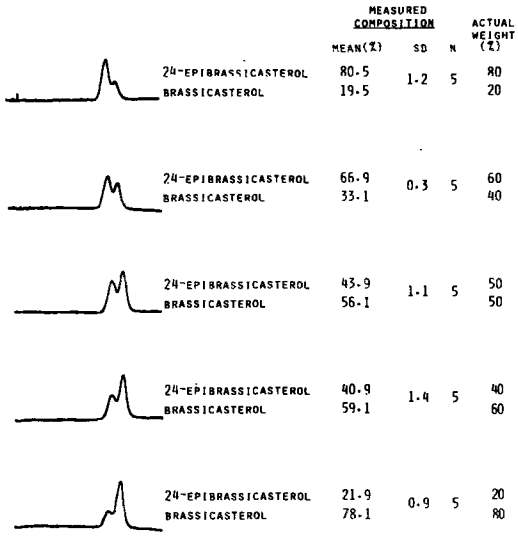

FIG. 3. Quantitative measurement by capillary GLC of weighed mixtures of 22-dehydrocampesterol (24-epibrassicasterol) and brassicasterol trimethylsilyl ethers (elution time from left to right).

for the separation, however, while slightly less than 3 hr for the C-24 epimeric pair of stearanes, was more than 8 hr for the stanol acetates.

The successful separation of pairs of epimeric sterols now permits studies with C-24 alkyl-substituted sterols in areas of research hitherto considered unapproachable. For example, it can now be ascertained whether certain fungi, insects, or other animals, in fulfilling their sterol requirements, selectively incorporate or utilize either the 24α- or 24β-substituted sterol in preference to the other. It can now be readily determined whether a sterol is epimerically pure at C-24. The method now permits rapid quantitative analyses of C-24 epimeric sterols of the *Atlantic scallop* (11) and of the oyster (12) as well as epimeric sterols from bryophtes and primitive and advanced tracheophytes (8,9).

PMR analysis of synthetic 22,23-dihydrobrassicasterol (16) indicated that it contained some 24α-epimer, and additional NMR analysis with a 220-MHz instrument indicated that it contained ca. 25% of the 24α-epimer (11), which was confirmed in this study. The 24α-isomer apparently originated during the catalytic hydrogenation of the Δ^{22}-bond since a similar hydrogenation of the Δ^{22}-bond in the presence of a different catalyst yielded dihydrobrassicasterol that contained only ca. 5% of the 24α-epimer (17). The effects can now be determined of various catalysts on the production of C-24 methyl epimers during the hydrogenation of the Δ^{22}-bond. Interestingly, the synthesis of sitosterol from stigmasterol, as in the preparation of 22,23-dihydrobrassicasterol, yielded only the 24α-epimer.

Our study indicates that very small quantities of TMS ethers of pairs of C-24 epimeric sterols can be separated by capillary GLC. However, even with the high precision of the retention ratio measurements obtained for the sterol TMS ethers (Table 2), the similarities in retention times suggest that the sterol purity should first be established by conventional GLC analysis methods before submitting the sterol to glass capillary GLC analyses for assignment of configuration at C-24. Although this paper can serve as a reference, we also suggest that both C-24 epimers should be available and analyzed by capillary GLC before configuration is assigned. Then, the method described here is a powerful technique in establishing the identity of a sterol and its absolute configuration at

TABLE 2

Precision of Relative Retention Time Measurements[a]

Sterol TMS ether	Retention time[b] (min)	RRT			
		N	Mean	SD	CV (%)
22-Dehydrocampesterol	115.9	5	1.125	0.0009	0.080
Ergostanol	135.4	3	1.304	0.0006	0.046
22,23-Dihydrobrassicasterol	136.5	10	1.316	0.0011	0.084
Campestanol	137.3	4	1.324	0.0013	0.098
Campesterol	138.3	4	1.335	0.0010	0.075
22,23-Dihydroporiferasterol	165.2	7	1.616	0.0018	0.110
Sitosterol	166.4	4	1.629	0.0006	0.037
25-Dehydrospinasterol	200.6	3	2.057	0.0017	0.083
25-Dehydrochondrillasterol	205.9	3	2.116	0.0031	0.147

[a]Relative to cholesterol TMS ether.

[b]Refer to text for chromatographic conditions.

C-24.

Whether SP-2340 is the best available phase for separating C-24 epimeric sterols, or whether other phases would afford a greater separation factor for sterols with the *E* Δ^{22}-bond, or support a larger sample size is another area of research that warrants further study. This work removes a significant obstacle to research studies with C-24 alkyl sterols, many of which were impractical to pursue.

ACKNOWLEDGMENT

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COMMUNICATIONS

Phospholipids and Component Fatty Acids of the Pigeon Liver

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ABSTRACT

The phospholipids from the livers of adult pigeons were separated by thin layer chromatography and the component fatty acids analyzed by gas liquid chromatography. They consisted of 53.0% phosphatidylcholine, 26.3% phosphatidylethanolamine, 8.6% sphingomyelin, 6.3% cardiolipin and 4.8% lysophosphatidylcholine. Phosphatidylethanolamine and phosphatidylcholine were characterized by a high concentration of long-chain polyunsaturated fatty acids with the highest percentages in the phosphatidylethanolamine. Sphingomyelin contained up to 64.5% saturated acids. About 80% of the fatty acids present in the cardiolipin fraction consisted of linoleic acid. The liver phospholipids had the same composition in lactating as in nonlactating pigeons, but differed in many respects from those available in the crop-milk.

Phospholipids are important constituents of the cell membranes, their composition more or less differing with species, tissue, age and diet (1-4). In birds, phospholipid synthesis occurs mainly, if not entirely, in the liver (5). So far, only the phospholipid composition of the chicken liver had been thoroughly investigated (1,6,7).

The ability of pigeons, males as well as females, to produce large amounts of lipid-rich crop-milk for the feeding of their hatchlings was a stimulus for a series of studies about the lipid metabolism of this bird (8-10). This paper deals with the amount and component fatty acids of the various phospholipid fractions in the liver. A comparison is made with the phosphatides present in the crop-milk (8,9).

Adult nonlactating, as well as lactating, pigeons of the species *Columba livia domestica* were investigated. No distinction between the sexes was made. The birds were killed by decapitation and bled by cutting a wing. The dissected livers were immediately frozen in liquid nitrogen and temporarily stored at -25 C. After extraction of the frozen tissue with chloroform/methanol (11), the phospholipid fractions were separated by thin layer chromatography on Silica Gel G with chloroform/methanol/water (75:25:4, by vol) and the methyl esters of the constituent fatty acids analyzed by gas liquid chromatography as described previously (8,9). The methylation procedures were described in detail by Christie (12). The quantity of each phospholipid class was calculated from the amount of the component fatty acids relative to an internal standard (methyl pentadecanoate) (13).

A first, important result was noted: although the liver of lactating pigeons was heavier and contained more lipids than that of nonlactating adults (unpublished results), no significant differences existed, neither in the proportions nor in the fatty acid composition of their various phospholipid fractions. So, we can limit ourselves to the results obtained for the normal adult livers (Table 1).

More than one-half of the liver phospholipids consisted of phosphatidylcholine (PC) comprising 53% of the total amount whereas phosphatidylethanolamine (PE) accounted for 26%. Other minor components were sphingomyelin (8.6%), cardiolipin (6.3%) and lysophosphatidylcholine (4.8%). The remaining phospholipid fraction (1-2%) was not further identified. The same phospholipid classes were previously found in the crop-milk (8), however, in different proportions. This secretion contains, e.g., relatively far more sphingomyelin (21.9%) but less PE (20.4%) and cardiolipin (2.5%). The phospholipid composition of the pigeon liver agrees, on the other hand, fairly well with that of the chicken liver (1,6,7). However, unlike in the chicken, phosphatidylinositol (PI) and phosphatidylserine (PS) were not detected in the pigeon liver. It is possible that, with the solvent we used, these components migrated on the thin layer plate with, respectively, PC and PE.

Fatty acids with 16 up to 24 carbons were detected with predominance of the C18 components. Each phosphatide had, however, its own characteristic fatty acid composition

TABLE 1

Amount and Fatty Acid Composition[a] of the Phospholipids from Adult Pigeon Liver

	Phosphatidylcholine	Phosphatidylethanolamine	Sphingomyelin	Cardiolipin	Lysophosphatidylcholine
Proportion of phospholipid (mol %)	53.0 ± 0.9	26.3 ± 0.2	8.6 ± 0.7	6.3 ± 0.3	4.8 ± 0.8
Fatty acids (wt %)					
16:0	14.7 ± 0.7	9.5 ± 0.4	20.5 ± 1.3	1.0 ± 0.1	13.5 ± 1.6
16:1	2.1 ± 0.2	0.9 ± 0.1	0.3 ± 0.1	3.7 ± 0.4	0.9 ± 0.2
18:0	28.3 ± 0.3	31.3 ± 1.2	33.2 ± 1.8	1.1 ± 0.2	42.6 ± 1.6
18:1	16.9 ± 1.0	12.7 ± 0.9	4.2 ± 0.3	10.4 ± 1.3	11.0 ± 1.6
18:2	23.2 ± 1.4	16.5 ± 0.5	6.4 ± 0.4	78.5 ± 1.9	15.8 ± 1.3
18:3 + 20:1	0.4 ± 0.1	0.6 ± 0.1	0.2 ± 0.05	1.0 ± 0.3	0.2 ± 0.08
20:0	0.1 ± 0.04	0.1 ± 0.04	3.8 ± 0.08	tr < 0.1	0.3 ± 0.1
20:2	0.3 ± 0.05	0.3 ± 0.1	0.5 ± 0.07	1.2 ± 0.3	1.4 ± 0.2
20:3	0.7 ± 0.06	1.1 ± 0.1	1.3 ± 0.6	0.7 ± 0.2	0.7 ± 0.2
20:4(n-6)	8.5 ± 0.4	20.1 ± 0.5	14.7 ± 0.6	0.9 ± 0.07	10.5 ± 1.5
20:5(n-3)	0.3 ± 0.1	0.9 ± 0.3	–	–	–
22:0	1.3 ± 0.1	0.4 ± 0.06	4.2 ± 0.7	–	–
22:4(n-6)	1.0 ± 0.06	1.3 ± 0.2	5.2 ± 0.09	–	–
22:5(n-6)	0.3 ± 0.03	0.7 ± 0.1	–	–	–
22:5(n-3)	0.8 ± 0.2	1.3 ± 0.3	–	–	–
22:6(n-3)	0.8 ± 0.09	2.1 ± 0.2	–	–	–
24:0	–	–	2.8 ± 0.5	–	–
Saturated	44.5 ± 0.7	41.3 ± 1.2	64.5 ± 1.4	2.0 ± 0.4	56.3 ± 0.7
Monoenoic	19.0 ± 1.2	13.6 ± 0.9	4.4 ± 0.5	14.1 ± 1.5	12.0 ± 1.6
Dienoic	23.5 ± 1.5	16.9 ± 0.6	6.9 ± 0.4	79.8 ± 1.9	16.9 ± 1.2
Polyenoic	12.5 ± 0.4	27.6 ± 1.1	23.6 ± 1.1	2.7 ± 0.6	14.2 ± 2.5

[a]Values are means (± SEM) of 5 determinations.

(Table 1). PC and PE showed the most complex patterns. Besides a high level of saturated acids, respectively, 44.5% and 41.3%, they contained several C20 and C22 polyenoic acids, among which arachidonic acid was the most abundant. Similar to observations in the crop-milk (9) and in the chicken liver (1,6,7), the percentages of these long-chain polyunsaturated acids, especially arachidonic and docosahexaenoic, in the pigeon liver were higher in the PE than in the PC fraction (27.6% and 12.5% or a proportion of 2.2 to 1, respectively). Sphingomyelin contained, as is typical for this class, a large proportion of saturated acids (64.5%), mainly stearic and palmitic, and in minor amounts 20:0, 22:0, as well as 24:0. This class is further characterized by the presence of a considerable amount of 20:4 (14.7%) and to a lesser extent, 22:4 (5.2%). The cardiolipin fraction from pigeon liver contained, as in the chicken (1,7), up to 80% linoleic acid. No acids higher than 20:4 were detected in this class as was also the case in lysophosphatidylcholine. This last component had a high concentration of saturated acids (56.3%), mainly stearic (42.6%), and a relatively high amount of arachidonic acid (10.5%).

When the acid composition of the liver phospholipids is compared with that of the crop-milk phosphatides (9), some striking differences appear. For instance, a considerably lower concentration of long-chain polyunsaturated acids was found in the PE (9.5%) and PC (4.4%) of the crop-milk, but the distribution proportion of these acids between the 2 cited classes was similar to that in the liver (2.2:1). Also, less arachidonic acid was present in the crop-milk sphingomyelin and lysophosphatidylcholine than in the same fractions of the liver. Cardiolipin from crop-milk contained only ca. 60% linoleic acid. On the other hand, all phospholipid classes from the crop-milk contained about twice as much oleic acid as the corresponding components of the liver. These differences show that the phospholipids present in the crop-milk secretion cannot be, as such, derived from the liver. If the liver is the major site of phospholipid synthesis (5), important changes must occur prior to deposition in the crop. The similarity between the phospholipid composition of the liver from lactating and nonlactating pigeons indicates, furthermore, that no specific phosphatide molecules are formed during the lactation period.

ACKNOWLEDGMENTS

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LETTERS TO THE EDITOR

Sir:

We wish to comment on the excellent analytical study recently offered in this journal by Lanza and Slover (1). The care employed in executing their laboratory work was not carried over into their literature survey and discussion. Of particular concern is the tone employed in the introduction where the cursory selection of references and discussion of related results might lead the uninitiated to view the presence of *trans*-isomers in dietary fats with some alarm. It would seem incumbent on such authors to objectively cover all aspects of the questions and not merely to introduce facts suggestive of sensationalism, particularly when they publicize the source of fats as to brand name and company.

One point of concern involves the levels of *trans*-isomers found in human tissue where only the 1958 work of Johnston et al. (2) was cited. Since that time, much improved analytical studies have suggested the older 12-14% estimates for *trans*-contents (2) are high. Heckers et al. (3) found 1-4% levels in depot fat of German males and Ohlrogge et al. (4) found 2-6% total *trans* in U.S. subjects' adipose tissue and 1-3% in their liver tissue.

Another point involves the suggestion that elevation of serum cholesterol is related to *trans*-isomers. The work of Vergroesen and Gottenbos (5) cited (1) has been reviewed carefully (6) and found wanting on several points. The changes supposedly related to diet are more likely the result of variation in serum cholesterol levels in humans and the analytical errors inherent in determining these levels. The second reference to McMillan et al. (7) is even more questionable because, as noted (6), considerable levels of *trans*-9,*trans*-12-octadecadienoate were introduced by the use of chemical isomerization with a concomitant elimination of essential fatty acid in the dietary fat employed. The known antagonistic nature of the *trans*-9,*trans*-12-isomer toward essential fatty acids is well-established, as reviewed (6).

This leads to another concern in discussing isomeric fats. These authors, as have others (cf. ref. in [6]), appear to talk interchangeably and collectively of *trans*-monoenes, *trans*,*trans*-dienes and *cis*,*trans*- or *trans*,*cis*-dienes as *trans*-unsaturated fats. This is sloppy science and only serves to confuse and mislead less-astute readers. We would urge that such discussions center on the correct lipid classes, as they all undoubtedly differ in their physiological activity or lack thereof.

Finally, it is disturbing to find reference here (1) to only the first suggestions of Enig et al. (8) regarding a possible epidemiological correlation between cancer and hydrogenated fats and not to the pointed criticisms of their work (9-11), plus their acknowledgment of those criticisms (12) and their reiteration that epidemiology *does not* establish a causal relationship as suggested here (1). Epidemiology may provide clues, but can never establish a causal relationship without direct experimental corroboration!

It is gratifying that these authors also found low levels of *trans*,*trans*-octadecadienes in foods containing hydrogenated fats. Perhaps they could have further reinforced these observations by adequately noting other work establishing such low levels of *trans*,*trans*-isomers in margarines (6,13).

In essence, our plea would be for completeness and exactness in such presentations, particularly where cursory inclusion and discussion of data and/or past findings tend to foster misunderstanding and misinterpretation and, more importantly, proliferation of these factors by future citation. As noted by Mahley (14), the consequences of not considering all available evidence are confusion of the public, a loss of credibility by the medical and scientific communities and an undermining of the validity of scientific evaluation in the eyes of the public and the media, as well.

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Sir:

Dr. Applewhite's principal criticisms of our paper on *trans*-fatty acid analysis (1) are that we did not thoroughly review the literature on the nutritional effects of *trans* fatty acids and that some of the references we did include were flawed. We agree completely that all available evidence must be considered in evaluating the nutritional effects of isomeric fatty acids. This, however, was not our purpose; we were describing analytical methodology and presenting compositional data. An extensive review would have been inappropriate and would probably have been unacceptable to the reviewers. The few references we cited in the introduction were given only to show that *trans* unsaturated fatty acid analysis is important, and that information on their amounts in foods is needed. The references we used were selected to illustrate the diversity in the results reported; others could have been quoted to achieve the same result. We tried to be unbiased, and cited both those who did and who did not report specific nutritional effects. We did not attempt a critical review.

We share his concern regarding sloppy nomenclature, and the need to be specific. We did not use the term "*trans*-unsaturated fats," but we did refer to *trans*-content, *trans* fatty acids, *trans* unsaturated fatty acids, and specific isomers. The separation and estimation of specific fatty acids was, in fact, discussed, and we reported data on the amounts of some of these in foods.

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Metabolism of Fatty Acids in Rat Brain Microsomal Membranes

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ABSTRACT

Using a technique in which substrate fatty acids are incorporated into microsomal membranes followed by comparison of their rates of desaturation with those of exogenous added fatty acids, it has been found that the desaturation rate may be greater for the membrane-bound substrate than for the added fatty acid. Moreover, the product of the membrane-bound substrate is incorporated into membrane phospholipid whereas the product of the exogenous substrate is found in di- and triacyl glycerols and in free fatty acids, as well. These and other findings point to a normal sequence of reaction of membrane lipids with membrane-bound substrates involving transfer of fatty acid from phospholipid to the coupled enzyme systems without facile equilibration with the free fatty acid pool.

INTRODUCTION

Most lipid metabolic reactions in the cells of living organisms involve membrane lipids and membrane-bound enzymes (1). However, only a few papers discussing the utilization of lipid substrates by their respective enzymes have attempted to describe the mechanism of the interactions among membranous enzymes and membranous lipid substrates (2).

Research from several laboratories has elucidated the biosynthetic mechanisms that determine the composition of the brain lipids. Reactions such as fatty acid elongation and desaturation occurring in brain membranes have been investigated in vitro with added fatty acids or fatty acyl-CoA thioesters, but with no assurance that this is the actual mechanism of the reaction as it proceeds in the membrane (3).

We have developed an in vitro technique for preparing brain microsomes labeled in the membrane phospholipids with [^{14}C] fatty acids. These membranes were then used as substrates for a study of desaturation of fatty acids as a new approach toward understanding the important reactions of membranous lipids as they probably occur in the membranes.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C] Linoleic (57 mCi/mmol), linolenic (56.2 mCi/mmol) and [2-^{14}C] 8,11,14 eicosatrienoic (55 mCi/mmol) acids were obtained from the Radiochemical Centre, Amersham, England. Radiopurity of the methyl esters was greater than 98% for all fatty acids. Nonradioactive fatty acids and lipid standards were purchased from Supelco, Inc., Bellefonte, PA, or Applied Science, State College, PA. The fatty acid substrates were prepared in micellar solution by dispersal in twice their molar amount of 0.1 N NH_4OH and diluting to the desired volume with 1% Triton WR 1339 (Ruger Chemical Co., Irvington, NJ). ATP, CoA, NADH, NADPH, glutathione and malonyl CoA were products of Sigma Chemical Co., St. Louis, MO. Pentex bovine albumin, fatty-acid-free fraction V, was a product of Miles Laboratories, Inc. All other reagents used were Baker analyzed reagents. Solvents were ACS grade and were redistilled before use.

Preparation of Microsomal Fractions from Rat Brains

Ten to 12 (4-5-day-old) male rats (Mission Labs, Rosemead, CA) were killed by decapitation and the brains quickly removed and washed with ice-cold 0.32 M sucrose. Brain tissues were homogenized with 0.32 M sucrose containing 0.1 M-phosphate buffer at pH 7.2 and 2 mM reduced glutathione (4 ml medium/g tissue) (4). Preparation of microsomes was as described by Gan-Elepano and Mead (5). All the above procedures were done at 4 C. Preparation of membranes and incubations were performed on the same day. Protein was determined by the method of Lowry et al. (6).

Incubations

For the incorporation of [^{14}C] fatty acids into the lipids of microsomal membranes, the incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.2, 4 mM-ATP, 0.2 mM CoA, 4 mM $MgCl_2$, 2 mM reduced glutathione, 0.05% Triton WR 1339, 7.5 to 17.5 μM-[^{14}C] fatty acids (52-57 mCi/mmol), and 2-4 mg of microsomal protein in a final vol of 1 ml. The incubation vials were capped with rubber stoppers and nitrogen was flushed through for at least 10 min, to ensure a nitrogen atmosphere. The vials were shaken at 37 C for 15 min.

For desaturation of exogenous fatty acids, the incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.2, 4 mM ATP, 0.2 mM CoA, 4 mM $MgCl_2$, 2 mM reduced glutathione, 1 mM NADH, 0.05% Triton WR 1339, 1.0 mg "soluble" protein, (50,000 x g x 2 hr supernatant), 7.5 to 15 μM [^{14}C]-fatty acids (52-57 mCi/mmol) and 2-8 mg microsomal protein in a final vol of 1 ml. Incubation was carried out for 20 min, at 37 C, under air, with shaking.

For desaturation of endogenous fatty acids, the [^{14}C] labeled microsomal membranes were washed with 1% BSA (bovine serum albumin) by resuspending the labeled microsomes in 1% BSA in 0.32 M sucrose and centrifuging at 50,000 x g for 2 hr, at 4 C.

The albumin-washed ^{14}C-labeled microsomal membranes were resuspended in 0.1 M-phosphate buffer, pH 7.2, containing 2 mM reduced glutathione and 0.32 M sucrose. The incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.2, 4 mM ATP, 0.2 mM CoA, 4 mM $MgCl_2$, 1 mM NADH, 10 mM $CaCl_2$, 1.0 mg of "soluble" protein, 2 mM reduced glutathione, 2-8 mg fresh, unlabeled microsomal protein and 2 to 4 mg of albumin-washed [^{14}C] microsomal membranes containing ca. 800,000 dpm in a final vol of 1 ml. Incubation was carried out for 10-30 min, under air, at 37 C, with shaking.

Extraction and Separation of Lipids

The total lipids from the reaction mixtures were extracted with 20 vol of chloroform/methanol (2:1, v/v). The suspension was centrifuged and to the supernatant was added 0.2 vol of water. The upper phase was removed, and the chloroform-rich lower phase was washed 3 times with Folch's theoretical upper phase (7). The lower phase, containing the lipids, was reduced to dryness under a stream of nitrogen at room temperature, resuspended in C/M (2:1, by vol) and an aliquot was taken for counting.

Neutral lipid classes were separated on precoated 0.25 mm Silica Gel G plates by development with a mixture of petroleum ether/diethyl ether/acetic acid (70:30:1, by vol). For the separation of polar lipids, the plates were developed with a mixture of chloroform/methanol/water (65:25:4, by vol). Areas containing the lipids were identified by comparison with known standards chromatographed at the same time. After a brief exposure to iodine vapors, they were scraped off the plates and transferred to counting vials to which 1.0 ml water and 10 ml Aquasol (New England Nuclear, Boston, MA) were added. Radioactivity was counted with a Beckman liquid scintillation spectrometer. Correction for quenching was made by the external standard method. Efficiency for [^{14}C] was about 70%.

The plates were also scanned for radioactivity using a Packard Radiochromatogram thin layer chromatography (TLC) scanner (Model 7200). Areas under the peaks were measured by triangulization.

Total phospholipids, free fatty acids and triacylglycerols were isolated by TLC with the solvent system petroleum ether/diethyl ether/acetic acid (70:30:1, by vol). Migration of monoacylglycerols was facilitated by the procedure described by Rousseau and Gatt (2). Bands were collected and methylated to obtain fatty acid methyl esters of the isolated lipid classes.

Total phospholipids were also subjected to hydrolysis by *Crotalus adamanteus* venom as suggested by Robertson and Lands (8). After hydrolysis, the mixture of free fatty acids and lysophospholipids was separated on TLC plates using the previously described solvent systems. The distribution of radioactivity among the lipid fractions was measured as already described.

Gas Liquid Chromatography

The total lipid extracts and lipid fractions isolated by TLC were subjected to methanolysis by treatment with 5% methanolic HCL solution at 80-90 C in screw-capped vials, under nitrogen, for at least 3 hr. The fatty acid methyl esters were extracted with hexane and washed 3 times with water. The hexane extract of radioactive fatty acids was taken to dryness under a stream of nitrogen.

Gas liquid chromatography (GLC) was carried out in a Packard Gas Chromatograph Model 7400 equipped with dual flame ionization detectors and a 4 mm x 6 ft coiled glass column. 10% Silar 10 C on 100-120 mesh Gas chrom Q was used for the analyses. The Packard Model 894 gas proportional counter was used for radioactivity determinations. Identification of the radioactive peaks was accomplished by comparison with known standard fatty acid methyl esters.

RESULTS

Incorporation Studies

The major aim of this study was to test the hypothesis that the alteration of membrane lipids, in this case desaturation, normally takes place within the membrane without release of intermediates to the external medium. The

TABLE 1

Distribution of Radioactivity among Lipid Fractions after Incubation of Rat Brain Microsomes with [^{14}C] Fatty Acids[a]

	Substrate					
	18:2		18:3		20:3	
	A	B	A	B	A	B
Total lipids[b]	100.0	8.90	100.0	8.10	100.0	10.70
Polar lipids (PL)	43.3	3.85	54.4	4.40	41.3	4.41
CPG	32.0	2.84	47.4	3.84	37.4	4.00
EPG	10.9	0.97	6.5	0.53	3.9	0.41
Other	0.4	0.03	0.5	0.04	–	–
Neutral lipids (NL)	56.7	5.04	45.6	3.70	58.7	6.28
DG	6.4	0.56	3.4	0.28	6.0	0.64
TG	15.0	1.33	14.0	1.13	22.9	2.45
FFA	35.3	3.14	28.2	2.29	29.8	3.18
Ratio PL/NL (TG+DG)	2.0		3.1		1.4	

[a]The incubation mixture contained: 0.1 M potassium phosphate buffer, pH 7.2, 4 mM-ATP, 0.2 mM-CoA, 4 mM-$MgCl_2$, 2 mM-reduced glutathione, 0.05% Triton WR 1339, 1.0-1.2 X 10^6 dpm of [1-^{14}C] fatty acids, and 2-4 mg microsomal protein, in a final vol of 1 ml. Incubation was carried out for 15 min at 37 C. Total lipids from the reaction mixture were extracted with 20 vol of C/M (2:1, v/v), and analyzed by radio-TLC. Values are given as (A) percentages of total radioactivity, and (B) nmol of substrate in the lipid fractions.

[b]Abbreviations: CPG-choline phosphoglycerides; EPG-ethanolamine phosphoglycerides, DG-diacylglycerols; FFA-free fatty acids; TG-triacylglycerols.

technique chosen for this trial involved the use of membranes labeled in the fatty acid moiety. The first step in the preparation of fatty acid-labeled microsomal membranes involved the incorporation of [1-^{14}C] fatty acids into the microsomal lipids. The distribution of radioactivity among lipid fractions after incubation of rat brain microsomes with [^{14}C] fatty acids is shown in Table 1. A significant proportion of the fatty acids added to the incubation mixture was incorporated into phospholipids, and to a lesser extent, into diacyl and triacyl glycerols. Among the phospholipids, CPG was several times more highly labeled than EPG, whereas in the neutral lipids, TG was preferentially labeled. A similar pattern of incorporation was found by Cook (4) with rat brain homogenates using desaturation conditions, although the level of free fatty acids remaining at the end of the incubation period was higher in his experiments.

No major differences were found stemming from the nature of the fatty acid substrates used. Incorporation into phospholipids was higher for 18:3, whereas the incorporation into triacylglycerols was greater for 20:3.

Increased incubation time or substrate concentration (up to 20 μM) did not substantially alter the amount or the pattern of incorporation. ATP and CoA were shown to be needed for the activation of the fatty acid substrates to acyl-CoA derivatives and their incorporation into membrane lipids. In the absence of ATP and CoA, between 80-95% of the substrate fatty acids added to the incubation mixture remained as free fatty acids at the end of incubation. The same effects were shown by adding to the incubation mixture unlabeled fatty acids, which would compete for the needed cofactors and binding sites.

[1-^{14}C] Linolenic acid was also shown to be largely incorporated into the 2-position of the phospholipids of the microsomal membranes. After hydrolysis of the labeled phospholipids with *C. adamanteus* venom, 66% of the initial radioactivity was recovered in the free fatty acid fraction whereas only 33% was found in the lysophospholipids.

Since our aim was primarily to obtain labeled microsomal membranes to be used as a source of endogenous fatty acid for desaturation studies, attempts were made to remove free fatty acids from these preparations by washing with 1% bovine serum albumin (BSA) and recentrifugation of the washed microsomes at 50,000 x g for 2 hr.

The distribution of radioactivity among lipid fractions of the ^{14}C-labeled membranes, after washing with 1% BSA, is shown in Table 2. In these and similar experiments, between 78 and 97% of the free fatty acids initially present in the labeled microsomes were removed by this procedure. Larger albumin concentrations were tested but they brought about no further decrease in the amount of free fatty acids left after washing.

TABLE 2

Distribution of Radioactivity among Lipid Fractions of ^{14}C-Labeled Microsomal Membranes after Washing with 1% Albumin[a] (Compare Table 1)

	Substrate					
	18:2		18:3		20:3	
	A	B	A	B	A	B
Total lipids	100.0	4.80	100.0	4.20	100.0	4.50
Polar lipids (PL)	66.2	3.17	62.5	2.62	46.6	2.09
CPG	52.5	2.52	56.9	2.39	38.5	1.73
EPG	13.7	0.65	4.9	0.21	7.3	0.32
Others	–	–	0.7	0.02	0.8	0.03
Neutral lipids (NL)	33.8	1.62	37.5	1.56	53.4	2.40
DG	5.8	0.27	2.7	0.11	3.8	0.17
TG	27.0	1.29	30.9	1.29	45.5	2.04
FFA	1.0	0.05	3.9	0.16	4.1	0.18
Ratio PL/NL (TG+DG)	2.0		1.8		0.9	

[a]Brain microsomes were incubated with [^{14}C] fatty acids as described in Table 1. The ^{14}C-labeled microsomal membranes were washed with 1% bovine serum albumin and recentrifuged at 50,000 X g for 2 hr at 4 C. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.2, containing 2 mM-reduced glutathione and 0.32 M sucrose, and extracted with 20 vol of C/M (2:1, v/v). Values are given as (A) percentages of total radioactivity, and (B) nmol of substrate in the lipid fractions. Abbreviations are as in Table 1.

Some other lipids apparently were lost during washing in addition to free fatty acids. Although the presence of lipids in the albumin wash fluid was not determined, the smaller amounts of labeled polar lipids recovered after washing as well as the decreased ratio of PL/DG-TG indicates that labeled polar lipids, mainly CPG and EPG, were preferentially lost.

Desaturation Studies

In vitro desaturation of exogenous fatty acids by rat brain microsomes is shown in Table 3.

Linoleic (18:2ω6), linolenic (18:3ω3) and 8,11,14-eicosatrienoic (20:3ω6) acids were converted into their corresponding desaturation products, 18:3ω6, 18:4ω3 and 20:4ω6. The relative desaturation activities for the 4-day-old rat brain microsomes were 18:3>20:3>18:2. Desaturation of 18:3ω3 was also found by Cook (4) to be greater than for the other fatty acids in whole brain homogenates.

Optimal incubation conditions for desaturation were determined with brain microsomes from 4-day-old rats only for linolenic acid (Fig. 1). The optimal pH was 6.5. Activities at pH 7.2, 7.5 and 6.0 were 12, 22 and 33% lower, respectively. The time response for the enzyme system was linear up to 20 min of incubation, and protein inhibition occurred between 6 and 8 mg of protein/ml. The highest rate of 18:4ω3 formation was reached at ca. 15 μM concentration.

In contrast to the results obtained with these

TABLE 3

Desaturation of Exogenous [1-^{14}C] Fatty Acids by Rat Brain Microsomes[a]

Substrate	A	B	Product	A	B	Desaturation rates
18:2ω6	75.1	8.41	18:3ω6	24.9	2.78	17.4
18:3ω3	50.8	7.36	18:4ω3	49.2	7.13	76.7
20:3ω6	66.5	4.98	20:4ω6	33.5	2.51	27.0

[a]The incubation mixture contained: 0.1 M potassium phosphate buffer, pH 7.2, 4 mM-ATP, 0.2 mM-CoA, 4 mM-$MgCl_2$, 2 mM-reduced glutathione, 0.05% Triton WR 1339, 1 mM-NADH, 1.0 mg "soluble" protein, 4-8 mg microsomal protein, and 11.2 (18:2), 14.5 (18:3) or 7.5 (20:3) μM-[^{14}C] fatty acids containing 0.9-1.8 X 10^6 dpm in a final vol of 1 ml. Incubation was carried out at 37 C for 20 min. Values were obtained by radio-GLC, and are given as (A) percentages of total radioactivity, and (B) nmol of substrate and product at the end of incubation. Desaturation rates are expressed as pmol/min/mg protein.

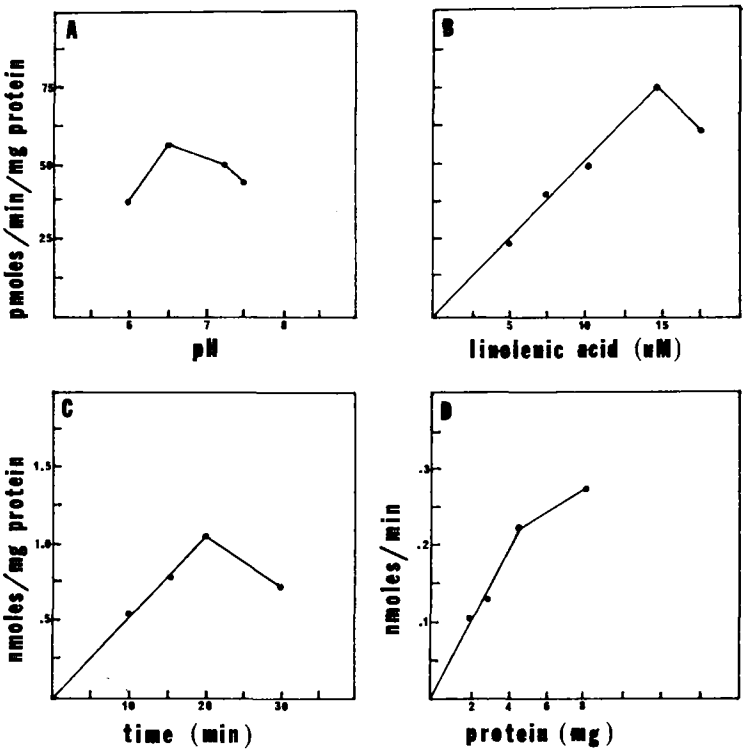

FIG. 1. Rate of desaturation of 18:3ω3 to 18:4ω3 by 4-day-old rat brain microsomes. (A) Effect of PH; (B) effect of substrate concentration; (C) effect of incubation time; (D) effect of microsomal protein concentration. The incubation mixture contained: 0.1 M potassium phosphate buffer, 4 mM-ATP, 0.2 mM-CoA, 4 mM-$MgCl_2$, 2 mM-reduced glutathione, 0.05% Triton WR 1339, 1.0 mg "soluble" protein, .10.5 μM [^{14}C] 18:3ω3, and 4.5 mg microsomal protein, in a final vol of 1 ml. Incubation was carried out at 37 C, for 20 min, with shaking. Change in the incubation mixture are indicated in the figure. Values are averages of duplicate determinations.

fresh, unlabeled microsomal preparations, the albumin-washed microsomes, under similar conditions, did not show any significant desaturation capacity for either added or endogenous [^{14}C] fatty acids. Only when fresh microsomes were incubated along with the albumin-washed labeled microsomal membranes, in the presence of the necessary cofactors, could desaturation of the endogenous fatty acids be demonstrated (Table 4). As with the exogenous substrates, the extent of desaturation of 18:3ω3 to 18:4ω3 was greater than conversion of 20:3 to 20:4 and 18:2 to 18:3.

Desaturation of both exogenous and endogenous fatty acids was dependent on the presence of added ATP and CoA to the incubation mixture. Addition of unlabeled fatty acids also decreased desaturation. However, in the absence of those cofactors, desaturation of exogenous fatty acids decreased to ca. 10% of the values obtained for the complete system in contrast to a reduction of only 40-50% for the desaturation of membrane-bound fatty acids.

As reported by Cook (4), we found that the desaturation capacity of the microsomal preparations obtained showed enough variation from one experiment to the others as to avoid a meaningful comparison between exogenous and endogenous substrates in separate experiments. However, when desaturation of both types of substrates was carried out under identical conditions using the same freshly obtained unlabeled microsomal enzyme preparation, as shown in Table 5 for desaturation of linolenic acid, it was observed that (a) with added fatty acid as substrates, the level of free fatty acids at the end of incubation decreased by almost 70%, whereas with the endogenous substrate, there were only minor changes or none; (b) in the absence of ATP and CoA, the exogenous substrate remained mostly as free

fatty acids during incubation, whereas there was a 3-fold increase in the level of free fatty acid with the endogenous substrate; (c) desaturation of the exogenous substrate was significantly more affected by the absence of ATP and CoA in the incubation mixture than was the endogenous substrate; and (d) the absolute amount of desaturation product formed from the endogenous substrate was at least twice as large as the amounts of free fatty acids available for desaturation at the start or at the end of the incubation period (with the complete system). Similar results were consistently obtained in several other experiments with linoleic or eicosatrienoic acid as substrates.

The distribution of the product of desaturation of linolenic acid in lipid fractions was also determined (Table 6). With added fatty acid as substrate, 67% of the total 18:4 synthesized was found in the phospholipid fraction (1.87 nmol), 22% in the free fatty acids (0.60 nmol) and 10% in the neutral lipids, DG and TG (0.28 nmol). Recovery was ca. 90% (2.75 nmol) of the product found in the total lipids (3.05 nmol). With membrane-bound linolenic acid, at least 97% (0.40 nmol) of the 18:4 formed was detected in the phospholipids, and very little, if any, in the free fatty acids and neutral lipids. Recovery was ca. 93% (0.41 nmol) of the 18:4 found in the total lipids (0.44 nmol).

DISCUSSION

The preparation of [^{14}C] fatty-acid-labeled microsomal membranes to study desaturation of endogenous fatty acids by rat brain microsomes required first the in vitro incorporation

TABLE 4

Desaturation of Endogenous [1-^{14}C] Fatty Acids by Rat Brain Microsomes[a]

Substrate	A	B	Product	A	B	Desaturation rates
18:2ω6	95.7	4.01	18:3ω6	4.3	0.19	1.1
18:3ω3	89.5	3.75	18:4ω3	10.5	0.44	5.5
20:3ω6	93.4	4.20	20:4ω6	6.6	0.29	3.2

[a]The incubation mixture contained: 0.1 M potassium phosphate buffer, pH 7.2, 4 mM-ATP, 0.2 mM-CoA, 4 mM-$MgCl_2$, 2 mM-reduced glutathione, 10 mM-$CaCl_2$, 1.0 mg "soluble" protein, 4-8 mg unlabeled microsomal protein, 1 mM-NADH, and 4.2 (18:2, 18:3) or 4.5 (20:3) μM-[^{14}C] fatty-acid-labeled microsomal membranes containing 0.6-0.8 X 10^6 dpm in a final vol of 1 ml. Incubation was carried out at 37 C for 20 min. Values were obtained by radio-GLC, and are given as (A) percentages of total radioactivity, and (B) nmol of substrate and product at the end of incubation. Desaturation rates are expressed as pmol/min/mg protein.

TABLE 5

Desaturation of Exogenous and Endogenous Linolenic Acid by Rat Brain Microsomes[a]

Substrate	I		II		III		IV
	A	B	A	B	A	B	
Exogenous 18:3ω3							
Complete system	100.0	10.5	28.2	2.9	37.7	3.9	49.5
−ATP and CoA	100.0	10.5	93.7	9.8	5.2	0.5	6.8
Endogenous 18:3ω3							
Complete system	3.9	0.4	5.7	0.6	10.6	1.1	13.9
−ATP and CoA	3.9	0.4	13.4	1.4	4.2	0.4	5.5

[a]The complete incubation mixture contained: 0.1 M potassium phosphate buffer, pH 7.2, 4 mM-ATP, 0.2 mM-CoA, 4 mM-$MgCl_2$, 2 mM-reduced glutathione, 1 mM-NADH, 0.05% Triton WR 1339, 1.0 mg "soluble" protein, 4.0 mg unlabeled microsomal protein, and 10.5 μM-[^{14}C] 18:3 (exogenous substrate) containing 1.2 X 10^6 dpm or ^{14}C-labeled microsomal membranes (endogenous substrate) containing 1.2 X 10^6 dpm in a final vol of 1 ml. Incubations were carried out at 37 C for 20 min. Values are given as (A) percentages of total radioactivity, and (B) nmol labeled fatty acids in total lipids. I. Labeled free fatty acids at the start of the incubation period (radio-TLC). II. Labeled free fatty acids at the end of the incubation period (radio-TLC). III. Desaturation product at the end of incubation (radio-GLC). IV. Desaturation rates expressed as pmol/min/mg protein.

TABLE 6

Desaturation of Exogenous and Endogenous Linolenic Acid By Rat Brain Microsomes. Distribution of Desaturation Product in Lipid Fractions[a]

	Total [^{14}C] FA[b]		18:4ω3–Product[c]	
	A	B	A	B
Exogenous substrate				
Total lipids	100.0	8.10	37.7	3.05
Polar lipids	54.5	4.41	42.5	1.87
Free fatty acids	28.2	2.28	26.5	0.60
Neutral lipids	17.3	1.40	20.4	0.28
Endogenous substrate				
Total lipids	100.0	4.20	10.6	0.44
Polar lipids	61.5	2.58	15.6	0.40
Free fatty acids	5.7	0.23	<1.0	0.00
Neutral lipids	32.8	1.37	<1.0	0.01

[a]Incubation conditions as in Table 5. Values are given as (A) percentages of total radioactivity, and (B) nmol of labeled fatty acids in the lipid fractions.

[b]Values obtained by radio-TLC.

[c]Values obtained by radio-GLC.

of labeled fatty acids into the membrane lipids. Although this was readily accomplished in our experiments as has already been shown in several tissues, including brain (4), a significant amount of free fatty acids remained attached to the microsomal membranes at the end of incubation, even after recentrifugation of the labeled microsomes. Albumin-washing of the labeled microsomal membranes was then carried out and proved to be successful in removing most of these free fatty acids. However, when incubated under desaturation conditions, these washed microsomal preparations were unable to convert their labeled membrane-bound fatty acids to the corresponding unsaturated products. Washed liver microsomes have been shown to lose a soluble protein factor that is necessary for the activity of the linoleic acid desaturase (9). This factor contains lipids, mainly CPG. It is possible that a similar loss of polar lipids shown in our washed brain microsomes could be related to the lack of desaturation capacity of these preparations. Moreover, the stimulatory effect of the post-microsomal supernatant on the brain microsomal desaturation of fatty acids, as reported by Cook (4), may support this possibility. Although the presence of a lipoprotein necessary for desaturation of fatty acids such as the one reported by Leikin et al. (9) in liver microsomes has not been investigated in the brain, it is very likely that a similar factor is present in this tissue as well.

Whole brain homogenate and microsomal in vitro desaturation of linoleic, linolenic and eicosatrienoic acids, with a peak of maximal activity at about 4 days of age in rats, has been reported previously (4,10) and was also demonstrated in our experiments. The lack of desaturation capacity already mentioned in the albumin-washed labeled microsomes necessitated the use of fresh microsomes as the source of enzymes to study desaturation of the endogenous membrane-bound fatty acids. Under these conditions, fresh microsomes were shown to desaturate endogenous 18:3ω3 to 18:4ω3, and to a lesser extent 20:3ω6 to 20:4ω6, and 18:2ω6 to 18:3ω6. This separation of enzyme and substrate on different membrane fragments has been shown by Rousseau and Gatt to promote reaction at a rate only slightly less than that with both on the same fragment (2). Although the rates of desaturation obtained by simply calculating the amount of products formed/min/mg of protein from the total amount of substrate (free plus bound or esterified fatty acids) initially present at the start of the incubation period were higher for the exogenously added fatty acids, several differences between the 2 systems must be considered in order to compare their desaturation rates.

With exogenous linolenic acid as substrate (Table 5), all of the added fatty acid (100% or 10.5 nmol) was available for desaturation from the start of the incubation, whereas with endogenous 18:3, only a small concentration of free fatty acid was initially present (3.9% or 0.4 nmol), the bulk of the substrate being sterified as part of the membrane lipids. During incubation, there were almost no changes in the concentration of labeled free fatty acids, suggesting either minimal hydrolysis of the membrane lipids or hydrolysis followed by

rapid reincorporation of the released fatty acids. The increase in free fatty acids observed in the absence of ATP and CoA at the end of incubation (13.4% or 1.4 nmol) favors the latter possibility, although in this case, hydrolysis was not followed by reincorporation because of the lack of cofactors.

The amount of 18:4 formed during incubation of the labeled microsomal membranes (10.6% or 1.1 nmol) was then twice as large as the amount of free fatty acids available for desaturation (3.9-5.7% or 0.4-0.6 nmol). If desaturation of endogenous fatty acids takes place only after hydrolysis and equilibration of the released fatty acids into the exogenous fatty acid pool, they would be desaturated at the same rate as the added fatty acids. The expected amount of 18:3 converted into 18:4 under these conditions (37.7% of 0.4-0.6 nmol or 0.2-0.3 nmol of product) would be much smaller than the amount actually found (1.1 nmol). On the assumption that the level of free fatty acids remaining at the end of the incubation period in the absence of ATP and CoA 1.4 nmol also represents maximal free fatty acid concentration in the complete system at any moment during incubation, the expected amount of desaturation (37.7% of 1.4 nmol or 0.6 nmol product) would be only half of the 1.1 nmol found.

These results may be explained by either of 2 mechanisms. One would involve hydrolysis of the membrane lipids, particularly the phospholipid fatty acids by a tightly coupled membrane phospholipase with coupling of the released fatty acids to a different fatty acid pool and desaturation system from the one known to operate with the exogenous fatty acids. This system also would be dependent on ATP and CoA for activation of the released fatty acids, and desaturation would take place at about twice the rate as that for exogenous fatty acids (78.5% of 1.4 nmol product; 103 pmol/min/mg protein against 49.5 pmol/min/mg protein) (Table 5). The other possibility is the occurrence of a direct desaturation mechanism by membrane-bound fatty acid desaturases, as has been shown in yeast and in rat liver microsomes (11). This system is not dependent on ATP and CoA, since acyl-CoA synthetases are not involved. We found that desaturation of endogenous fatty acids was only partially dependent on the presence of those cofactors. Desaturation of membrane-bound fatty acids could thus be the result of direct desaturation plus desaturation of any released fatty acids after activation to their acyl-CoA derivatives in the presence of ATP and CoA.

The existence of both these mechanisms is further supported by the finding (Table 6) that the product of membrane-bound linolenic acid was incorporated only into membrane phospholipids, whereas the product of exogenous 18:3 was found in diacyl- and triacyl glycerols and free fatty acids, as well. These results are consistent with direct desaturation of membrane-bound phospholipid fatty acids or with transfer of fatty acid between tightly coupled enzymes. On the other hand, desaturation of endogenous fatty acids involved very low levels of free fatty acids. The absence of any significant synthesis of neutral lipids from the desaturated fatty acids may reflect a higher affinity of the PL-synthesizing enzymes for available acyl-CoA under physiological conditions. In contrast, desaturation of much higher levels of exogenous fatty acids may stimulate TG formation, as has been observed in many different cells, through lower affinity NL-synthesizing enzymes. However, this may not be the physiological mechanism occurring in the brain tissues. The elevated levels of labeled triacyl glycerols formed during desaturation of exogenous fatty acids were indeed unexpected, since TG are almost absent in brain lipids.

Further experiments are needed to support fully the existence of either of these 2 mechanisms in rat brain microsomal membranes. We believe our studies to be significant, also, as a new approach to the elucidation of the mechanisms involved with membrane-bound enzymes acting on membrane-bound lipids as they actually occur in the organisms.

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Lysophosphatidylcholine Acyltransferase Activity during Experimental Cholelithiasis

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ABSTRACT

The accumulation of (1-palmitoyl)lysophosphatidylcholine, lysolecithin, in gallbladder bile was observed during the first week of cholesterol-induced experimental cholelithiasis using the prairie dog model for cholesterol gallstone formation. Gallbladder fluid transport function decreased as bile lysolecithin concentration increased. These observations suggest that lysolecithin plays an important, early role in the etiology of gallstone disease. Furthermore, the relative activities of hepatic and gallbladder mucosa lysophosphatidylcholine acyltransferase and acylcoenzyme A hydrolases may be responsible for the turnover of gallbladder bile lysolecithin.

INTRODUCTION

The pathologic relationship(s) between cholecystitis and cholelithiasis are unclear. Chronic cholecystitis is an inflammatory condition of the gallbladder for which complications are sometimes fatal (1). The disease is usually found associated with the presence of gallstones. Lysophosphatidylcholine (LPC; lysolecithin) has been implicated as a major factor in the etiology of cholecystitis since elevated levels of this lytic agent have been found in the gallbladder biles of patients suffering from the disease (2). Acute cholecystitis may also be induced experimentally in animals by the instillation of the phospholipid within the gallbladder (3). It has also been demonstrated that the formation of lysolecithin from lecithin in bile supersaturated with cholesterol lowers the effective concentration of lecithin and leads to cholesterol precipitation, since the cholesterol-solubilizing capacity of lysolecithin is much less than that of lecithin (4). Thus, it is possible that lysolecithin plays an important role in the etiology of gallstone disease (cholelithiasis). However, the origin of lysolecithin in bile is unknown (5). Recently, Neiderhiser and Harmon (6) have shown that lysolecithin, instilled at a subtoxic dose within the guinea pig gallbladder, is absorbed from the bile into the mucosa where it is converted to lecithin through an acylation reaction catalyzed by the enzyme, acylcoenzyme A: lysophosphatidylcholine acyltransferase (EC 2.3.1.23). Their data suggests that, under normal conditions, a protective mechanism exists for the disposition of potentially toxic lysolecithin accumulation. Yet, there is little information available concerning the action of this enzyme during cholelithiasis. This study examines the activity of acyl coenzyme A: lysophosphatidylcholine acyltransferase (LPC-AT) of the gallbladder mucosa during experimental induction of cholelithiasis using the prairie dog-cholesterol gallstone model (7). This animal, when fed a cholesterol diet, will produce cholesterol gallstones within 6 weeks. Our results indicate that an early rise in gallbladder bile lysolecithin concentration is correlated with gallbladder membrane dysfunction. Furthermore, the relative activities of hepatic and gallbladder mucosal acyltransferase may be responsible for the early accumulation of lysolecithin in gallbladder bile.

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EXPERIMENTAL PROCEDURES

Materials

L-1-[palmitoyl-1-^{14}C] lysopalmitoyl phosphatidylcholine, specific activity (sp act) 49.1 mCi/mmol, was purchased from New England Nuclear at >99% radiopurity. Unlabeled phospholipids were purchased from Sigma Chemical Co. at 98-99% chemical purity verified by thin layer chromatographic (TLC) analysis. Oleoylcoenzyme A, potassium salt, was also obtained from Sigma. Sodium 5,5'-dithiobis-(2-nitrobenzoic acid), DTNB, 99% purity, was obtained from Aldrich and neutralized with sodium bicarbonate prior to use. All additional reagents were obtained as the highest purity available from commercial sources. Solvents were glass-distilled.

Animal Studies

Adult, male prairie dogs (*Cynomys ludovicianus*, trapped in the wild state and obtained from O.M. Locke, New Branfels, TX), weighing

ca. 1 kg, were caged individually in a thermoregulated (23 C) room. Study groups were fed a 0.4% cholesterol-enriched chow (Teklad Test Diet, TD73177) from 1-5 weeks whereas control animals were fed a trace-cholesterol chow (Purina Rodent Laboraotry Chow 5001, 0.03% cholesterol). All animals were initially fed the control diet 2 weeks prior to the beginning of a study. The test diet induces cholesterol crystals within 3 weeks and gallstones in 6 weeks (8). After a 16-hr fast, prairie dogs were anesthetized with 100 mg ketamine -HCl/kg body weight administered by intramuscular injection. Sedation was maintained by 25-50 mg doses/hr. The abdomen was opened, the cystic duct ligated and the common duct cannulated with a PE 50 polyethylene tubing catheter. Common duct bile was collected over a 3-hr period. Termination was accomplished by exsanguination and the blood saved for serum cholesterol determination. In cases where common duct bile was not collected, the cystic duct was clamped and the animal sacrificed immediately. Finally, the liver and gallbladder were removed intact and rinsed with sterile saline. The gallbladder was freed from its hepatic bed and its contents were aspirated by syringe and stored on ice prior to analysis. The liver and the gallbladder were rinsed then stored at 4 C in Ca^{+2}-free Krebs-Ringers phosphate buffer, pH 7.4, prior to the preparation of cell fractions in order to avoid the possible activation of Ca^{+2}-dependent phospholipase A_2 activity (9).

Microsomal Preparations

Gallbladders were excised and placed in ice-cold 0.05 M calcium-free Krebs-Ringer phosphate buffer, pH 7.4. The epithelia was exposed and rinsed several times to remove mucus and bile or cholesterol crystals. The gallbladders were then blotted dry and the mucosal cell layer was removed by scraping the epithelium with a glass microscope slide. The mucosal cells from 5 gallbladders were pooled and then homogenized in 1.0 ml 0.25 M sucrose at 4 C in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle (15 strokes, medium speed). The homogenate was then diluted with an additional 1.0 ml 0.25 M sucrose and centrifuged at 500 × g for 10 min (Sorvall RC-5, SS34 rotor). The supernatant was next centrifuged at 12,000 × g for 25 min, and its resulting supernatant was centrifuged at 100,000 × g for 90 min (Beckman Model L2-50, Type 40 rotor) to obtain the microsomal pellet (10).

Pellets were stored at -20 C until assayed. They were then resuspended in 0.25 M sucrose with analysis conducted on freshly resuspended pellets. Finally, a portion of the suspension was fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, and subsequently examined by electron microscopy to verify the purity of the microsomal preparations. No appreciable level of suborganelle contamination was observed by electron microscopy. Hepatic microsomes were prepared in a similar manner except a 1-g wedge was first minced over ice, then homogenized in 4:1 (v/w) of 0.25 M sucrose. The resulting homogenate was then diluted with 2.0 ml sucrose and fractionated as just described.

Enzyme Assays

The spectrophotometric assay of Lands and Hart (11) was used to measure both lysophosphatidylcholine acyltransferase (LPC-AT) and oleoylcoenzyme A hydrolase (CoA-H) activities of the gallbladder mucosal and hepatic cell microsomal preparations. A typical incubation mixture consisted of 0.01-0.15 mg microsomal protein (ca. 1 mg/ml 0.25 M sucrose), 40 nmol oleoylcoenzyme A, 1.0 μmol DTNB, and 50 μmol Tris-HCl buffer, pH 7.4, adjusted to a final vol of 1.0 ml with distilled water. The reaction was initiated by the addition of oleoylcoenzyme A and the change in absorbance at 413 nm with time was recorded using a Gilford-Beckman Model DU recording spectrophotometer. Conditions were at ambient temperature.

A molar extinction coefficient of 13,600 M^{-1} cm^{-1} at 413 nm was used to calculate the number of nmol free coenzyme A released and subsequently complexed with DTNB. The oleoylcoenzyme A hydrolase activity was then defined as the rate of release prior to the addition of lysophosphatidylcholine, measured as the initial velocity. Finally, 40-100 nmol lysophosphatidylcholine (in 10 μl absolute ethanol) was added and the increase in absorbance at 413 nm was recorded. The difference in rates before and after the addition of lysophosphatidylcholine was thus used to define lysophosphatidylcholine acyltransferase activity. All activities were determined from the measurement of initial velocity, whereas sp act were obtained where sp act remained constant with protein concentration. In some instances, [^{14}C]lysopalmitoylphosphatidylcholine (100 nmol; sp act 491 dpm/nmol) was used to verify the spectrophotometric monitor of lysophosphatidylcholine (LPC) to phosphatidylcholine (PC) conversion. Recovery of [^{14}C]phosphatidylcholine by Bligh and Dyer extraction and TLC indicated ca. 95% agreement.

In the case of the radioactive assay, lysophosphatidylcholine acyltransferase activity was defined at the rate of conversion of [1-^{14}C]-

palmitoyl]lysophosphatidylcholine to radiolabeled phosphatidylcholine. The incubation mixture was essentially as that just described except that DTNB was not added and the reaction conditions were set at 37 C for 10 min with gentle agitation. The reaction was terminated by the addition of 5 ml chloroform/methanol (1:4, v/v) and the phospholipids were extracted by the Bligh and Dyer method, then separated by TLC as described by Neiderhiser and Harmon (6,10). Radioactivity (dpm) was determined using a Searle Mark III liquid scintillation counter set in the variable quench mode. TLC material was mixed first with 1 ml methanol, to which was added 15 ml Aquosol 2 (New England Nuclear) liquid scintillation cocktail.

Phospholipid Determination

Gallbladder and common duct bile were extracted by the Bligh and Dyer method and the phospholipid species was separated by Silica Gel G TLC using a solvent system consisting of chloroform/methanol/water (65:25:4, v/v). Spots corresponding to standard lysophosphatidylcholine and phosphatidylcholine were visualized by iodine vapor, then scraped and transferred into Teflon screw-capped culture tubes. The individual phospholipids were subjected to mild alkaline deacylation as described by Kates (12) using the methanolic sodium hydroxide transesterification reaction to form the corresponding fatty acid methyl esters. Since the predominant acyl moiety of prairie dog bile phosphatidylcholine and lysophosphatidylcholine was determined to be palmitoyl, the concentrations of these phospholipids were estimated on the basis of their corresponding palmitic acid contents. Thus, the derived fatty acid methyl esters were analyzed by gas liquid chromatography (13). A Varian Model 940 GC equipped with a 1.5% OV-101 column was used with the column; flame-ionization detector and injector temperatures were set at 210 C, 250 C and 240 C, respectively.

Cholesterol Determination

The cholesterol content in bile was determined by the ferric chloride reagent-colormetric test of Courchaine et al. as described by Kates (14). Cholesterol samples were recovered from the thin layer chromatographs used to separate the biliary phospholipids just described, evaporated to dryness under a nitrogen stream and redissolved in glacial acetic acid. Ferric chloride reagent was added with color development at 550 nm. Cholesterol values were estimated by comparison to a standard curve obtained by analysis of pure cholesterol standard and expressed as mg cholesterol/ml original bile sample. Cholesterol content in liver was also determined by this method following the extraction of a 1.0-g tissue wedge. Liver cholesterol values were expressed as mg cholesterol/g of liver tissue. Serum cholesterol was determined on a Technicon SMAC automated biochemical analyzer and expressed in mg/100 ml.

Noneverted Explanted Gallbladder Model

The rate of luminal fluid transport from explanted prairie dog gallbladders was determined as described by Lee (15). Animals were anesthetized with ketamine •HCl, the abdomen opened and the gallbladder's cystic duct clamped. The liver was immediately removed with the gallbladder intact. After a saline rinse, the gallbladder was freed from its hepatic bed and any adhering liver tissue removed. An incision near the ampulla was made and a PE 50 polyethylene tube inserted and secured by silk ligatures. Gallbladder bile was allowed to drain through the catheter and saved for further analysis. The gallbladder was then rinsed several times in standard Krebs-Ringer phosphate buffer containing calcium, pH 7.4. Finally, the gallbladder was distended with this buffer and the catheter closed with silk ligatures. The gallbladder was emersed in a 37 C constant temperature water bath containing Krebs-Ringer buffer to which was supplied continuous oxygenation. In preliminary studies, we confirmed the observations of Lee (15) and Diamond (16) that the rate of luminal fluid loss can be accurately measured in a reproducible, less tedious manner by following the decrease in gross gallbladder weight with time. Therefore, loss in luminal fluid was determined indirectly by reweighing the gallbladder in 5-min intervals for 75-100 min. At the cessation of the experiment, the gallbladder was drained, the catheter removed, and the blotted gallbladder reweighed. Results were expressed as the linear rate of fluid transport/hr and normalized to a 100-mg gallbladder.

Statistical Analysis

Results are expressed as mean ± SD with statistical evaluation by the unpaired Student's t-test. Linear correlation of data was expressed as the correlation coefficient, r.

RESULTS AND DISCUSSION

Prairie dogs subjected to a medium (0.4%) cholesterol diet show dramatic increases in both

TABLE 1

Change in Cholesterol Content during Cholesterol Feeding

Weeks on cholesterol	Cholesterol levels*			
	Serum (mg/100 ml)	Liver (mg/g)	Gallbladder bile (mg/ml)	Common duct bile (mg/ml)
0	175 ± 66	1.45 ± .06	.92 ± .46	.11 ± .01
1	513 ± 144[a]	2.49 ± .07[d]	2.5 ± 1.5[ns]	.27 ± .16[ns]
3	1198 ± 167[c]	2.50 ± .23[c]	2.35 ± .75[a]	.37 ± .02[d]
5	985 ± 35[d]	1.38 ± .10[ns]	3.44 ± 1.2[b]	.39 ± .06[c]

*Values represent the mean ± SD for n = 3 prairie dogs in each cholesterol feeding week. Significantly different from untreated (0 weeks on cholesterol diet) prairie dogs, by the unpaired *Student's t-test*: [a]$p < .05$; [b]$p < .02$; [c]$p < .01$; [d]$p < .001$.

ns- not significant, $p > .05$.

serum and biliary cholesterol levels (Table 1). Liver cholesterol content, as well as gallbladder and common duct bile cholesterol contents, appear to be a reflection of the elevation in serum cholesterol levels (Fig. 1). Such changes are clearly observable within 1 week on this diet and are similar to those reported by Brenneman et al. (7) in their early studies with prairie dogs placed on a high (1.2%) cholesterol diet.

More interesting, however, is the variation in biliary phospholipid content and especially that of the LPC content in gallbladder bile. As can be seen in Figure 2, a 3-fold increase in LPC concentration occurs within the first week on cholesterol ($p < 0.01$) followed by a return to the control level within 3 weeks. A less dramatic change in PC level is observed, thus resulting in an increased ratio of LPC/PC in gallbladder bile (Fig. 3). As in the case of LPC-fluctuation, the LPC/PC ratio was significantly different only during the first week of cholesterol feeding ($p < 0.05$) relative to controls. Fluctuation in PC content ($r = 0.5721$) and LPC ($r = 0.0913$) rise does not correlate well with that of serum cholesterol level during this 3-week period. Although a strong correlation ($r = 0.9874$) between gallbladder bile PC and cholesterol levels for the first 3 weeks on cholesterol diet was observed, this trend was unsubstantiated in the case of gallbladder LPC ($r = 0.444$). Moreover, since the ratio of LPC/PC in bile did not remain constant, our data does not support a precursor-product

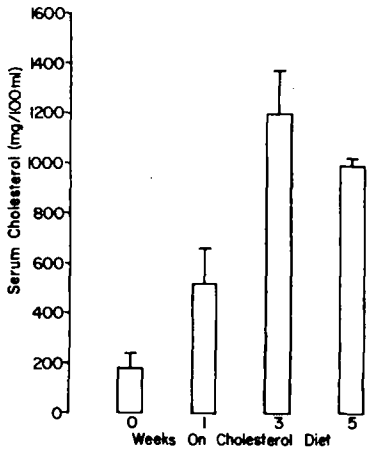

FIG. 1. Change in serum cholesterol level upon cholesterol feeding. Values represent the mean ± SD for n = 3 prairie dogs in each cholesterol feeding week. Statistical significance relative to untreated animals, in wk: (1) $p < .05$, (3) $p < .01$, and (5) $p < .001$.

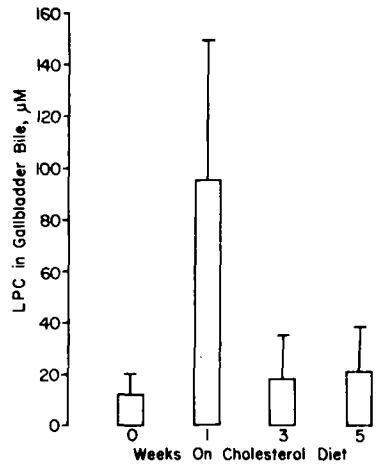

FIG. 2. Appearance of lysophosphatidylcholine (LPC) in gallbladder bile during cholesterol feeding. Values represent mean ± SD for n = 6 determinations. *Statistical significance, in wk*: (1) $p < .01$, (3) NS and (5) NS.

relationship between LPC and PC.

Lysophosphatidylcholine Acyltransferase Activity (LPC-AT)

Neiderhiser and Harmon (6,10) have shown that, in guinea pig gallbladders, the ability to convert LPC to PC is catalyzed by a microsomal lysophosphatidylcholine acyltransferase. They suggest that, under normal conditions, this enzyme protects the mucosal membrane from LPC-directed damage by converting LPC to PC with subsequent incorporation of PC into the mucosal membrane. It was therefore of interest to measure the gallbladder mucosal LPC-AT activity during the course of LPC fluctuation in gallbladder bile. Since the origin of biliary phospholipids and, especially, LPC is uncertain (5), we also monitored hepatic LPC-AT activity.

We observed no LPC-AT activity ([^{14}C]LPC conversion to [^{14}C]PC) in liver or gallbladder microsomal preparations in the absence of oleoylcoenzyme A. In addition, we were unable to demonstrate significant radiolabeled PC hydrolysis in gallbladder homogenates, suggesting the absence of phospholipase activity (A. Rutledge and R. Matson, unpublished observations). However, since these gallbladders had been rinsed in calcium-free Krebs-Ringer phosphate buffer prior to homogenization, the occurrence of a calcium-dependent phospholipase A activity cannot be dismissed (9). Optimal LPC-AT activity was obtained using oleoylcoenzyme A and (1-palmitoyl)lysophosphatidylcholine. Substitution of the 1-acyl moiety resulted in the following order of activity: 16:0 LPC>14:0 LPC≥18:1 LPC>> 18:0 LPC. Because the activity of acylcoenzyme A hydrolase (CoA-H) could appreciably influence the effective cofactor concentration in our experiments, it was also necessary to determine its relative activity. The gallbladder mucosal activity of both LPC-AT and CoA-H have been found to vary among animal species (10). As indicated in Table 2, however, the sp act of CoA-H was relatively constant in gallbladder mucosal microsomal preparations obtained from prairie dogs maintained on a control diet 4-6 weeks or those placed on a cholesterol diet for 4 weeks. In contrast, gallbladder LPC-AT sp act were elevated 2- to 3-fold for those animals placed on a cholesterol diet for 4 weeks. For control animals during this period, LPC-AT sp act was 2- to 6-fold

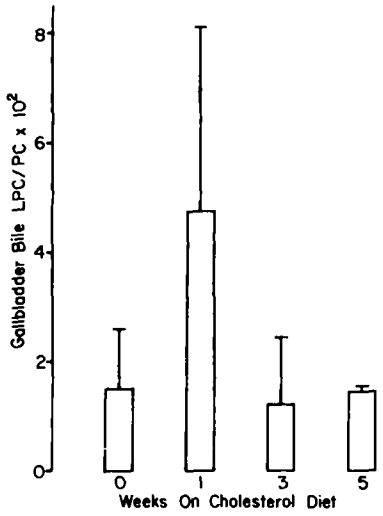

FIG. 3. Fluctuation in gallbladder bile LPC/PC ratio. Values represent mean ± SD for n = 6 determinations. Statistical significance, in wk: (1) $p < .05$, (3) NS and (5) NS.

TABLE 2

Analysis of Prairie Dog Gallbladder Mucosal Enzymes

Weeks on cholesterol	Serum cholesterol[a] (mg/100 ml)	LPC-AT[b] (nmol/min/mg microsomal protein)	CoA-H[b] (nmol/min/mg microsomal protein)
0	186 ± 48	6.66 ± .98	7.96 ± .92
4[c]	555 ± 162[d]	15.89 ± 2.46[d]	8.04 ± 2.82[ns]
12[e]	722 ± 221[f]	nd	nd

[a]Values represent the mean ± SD for n = 5 prairie dogs in each cholesterol feeding week.

[b]Mean ± SD for n = 4 determinations.

[c]Gallbladder and gallbladder bile normal in appearance.

[d]$p < .001$.

[e]Gallbladder bile saturated with cholesterol crystals, gallstones present and gallbladder wall appears thickened.

[f]$p < .01$; ns - not significant, $p > .05$.

nd–enzymatic activities below level of detection (≤ 0.368 nmol/min).

less than that reported for guinea pig, whereas CoA-H sp act was at a similar level (10).

Analysis of hepatic microsomal levels of LPC-AT and CoA-H activities were in sharp contrast to those obtained from the gallbladder. As shown in Figure 4, both LPC-AT and CoA-H sp act increased markedly with the duration on cholesterol diet. Strong correlations for LPC-AT (r = 0.9646) and CoA-H (r = 0.9523) sp act and serum cholesterol levels during the 3-week cholesterol feeding interval were observed. More important, however, hepatic microsomal CoA-H activities were substantially greater than LPC-AT activities during this period. This could explain the sudden rise in the absolute concentration of gallbladder LPC and the increase in the LPC/PC ratio. It is possible that hepatic CoA-H might effectively limit the acylcoenzyme A pool normally available to LPC-AT, resulting in lowered capacity to convert LPC to PC in the liver. As a result, excess hepatic LPC may be transferred into the bile. Increased LPC concentration in bile apparently stimulates gallbladder mucosal LPC-AT activity, but not that of CoA-H of the gallbladder mucosa. This would account for the decrease in LPC levels in gallbladder bile within the 3-week period.

As indicated in some of the figures and tables, there is a dramatic drop in gallbladder bile phospholipid content and the hepatic LPC-AT and CoA-H activities after the first 3-week cholesterol feeding period. This may signal the initiation of cholesterol gallstone formation. At 5 weeks on cholesterol diet, gallbladder biles contain cholesterol crystals and gallstones. Furthermore, we failed to detect appreciable levels of these enzymatic activities in mucosal preparations derived from the gallbladders of animals during the acute stages of cholelithiasis (Table 2).

Gallbladder Dysfunction

It is well known that lysophosphatidylcholine is a lytic agent which, under certain conditions, can lead to membrane damage resulting in the loss of membrane integrity (2,17). It was, therefore, of particular interest to determine whether the sudden rise in gallbladder bile LPC might have a deleterious effect on gallbldder function. One assessment of the absorptive function is the measurement of fluid loss using the noneverted gallbladder model (16). A normal, uninjured gallbladder filled and suspended in an isotonic solution will pump fluid from the luminal side into the surrounding media at a linear rate (15,16). This process has been demonstrated to be energy-linked since the addition of metabolic inhibitors of oxidative phosphorylation inhibit the rate of transport (15). Using this model with prairie dog gallbladders obtained from animals maintained on a cholesterol diet for 1-5 weeks, an apparent loss in gallbladder function is observed with increasing duration on the cholesterol diet (Fig. 5). As shown, this loss is most dramatic

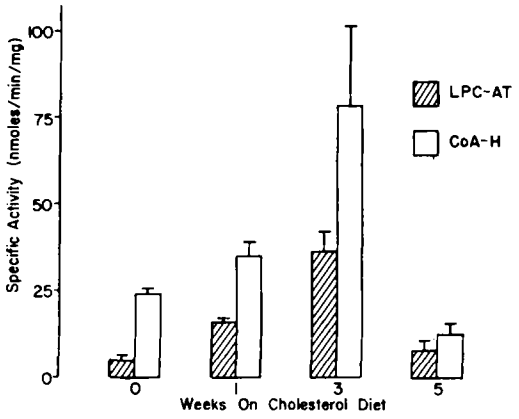

FIG. 4. Analysis of prairie dog hepatic enzyme levels. Values represent mean ± SD for n = 3 determinations. Statistical significance for LPC-AT sp act, in wk: (1) $p < .001$, (3) $p < .001$ and (5) NS. Statistical significance for CoA-H sp act, in wk: (1) $p < .01$, (3) $p < .02$ and (5) $p < .01$.

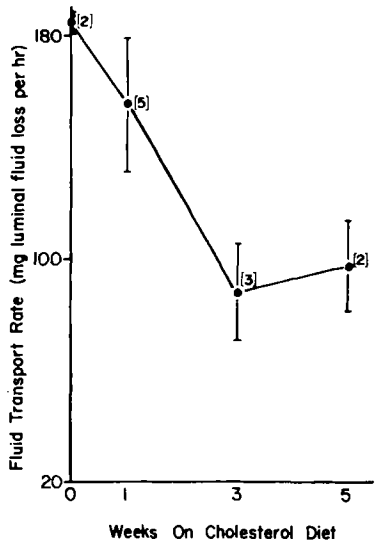

FIG. 5. Noneverted gallbladder fluid transport during experimental cholelithiasis. Values represent mean ± SD, whereas numbers in brackets indicate the number of determinations (n) in each set. Statistical significance, in wk: (1) NS, (3) $p < .01$ and (5) $p < .02$.

after 1 week on cholesterol diet corresponding to the period in which the elevated LPC concentration in gallbladder bile could cause mucosal membrane damage.

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ω- and (ω-1)-Hydroxylation of 1-Dodecanol by Frog Liver Microsomes

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ABSTRACT

Frog liver microsomes catalyzed the hydroxylation of 1-dodecanol into the corresponding ω- and (ω-1)-hydroxy derivatives. The hydroxylation rate for 1-dodecanol was much lower than that for lauric acid. Both NADPH and O_2 were required for hydroxylation activity. NADH had no effect on the hydroxylation. The hydroxylating system was inhibited 49% by CO at a $CO:O_2$ ratio of 4.0. The formation of ω-hydroxydodecanol was more sharply inhibited by CO than was the formation of (ω-1)-hydroxydodecanol, implying that more than one cytochrome P-450 was involved in the hydroxylation of 1-dodecanol and that CO has a higher affinity for the P-450 catalyzing the ω-hydroxylation. The formation of laurate during the incubation of 1-dodecanol with frog liver microsomes suggests that a fatty alcohol oxidation system is also present in the microsomes. NAD^+ was the most effective cofactor for the oxidation of 1-dodecanol and $NADP^+$ had a little effect. Pyrazole (an inhibitor of alcohol dehydrogenase) had a slight inhibitory effect on the oxidation and sodium azide (an inhibitor of catalase) had no effect.

INTRODUCTION

In a previous paper (1), we described the substrate specificity and kinetic properties of a NADPH-supported cytochrome P-450-dependent monoxygenase system in frog liver microsomes, which carried out the hydroxylation of fatty acids in both ω- and (ω-1)-positions. Although there are many studies on the hydroxylation of fatty acids by a number of the hydroxylating systems in various organisms (2-9), little information is available concerning the hydroxylation of fatty alcohols which are the analogs of fatty acids and important precursors in the formation of ether-linked lipids and waxes (10). We have now found that 1-dodecanol (lauryl alcohol) as well as fatty acids can be hydroxylated in both ω- and (ω-1)-postions by the cytochrome P-450-dependent monooxygenase in frog liver microsomes. We have also confirmed that a portion of the substrate (1-dodecanol) was oxidized to lauric acid by an alcohol oxidation system which is present in frog liver microsomes during the incubation.

MATERIALS AND METHODS

Substrates and Standards

1-[1-^{14}C]Dodecanol (sp act 30 mCi/mmol) and [1-^{14}C]lauric acid (sp act 32 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.) and had a radiopurity of 98%. 1,12-Dodecanediol, dodecane-1,12-dioic acid and pyrazole were obtained from Tokyo Kasei Chem. Co. (Tokyo, Japan). The ω- and (ω-1)-hydroxylauric acids were synthesized chemically (7). 1,11-Dodecanediol was synthesized by the reduction of methyl (ω-1)-hydroxylaurate with $LiAlH_4$. NADPH, NADH, $NADP^+$ and NAD^+ were from Sigma Chem. Co. (St. Louis, MO). The organic solvents were reagent grade and freshly distilled before use.

Preparation of Microsomes

Japanese bullfrogs (*Rana catesbeiana*) weighing 200-250 g were obtained from Tokyo Experimental Animal Laboratories (Tokyo, Japan). The preparation of frog liver microsomes was done as previously described (11). The microsomes were washed twice with 0.25 M sucrose and resuspended in the same medium. The protein concentration of the microsomal suspension was determined by the method of Lowry et al. (12).

Isolation and Identification of ω- and (ω-1)-Hydroxydodecanol

The standard incubations contained microsomes (3.38 mg), 1 mmol potassium phosphate buffer (pH 7.5), 2 μmol NADPH, and 0.15 μmol of 1-[1-^{14}C]dodecanol (4.9×10^5 cpm). The final vol was 6.25 ml; incubation time was 15 min at 37 C. The reaction was stopped by the addition of 2 vol of $CHCl_3/CH_3OH$ (2:1, v/v) followed by the addition of cold 1-dodecanol, 1,12-dodecanediol and 12-hydroxylauric acid. The $CHCl_3$ phase was removed and saved while the aqueous phase was acidified with 3 N HCl and extracted 3 times with diethyl ether. The $CHCl_3$ and ether phases were combined, dried over anhydrous Na_2SO_4, and filtered. The solvents were removed from the filtrate under nitrogen, and treated with diazomethane in

diethyl ether. A portion of the sample was analyzed by thin layer silica gel chromatography (TLC) (solvent: hexane/diethyl ether/acetic acid, 50:50:1, v/v/v), and the distribution of radioactivity was determined by scintillation counting of the silica gel zones scraped from the plate. The counting procedures were the same as described previously (1). For the final determination of the percentage hydroxylation of 1-dodecanol, the remaining residue was chromatographed on a small column packed with 2 g of silicic acid (Mallinckrodt Chemical Works, St. Louis, MO) and 1 g of Hyflo Super-Cel (Johns Manville Corp., Denver, CO). The methyl ester of lauric acid (oxidation product of 1-dodecanol) was eluted with 3% diethyl ether in hexane; the unchanged 1-dodecanol was eluted with 7% diethyl ether in hexane. Hydroxylaurates were eluted with 20% diethyl ether in hexane whereas the hydroxylated dodecanols (dodecanediols) were eluted with 100% diethyl ether. The radioactivity in each fraction was determined by scintillation counting to determine the percentage hydroxylation. A portion of each fraction was subjected to TLC with the same solvent system as described above. Another portion of 20% diethyl ether fraction was treated with acetic anhydride/pyridine as described previously (8). The remaining portion of 100% diethyl ether fraction was divided into 2 portions. One portion was treated with acetic anhydride/pyridine whereas the second portion was treated with CrO_3 in 90% acetic acid followed by reaction with diazomethane as described previously (8). The O-acetyl derivatives of polar products in 20% and 100% diethyl ether fractions and the keto acid derived from the conversion of dodecanediols with treatment of CrO_3/acetic acid were analyzed by radio-gas chromatography as described next.

Radio-Gas Chromatographic Analyses

The general procedures used for radio-gas chromatographic analyses of the methyl ester of lauric acid, the O-acetyl derivatives of hydroxylauric acid, O-diacetyl derivatives of dodecanediol, and keto acids have already been described (1). All analyses were done on a coiled glass column (3 mm x 1.5 m) packed with 15% EGSS-X on Celite 545 (60-80 mesh) (Gasukurokogyo, Inc., Tokyo, Japan). The flow rate of the helium gas was 40 ml/min at 180 C.

RESULTS

Identification of ω- and (ω-1)-Hydroxydodecanol

After the incubation of ^{14}C-labeled dodecanol with frog liver microsomes and subsequent extraction, the sample was analyzed by TLC (Fig. 1). Three radioactive products were detected on a silica gel plate, and had chromatographic properties of 1,12-dodecanediol, methyl 12-hydroxylaurate and methyl laurate. These radioactive products were separated by silicic acid column chromatography (c.f., Materials and Methods). Each product gave only one radioactive spot when subjected to TLC with hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The radioactive product migrating with 1,12-dodecanediol was treated with acetic anhydride/pyridine and the diacetoxy derivatives thus obtained were analyzed on an EGSS-X column at 180 C. The distinct radioactive peaks were observed, and the one which contained about 50% of the ^{14}C activity corresponded exactly to unlabeled 1,11-diacetoxydodecane; the other, which contained about 50% of the ^{14}C-activity, coincided with unlabeled 1,12-diacetoxydodecane. In order to confirm the structures of the enzymatically produced hydroxydodecanols, the conversion of the dodecanediols to the analogous keto acids was done with CrO_3 in 90% acetic acid, and the keto acids were analyzed by radio-gas chromatography as described previously (13). The radioactive keto methyl esters gave essentially the same chro-

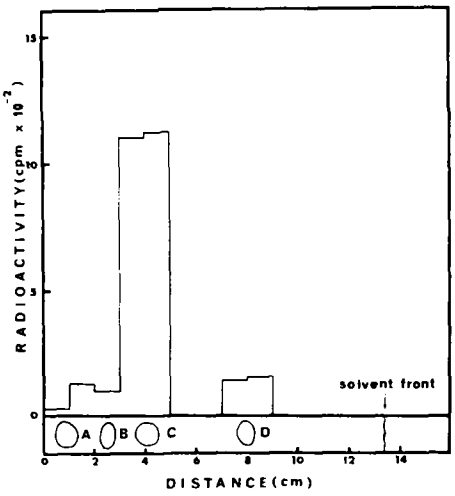

FIG. 1. Thin layer silica gel chromatography of the enzyme reaction products formed during incubation of frog liver microsomes with 1-[1-^{14}C]dodecanol. A portion of the products was treated with diazomethane and then applied along with appropriate standards to a thin layer plate coated with silica gel. The plate was developed in hexane/diethyl ether/acetic acid (50:50:1, v/v/v). Spots A, B, C and D were 1,12-dodecanediol, methyl 12-hydroxylaurate, 1-dodecanol and methyl laurate, respectively.

matographic picture as diacetoxy derivatives. Again, the enzymatically produced materials gave 2 peaks; one corresponded to unlabeled methyl 11-ketolaurate and the other coincided with unlabeled dimethyl dodecane-1,12-dioate.

The radioactive product migrating with methyl 12-hydroxylaurate was analyzed as already described by using authentic standards of unlabeled methyl 11- and 12-hydroxylaurate, and it was found that the radioactive product was a mixture of 11-hydrolaurate (73%) and 12-hydroxylaurate (27%). Radioactive methyl laurate was also confirmed by radio-gas chromatography. We concluded from these results that the 1-[1-^{14}C]dodecanol substrate had been enzymatically hydroxylated to give a mixture of 2 isomeric dodecanediols which included 11- and 12-hydroxy-[1-^{14}C]-dodecanol. However, it was observed that laurate and hydroxylaurates were also formed during incubation of frog liver microsomes with 1-dodecanol. The amounts formed of dodecanediols, hydroxylaurates and laurate were 2.78, 1.70, and 4.85 nmol/mg/15 min, respectively.

Properties of the Microsomal ω- and (ω-1)-Hydroxylation of 1-Dodecanol

After it was found that 1-[1-^{14}C]dodecanol could be hydroxylated in the ω- and (ω-1)-positions by frog liver microsomes, various pyridine-nucleotides were examined for the hydroxylation of 1-dodecanol (Table 1). NADPH was the only cofactor required for the hydroxylation of [1-^{14}C]dodecanol. NADH, $NADP^+$ or NAD^+ had no effect on the hydroxylation of 1-dodecanol. In addition to NADPH, O_2 was also required for the hydroxylation. However, NAD^+ was the most active cofactor for the oxidation of 1-dodecanol to lauric acid, and NADPH, NADH and $NADP^+$ had a little effect on the oxidation. Although hydroxylaurates were formed when 1-dodecanol was incubated with frog liver microsomes in the presence of NADPH, the hydroxylation rate was much lower than that when lauric acid was used as a substrate in the same experiment (Table 1).

Under an atmosphere containing 80% carbon monoxide, the hydroxylation activity (the sum of 11- and 12-hydroxydodecanol) of 1-dodecanol was significantly decreased (Table 2). Moreover, the ω/(ω-1)-hydroxylation ratio for the incubation under carbon monoxide was remarkably altered compared to that for control incubation; the hydroxylation rate of ω-hydroxydodecanol was sharply decreased (Table 2). A similar result was obtained for both the hydroxylation rate (the sum of 11- and 12-hydroxylaurate) of laurate and the ω/(ω-1)-hydroxylation ratio (Table 2).

Since the formation of laurate from 1-dodecanol implies the involvement of alcohol and aldehyde dehydrogenases activity toward 1-dodecanol in frog liver microsomes, the effect of pyrazole (an inhibitor of alcohol dehydrogenase) was examined (Table 2). Pyrazole (1 mM) had little inhibitory effect (10% inhibition) on the formation of laurate. No effect was observed with sodium azide (1 mM), a catalase inhibitor (Table 2).

DISCUSSION

In previous papers (1,11), we reported the hydroxylation of fatty acids in both ω- and

TABLE 1

Pyridine Nucleotide and O_2 Requirement for the Hydroxylation of 1-Dodecanol by Frog Liver Microsomes

Substrate	Pyridine nucleotide[a]	Products (nmol/mg/15 min)		
		Hydroxydodecanol	Hydroxylaurate	Laurate
1-Dodecanol	NADPH	3.27	2.09	5.19
↓	NADH	0.38	0.09	3.42
	$NADP^+$	0.52	0.14	6.31
	NAD^+	0.51	0.89	37.68
	NADPH, anaerobic[b]	0.39	0.29	0.96
	NADPH, boiled enzyme	0.49[c]	0.39[c]	0.34
Lauric acid	NADPH	–	7.13	–
↓	NADPH, anaerobic[b]	–	0.68	–
	NADPH, boiled enzyme	–	0.73[c]	–

[a]The concentration of all the pyridine nucleotides was 0.32 mM.

[b]The incubation was carried out under 1 atm of helium.

[c]Although the products obtained from the boiled enzyme controls had chromatographic properties of hydroxydodecanol and hydroxylaurate on TLC, no radioactive peaks were observed when the O-acetyl derivatives of the products were analyzed by radio-gas chromatography on an EGSS-X column at 180 C.

(ω-1)-positions and the effect of detergents on these hydroxylations by frog liver microsomes. The present work confirms that frog liver micrsomes also catalyze the hydroxylation of 1-dodecanol in both ω- and (ω-1)-positions. However, the fatty alcohol hydroxylase of frog liver microsomes described here differs in several respects from the fatty alcohol hydroxylase from *Bacillus megaterium*, which was reported by Miura and Fulco (13). The hydroxylating system from *B. megaterium* is readily obtained in soluble form and hydroxylates various fatty alcohols for which chain lengths were from 8 to 16 carbon atoms in the ω- and ω-2, and ω-3 positions, but never in the ω-position (13), whereas the hydroxylase of frog liver microsomes was obtained as a particulate fraction. The sp act for hydroxylation of 1-dodecanol in frog liver is much higher than in *B. megaterium*. Although the oxidation of 1-dodecanol to laurate was observed during incubation of frog liver microsomes with 1-[1-^{14}C]dodecanol, there was no significant oxidation of the fatty alcohol substrate to fatty acids for a soluble hydroxylase system from *B. megaterium*.

The hydroxylation of the laurate formed from the oxidation of 1-dodecanol was observed (Table 1), but the hydroxylation activity was much lower than when laurate was used as a substrate in the same experiment. No attempts were made to trap an aldehyde intermediate in the reaction of this study. However, as Lee has reported that an aldehyde intermediate is involved in the conversion of hexadecanol to palmitic acid by rat liver microsomes (14), 1-dodecanol may be oxidized to laurate by following oxidation processes during incubation of frog liver microsomes with 1-dodecanol: 1-dodecanol → (1-dodecanal) → 1-dodecanoic acid (lauric acid)—before the hydroxylation takes place. Therefore, the lower activity may be explained by the lag in substrate (laurate) formation and the lower level of this substrate available for hydroxylation.

Marked sensitivity of the hydroxylation of 1-dodecanol to carbon monoxide and the requirement of molecular oxygen for the hydroxylation (Table 2) suggest the involvement of cytochrome P-450 in the hydroxylating system of frog liver microsomes. The decrease of the formation of 11- and 12-hydroxylaurate under carbon monoxide also suggest the contribution of cytochrome P-450 in the hydroxylation (Table 2). It was postulated that more than one cytochrome P-450 is involved in the hydroxylation of 1-dodecanol in ω- and (ω-1)-positions because the ω/ω-1-hydroxylation ratio for the control incubation

TABLE 2

Effect of Pyrazole, NaN_3, and CO on the Hydroxylation and Oxidation of 1-Dodecanol by Frog Liver Microsomes

	Products (nmol/mg/15 min)						
	Hydroxydodecanol		Hydroxylaurate			ω/ω-1-Hydroxylation	
	ω	ω-1	ω	ω-1	Laurate	Hydroxydodecanol	Hydroxylaurate
Control	1.33(100)	1.45(100)	0.46(100)	1.24(100)	5.19(100)	0.92	0.37
CO-O_2 (8:2, v/v)	0.13(10)	1.29(89)	0.00(0)	0.62(50)	3.90(75)	0.10	0.00
Pyrazole (1 mM)	0.95(71)	1.26(87)	0.34(74)	1.06(85)	4.86(94)	0.75	0.32
NaN_3 (1 mM)	1.24(93)	1.35(93)	0.34(74)	1.36(110)	5.33(103)	0.92	0.25
Boiled enzyme	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.34(7)	–	–
Laurate as a substrate			2.88(626)	3.52(284)	–	–	0.82
Boiled enzyme			0.00(0)	0.00(0)	–	–	–

Numbers in parentheses are percentages of control.

was quite different from that for the incubation under carbon monoxide. It seems that carbon monoxide specifically inhibited the hydroxylation by the cytochrome P-450 which is responsible for the ω-hydroxylation of 1-dodecanol or laurate, as there was a great decrease of ω-hydroxydodecanol or ω-hydroxylaurate formation.

The oxidation of 1-dodecanol to laurate was observed in the incubation of the substrate with frog liver microsomes, suggesting the presence of a fatty alcohol oxidizing system in the microsomes. As this fatty alcohol oxidizing system is almost insensitive to inhibition by pyrazole (Table 2), a proven inhibitor for a soluble alcohol dehydrogenase (15), the oxidizing system in frog liver microsomes may be similar to the ones present in the microsomes of rat liver (14) and mouse preputial gland tumors (16). However, the sp act in frog liver is lower than that in rat liver, but much higher than that in mouse preputial tumors. This may be due to different assay conditions or tissue specificity. Catalase is known to function peroxidatively in the presence of ethanol and H_2O_2 to form acetaldehyde (17), and it was plausible that catalase could be responsible for the oxidation of 1-dodecanol to laurate. However, the involvement of catalase seems unlikely since the formation of laurate was not inhibited by sodium azide (an inhibitor of catalase). On the other hand, Miwa et al. reported the direct oxidation of ethanol by a catalase- and alcohol-dehydrogenase-free reconstituted system containing cytochrome P-450 from rat liver (18). The decrease in the formation rate of laurate in the incubation mixture in the presence of carbon monoxide or under helium, when compared to that of the control incubation (Table 2), may support this hypothesis. Teschke et al. reported also that a hepatic ethanol metabolizing system from rat liver could be partially inhibited by carbon monoxide (19). Therefore, it seems that the microsomal oxidoreductase which is insensitive to pyrazole, and the oxidation system containing cytochrome P-450, are involved for the oxidation of 1-dodecanol to lauric acid in frog liver microsomes. Bosron et al. recently described a new and distinctive form of human liver alcohol dehydrogenase, π-ADH, which is insensitive to pyrazole (20). Further experimental work is needed to more clearly elucidate the hydroxylating system of fatty alcohol in frog liver microsomes.

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Ceramide Structure of Sphingomyelin from Human Milk Fat Globule Membrane

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ABSTRACT

Sphingomyelin was purified from human milk fat globule membrane and submitted to phospholipase C to yield ceramide. The structure of this ceramide was investigated by gas liquid chromatographic analyses of its components, fatty acids and sphingoid bases. The structure of the native ceramide was confirmed by direct-inlet mass spectrometry. It was shown to contain a major base C_{18}-sphingosine associated with a high proportion (60%) of C_{20}, C_{22}, C_{24} and $C_{24:1}$ nonhydroxylated fatty acids. As these very long-chain fatty acids might be of nutritive importance, the concentration of sphingomyelin in human milk and its distribution in cream and skim milk were established.

INTRODUCTION

Lipids are secreted by the mammary gland as fat droplets containing a triglyceride core surrounded by a thin lipoprotein membrane made of elements from the Golgi apparatus as well as from the plasma membrane of acinar cells (1-3). Thus, the major part of the lipids commonly found in membranes, i.e., phospholipids, glycolipids and cholesterol, occur in milk as components of the fat globule membrane (4,5). These lipids account for only a few percent of the total milk lipids but they may be of great nutritive importance in the neonatal period as milk is the only natural nutrient at this crucial time of early development.

Recent studies on fatty acid synthesis in brain of neonatal mouse suggest that a part of the long chain fatty acids required for normal myelin formation may have a nutritional origin (6,7). If such a requirement exists in human infancy, milk sphingolipids are the best candidate to fulfill it.

We have elucidated the structure of human milk cerebroside (8). In the present study, we investigated the structure of sphingomyelin, the major sphingolipid of human milk. As previous reports on the matter display some discrepancy (9,10) and as reliable data might be of importance for nutritive balance of artificially composed milk, it appeared that the fatty acid composition of human milk sphingomyelin deserved a new investigation.

MATERIALS AND METHODS

Milk Fat Globule Membrane Preparation

Human milk (750 ml), collected and refrigerated at a local lactarium, was processed on the day of collection. Milk was centrifuged at 17 C for 30 min at 45,000 x g. The cream (28 g) was collected by aspiration and gently suspended in 20 ml/g of 0.1 M Tris-HCl buffer, pH 7.2. The suspension was centrifuged again under the same conditions. The washed cream was suspended in 5 ml/g of Tris-HCl buffer and churned with a Polytron homogenizer at low speed (speed setting 2). The buttermilk was centrifuged at 4 C for 1 hr at 100,000 x g in order to obtain pellets of milk fat globule membranes. Membranes were dispersed in 20 vol of chloroform/methanol (2:1, v/v) at room temperature and then reextracted with 10 vol each of chloroform/methanol (1:1, v/v) and (1:2, v/v). Lipid extract was weighed (71 mg) as well as the nonlipid residue (48 mg).

Lipid Extraction from Crude Milk and Milk Subfractions after Centrifugation

Frozen human milk was used in this experiment. Milk was allowed to thaw at 4 C overnight. Part of it was shell-frozen and lyophilized. The other part was centrifuged at 70,000 x g for 45 min. At the end of the run, the centrifuge tubes were frozen at -20 C and then in liquid nitrogen. The upper layer of cream was fractured as well as the pellet. This procedure avoided contamination of the aqueous phase (skim milk) by the cream or by the particles of the pellet. The cream and the skim milk were weighed and lyophilized. Pellets were suspended in saline, pooled and centrifuged.

The pellet was suspended in a small volume of saline and dispersed in 20 vol of chloroform/methanol (2:1, v/v). After a first extraction, the residue was reextracted twice as described for the fat globule membrane. Lyophilized crude milk and skim milk were weighed. A volume of water equal to the dry weight was added and lipids were extracted with 20 vol of chloroform/methanol (2:1, v/v)/g of hydrated material. Residues of the first extraction were

extracted again in 10 vol of chloroform/methanol (1:1, v/v) and in 10 vol of chloroform/methanol (1:2, v/v). Lyophilized cream was first homogenized in 10 vol of chloroform/g of dry weight. The residue was then extracted successively in 4 vol of chloroform/methanol/water (40:20:3, v/v/v), in 2 vol of chloroform/methanol (1:1, v/v) and in 2 vol of chloroform/methanol (1:2, v/v).

Sphingomyelin Purification

Combined lipid extracts of each sample were dried and weighed. Lipids were dissolved in chloroform/methanol (2:1, v/v) and partitioned according to Folch et al. (11). Lipid phosphorus was assayed on the purified lipid extracts by Bartlett's method (12). Purified lipids were separated into classes by chromatography on a silicic acid column (Biosil-A Bio-Rad Lab., Richmond, CA). Phospholipids were eluted with methanol and submitted to alkaline methanolysis in the presence of 0.3 N NaOH in chloroform/methanol (2:1, v/v) for 1 hr at room temperature. Then, the solution was neutralized with 10% acetic acid in methanol and partitioned after addition of water. The alkali-resistant phospholipids were separated by thin layer chromatography on silicic acid with chloroform/methanol/water (60:25:4, v/v/v). Individual spots, detected with a water spray, were assayed for phosphorus content.

Ceramide Isolation

The alkali-resistant phospholipids, which contained sphingomyelin, were submitted to enzymatic hydrolysis according to Karlsson (13) in order to obtain ceramide from sphingomyelin. Phospholipids (3-5 μmol) were dried at the bottom of a screw-capped, Teflon-lined, 10-ml tube. Two ml of a 1 mM $CaCl_2$-50 mM Tris-HCl buffer, pH 7.2, 0.5 mg of phospholipase C from *Clostridium welchii* (Sigma Chemical Co., St. Louis, MO) and 2 ml of diethyl ether were added. Tubes were shaken overnight at room temperature. Ceramides were extracted 4 times with 2 ml of diethyl ether. The resulting ceramide was purified by Biosil-A column chromatography as already described (14). Under these conditions, the recovery of free ceramide was quantitative.

Ceramide Hydrogenation and Hydrolysis

Ceramide (1 mg) was hydrogenated in methanol (1 ml) in the presence of Adams' platinum oxide catalyst (0.5 mg). Hydrogen was bubbled through the suspension for 5 min. The tube was capped and left overnight with gentle stirring.

Native, as well as hydrogenated, ceramides were hydrolyzed in methanol/conc. HCl/water (83:9.6:10.3, v/v/v) for 18 hr at 80 C. Fatty acids, extracted with hexane, were converted to methyl esters with anhydrous methanolic 0.8 N HCl before separation into nonhydroxylated and 2-hydroxylated esters (15). Long-chain bases were converted into aldehydes by periodate oxidation (16).

Analytical Methods

Gas liquid chromatography of fatty acid methyl esters and of aldehydes derived from sphingoid bases were performed according to published procedures (14).

Mass spectrometry of the trimethylsilylated ceramides was performed on a V.G. Micromass M.M. 305-F instrument. Electron energy was 70 eV, acceleration voltage was 8 KV and ionization current 100 μA. The ion source temperature was set at 200 C and the probe was heated from ambient temperature to 250 C. Mass spectra were recorded every 3 sec on the data acquisition system (V.G. Data System 2050).

RESULTS

Sphingomyelin was purified from 3 different batches of milk fat globule membranes and their fatty acids were analyzed (Table 1). Hydroxy fatty acid methyl esters were not detected and considered to occur below the 1% level. Small fluctuations of normal fatty acid composition appeared from sample to sample. The only main unsaturated fatty acid was nervonic acid. Nervonic acid and its saturated homolog, lignoceric acid, occurred in nearly equal quantities. Both C_{24} fatty acids contributed 30-40% of the human milk sphingomyelin fatty acids. From these analyses, it was evident that, in sphingomyelin of human milk fat globule membrane, two-thirds of the fatty acid residues had a chain length of 20 or more carbon atoms. The other third was contributed mainly by palmitic and stearic acids in similar proportions.

The sphingoid bases, analyzed as their aldehyde derivatives after periodate oxidation, displayed a chromatographic profile of multiple peaks. However, a prominent peak had a retention time equal to octadecanal. Upon hydrogenation, the entire profile shifted toward lower molecular weights as expected if aldehydes had lost 2 carbon atoms. This is the characteristic chromatographic behavior of a molecule with a *trans* double bond before and after hydrogenation (17). The major peak was then identified as hexadecenal. The parent base,

C_{18}-sphingosine, accounted for 60% of the sphingoid bases of human milk sphingomyelin. A similar proportion was found in the monohexosylceramide (Table 2). In sphingomyelin, a small amount of saturated base C_{18}-sphinganine was also detected and estimated to account for 7.1% of the bases.

Analyses of ceramide components after hydrolysis were confirmed by mass spectrometry of the trimethylsilylated genuine ceramide (Fig. 1). The most intense ions detected on the spectrum were easily interpreted as fragments arising from association of C_{18}-sphingosine with the major fatty acids listed in Table 1 (Fig. 2). Peaks at m/z 311 (M-a), 426 (M-(b+1)) and 336 (M-(b+1+90)) were characteristic fragments of the long-chain base sphingosine (14,18). The association of sphingosine with 24:0 or 24:1 gave rise to twin ions at m/z 778-776 (M-15), 703-701 (M-90), 690-688 (M-103) and 600-598 (M-103-90). Ions with similar formation were also detected for C_{23}, C_{22} and C_{20} N-acylsphingosine.

Fatty acid fragments M-a and M-(a-73), which were produced by fragmentation of ceramide containing either sphingosine or sphinganine, were found to be prominent in the spectrum as exemplified by the twin ions of 24:1 and 24:0 acylamides at m/z 480-482 and 553-555. Minor unsaturated bases gave a peak common with C_{18}-sphingosine at m/z 243 (M-(b+1+c)) and specific peaks (fragment a) at m/z 297 (C_{17}-sphingosine) and 283 (C_{16}-sphingosine). The presence in human milk sphingomyelin of ceramides containing C_{18}-sphinganine was confirmed by the mass spectrum; a peak at m/z 313 coming from fragment "a" of sphinganine had an intensity 10 times lower than the homolog peak of sphingosine. It was of the same order of magnitude as that calculated from the analysis of aldehyde profile before and after hydrogenation (Table 2). Fatty acid fragments M-(a+89), typical of ceramide containing sphinganine, were also identified at m/z 391-393 (24:1 and 24:0), 365 (C_{22}), 337 (C_{20}), 309 (C_{18}) and 281 (C_{16}).

These data suggested that fatty acid distribution was about the same when associated with sphinganine as when associated with sphingosine.

As long-chain fatty acids might be important nutrients in early infancy, the sphingomyelin content of human milk was determined as well as its distribution in cream and skim milk (Table 3). It appeared that 70% of the phospholipids were associated with cream, 26% with skim milk and 4% in the pellet. The pellet was made of cellular debris and of milk fat globule membranes liberated when the frozen milk was thawed (3). Sphingomyelin contributed 28-29% of the milk phospholipids and this proportion was maintained in cream and in skim milk. Sphingomyelin purified from crude milk, cream or skim milk displayed an identical fatty acid composition. The concentration of sphingomyelin in human milk was found to be 11 μmol/100 ml. As two-thirds of its fatty acids had 20 or more carbon atoms, the human milk contained at least 7.25 μmol of long chain fatty acids/100 ml.

TABLE 1

Fatty Acid Composition (mol %) of Human Milk Sphingomyelin

Fatty acid	1[a]	2[a]	3[a]	4[b]
14:0	1.7	2.1	2.4	2.0
14:1	.1	–	–	–
15:0	.1	.1	.1	.1
15:1	.2	–	–	–
16:0	15.8	12.7	16.1	12.8
16:1	.3	.1	.6	.6
17:0	.5	.6	.2	.5
18:0	9.1	15.2	12.1	11.8
18:1	.7	.5	.4	1.0
18:2	–	–	–	.3
19:0	.2	.7	.4	.4
20:0	8.3	10.8	9.4	8.9
20:1	–	.3	.4	.5
21:0	–	.6	.5	.8
22:0	16.2	19.1	16.4	19.5
22:1	.7	.9	1.1	1.6
23:0	4.2	4.0	3.6	4.0
23:1	–	.3	.2	–
24:0	20.0	17.2	15.4	19.5
24:1	19.7	13.3	19.3	15.4
25:0	.4	.3	.4	–
25:1	.8	1.2	.3	–
26:0	.3	–	.2	–
26:1	.7	–	.5	–

[a]Analyses of 3 different batches of human milk sphingomyelin.

[b]Data from Morrison and Smith (9).

TABLE 2

Sphingoid Base Composition (wt %) of Human Milk Sphingomyelin and Glycosylceramide

Sphingoid base	Sphingomyelin	Glycosylceramide[a]
12:1	1.6	1.4
13:1	2.8	3.1
14:1	6.3	8.3
15:1	2.9	4.0
16:1	4.8	7.8
17:1	5.7	6.6
18:0	7.1	–
18:1	61.8	60.0
19:1	1.6	2.0

[a]Monoglycosylceramide containing galactosyl and glucosylceramide.

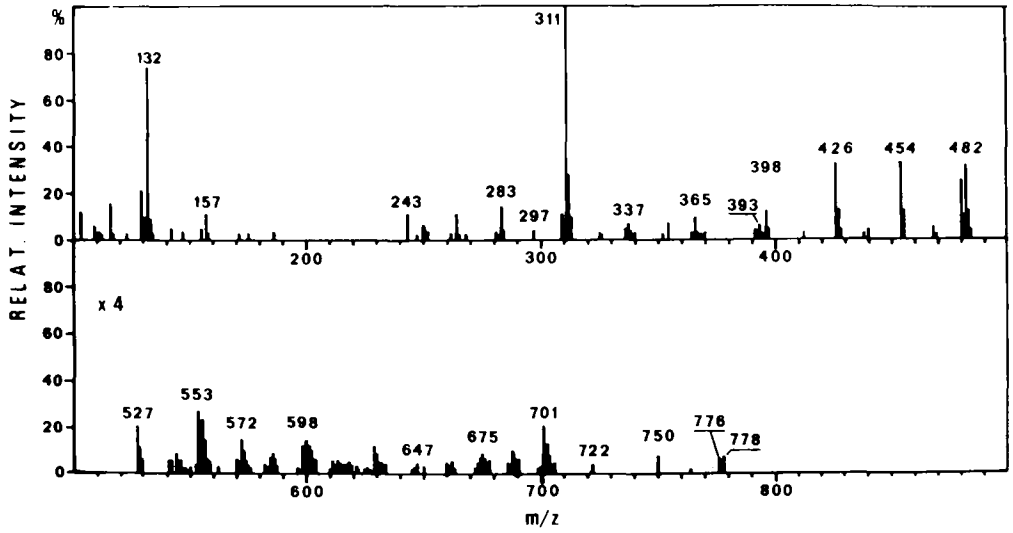

FIG. 1. Mass spectrum of ceramide liberated from human milk sphingomyelin (Table 1, sample 2). Ceramide was trimethylsilylated and analyzed by direct-inlet electron-impact mass spectrometry. The spectrum was recorded at a probe temperature of 210 C for the maximal intensity of the peak at m/z 311.

DISCUSSION

Bracco et al. (10) have reported that sphingomyelin of human milk fat globule membranes contains large amounts (31%) of 18:1 fatty acid. Our own gas chromatographic analyses, with or without hydrogenation of the ceramide, demonstrated that all but one of the fatty acids, nervonic acid, were saturated. Mass spectrometry of the ceramide liberated by enzymatic hydrolysis of sphingomyelin confirmed these findings and ruled out any significant contribution of a possible N-oleyl-sphingosine.

The fatty acid composition of sphingomyelin from human milk fat globule membrane which was reported here was in good agreement with the data published by Morrison and Smith (9). Furthermore, the present study demonstrated that 2-hydroxy fatty acids, which were not assayed by previous investigators, accounted for less than 1% of the sphingomyelin fatty acids.

Our results showed that human milk sphingomyelin differed also from cow milk sphingomyelin in its sphingoid base composition. Relative proportions of sphinganine vs sphingosine bases were similar but cow milk sphingomyelin was more heterogeneous and contained a higher proportion of C_{16}-sphingosine and branched chain bases (17).

It has been demonstrated that sphingomyelin (19) and cerebroside (20) are hydrolyzed in rat intestinal lumen prior to absorption of the constituent sugar, fatty acid and sphingosine. An enzyme activity associated with the lactase of the brush border was shown to hydrolyze glycolipids in rabbit intestine (21). Assuming that similar mechanisms of digestion occur during infancy and that long-chain fatty acids of nutritional origin are likely to be involved in myelin synthesis (6,7), it was of interest to determine the amount of sphingomyelin in human milk. The phospholipid concentration was found to be 39 μmol or 30 mg/100 ml of milk (Table 3). This result compared favorably with recent data obtained by Lauber and Reinhardt (22), showing that breast milk from Ivory Coast mothers contained 20 to 35 mg of phospholipids/100 ml. The relative proportion of sphingomyelin (29% of phospholipids) (Table 3) was alo in good agreement with previous results: 26.2% (10) and 31.2% (23). This proportion was the same in cream as in skim milk and, in both constituents, the fatty

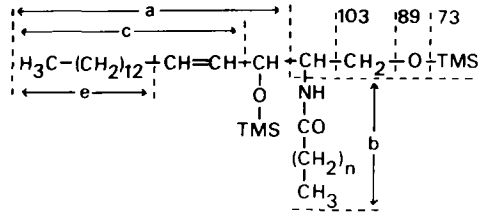

FIG. 2. Fragmentation of trimethylsilylated ceramide according to Samuelsson and Samuelsson (18).

TABLE 3

Sphingomyelin Content of Human Milk

	Volume (ml)	Dry wt (g)	Lipid wt (g)	Phospholipids (μmol)	μmol % of total phospholipids		Sphingomyelin (μmol/100 ml)
					Alkali-resistant phospholipids	Sphingomyelin	
Crude milk	175	21.5	6.6	68.8	41.0	28.1	11.0
Centrifuged milk	450	–	–	–	–	–	–
Cream	nd[a]	15.0	13.3	120.0	40.0	29.2	7.8
Skim milk	407	39.5	1.1	44.8	40.4	27.9	2.8
Pellet	nd	nd	nd	4.7	nd	nd	–

[a]nd = not determined.

acid composition of sphingomyelin was identical. Similar observations have already been reported in cow milk (24).

These findings demonstrated that 100 ml of human milk contained 7.25 μmol of long chain fatty acids as components of sphingomyelin and that two-thirds of this amount was located in the cream.

Cerebrosides, i.e., galactosylceramide, glucosylceramide and lactosylceramide, also contribute to the long-chain fatty acid content of human milk. We have already shown that there is 1 molecule of cerebroside/27 molecules of phospholipids in human milk fat globule membrane and that cerebrosides have the same proportion of long-chain fatty acids as sphingomyelin (8). These results show their contribution to the long-chain fatty acid content of human milk to be 0.9 μmol/100 ml of milk. From the present study, we conclude that 100 ml of human milk could supply at least 8 μmol of fatty acids with 20 or more carbon atoms as constituents of sphingomyelin or cerebrosides.

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Effects of Clofibrate and Tiadenol on the Elimination of Lipids and Bile Acids in Rat Bile

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ABSTRACT

The aim of the work presented here was to compare the biliary elimination of cholesterol and the different bile acids of rats that had been made hypolipidemic by short-term treatments with clofibrate or tiadenol. Both treatments induced a significant decrease in cholesterol output in the bile. The analysis of the different bile acids showed a decrease in dihydroxylated acids elimination (especially CDC acid) without any difference between the 2 sexes. This decrease was associated with an increase in cholic acid excretion. These results are directly correlated with the dose of the administered hypolipidemic drug. The drugs caused a significant increase in the ratio of trihydroxylated acids to dihydroxylated acids. The maximal effect on the concentration of the biliary acids of the bile and on the output was obtained, for both drugs, with a treatment of 200 mg/kg/day. Clofibrate had a greater effect than tiadenol at this dose. Both drugs show a greater effect on lowering serum lipid levels in female animals when compared to males, whereas elimination of bile cholesterol and modifications of bile acids were greater in male animals than female animals.

INTRODUCTION

Clofibrate (ethyl-α-p-chlorophenoxyisobutyrate) is widely used in man to lower the level of plasma lipids (1,2), and it has also been observed to lower the level of triglycerides and cholesterol in rats (3,4). The way in which clofibrate lowers the level of triglycerides is now well understood: it reduces fatty acids in the liver which reduces the synthesis of triglycerides and, consequently, the production and secretion of very low density lipoprotein (VLDL) (5). On the other hand, the hypocholesterolemic effect is not so well established; we know it reduces the synthesis of hepatic cholesterol by inhibition of hydroxy methylglutaryl coenzyme A reductase and that it promotes the excretion of acid steroids in man (6) and rats (7-10). The excretion of neutral steroids in rats remains unchanged (10) although the excretion of cholesterol through bile and feces in man is increased (11).

Tiadenol (2,2'-(decamethylene dithio) diethylethanol), which has recently been introduced as a therapeutic drug, is more effective than clofibrate in reducing the total lipemia and cholesterolemia in man. Both drugs, however, appear to have the same hypotriglyceridemic effect (12-14). The hypolipidemic and hypocholesterolemic effects of tiadenol on normolipemic rats have been demonstrated (15,16). Long-term treatment does not alter the growth of the rats but causes hepatomegaly (17), choleresis, a slight decrease in the concentration of bile salts excreted in the bile (16), and a decrease in bile cholesterol (15). Tiadenol, like clofibrate, increases the bile-salt-independent fraction of bile (i.e., salts) and increases the ratio of bile salts plus phospholipids to cholesterol.

In a recent publication (18), Fredj et al. reported a comparative study of the effects of the 2 hypolipidemic drugs on the elimination of cholesterol and total bile acids in the bile after the administration of radioactive cholesterol. The excretion of radioactive bile salts is increased by both drugs, whereas the excretion of radioactive cholesterol is increased by clofibrate but tends to be decreased by tiadenol. Consequently, the ratio of radioactive bile salts to radioactive cholesterol is higher with tiadenol than with clofibrate. It seems, therefore, that both drugs affect not only the synthesis of cholesterol but also its conversion into bile acids by the liver. The aim of the present study was to compare more precisely the biliary elimination of cholesterol and different bile acids in rats which have been rendered hypolipidemic after a short treatment with different doses of each of these 2 drugs.

MATERIALS AND METHODS

We used normolipidic, laboratory-bred Wistar rats of both sexes. Before and during treatment the rats had free access to drinking water and received ad libitum a standard commercial diet in which the quantity of cholesterol was less than 0.07%; this complete vitamin-rich diet is low in cholesterol.

Clofibrate and tiadenol were administered daily for 7 days through gastric tubes. A solution of 2 g of the drug in 100 ml of gum arabic water was given so that each rat received

100 or 200 mg/kg/day of hypolipidemic substance. Control rats received the same amount of gum arabic water at the same time under the same conditions.

On the 8th day, i.e., the day following the last treatment, female rats (weighing an average of 200-250 g) and male rats (weighing an average of 250-300 g) were fasted for 12 hr. The rats were anesthetized with ethylurethane and a bile fistula was formed. Body temperature was allowed to normalize for 30 min and then a sample of bile was collected for 60 min in a tube held in an ice-bath. The rats were then bled to death by severing the carotid artery. The plasma was immediately collected and analyzed for the level of total cholesterol (19), phospholipids (20) and total lipids (Boehringer biochemica test 15.911).

Bile was tested for the level of cholesterol by gas chromatography (21), for phospholipids by measuring total phosphorus after mineralization with perchloric acid and hydrogen peroxide (Boehringer biochemica test 15.620), and for total bile acids by the 3α-hydroxysteroid dehydrogenase method (22). The different bile acids from each sample of bile were separated and analyzed by gas chromatography. Hydrolysis of conjugated bile acids was accomplished by cholylglycine hydrolase by the following procedure: 35 μl of bile, 15 μl of 9‰ NaCl, 500 μl of acetate buffer and 50 μl of enzyme were mixed. Tubes were incubated at 37 C for 150 min. The acetate buffer was 0.2 M in sodium acetate, pH 5.6, with added 20 μM β-mercaptoethanol and 20 μM EDTA. Internal standards were introduced at the end of the incubation. The solutions were then acidified with 0.5 ml of 5 N HCl and 1 ml of a saturated solution of NaCl was added. The bile acids were extracted with ethyl-acetate and the methylated derivatives were obtained by treatment with a solution of diazomethane in ether.

Bile acids were analyzed by gas chromatography (Packard Becker 417) with glass columns and flame ionization detector. The columns, 150 cm long with an internal diameter of 0.3 cm, were filled with acid-washed, DMCS-treated Chromosorb W (100 and 120 mesh). This support was impregnated at 2% with a methyl phenyl silicone (SP 2250, Supelco) which has the double advantage of lower viscosity and thermal stability greater than other tested silicones (OV 17-QF 1 or OV 210). The temperature of the column was fixed at 250 C, carrier gas (nitrogen U) at 45 ml/min, hydrogen flow 30 ml/min and compressed air 300 ml/min.

Quantitative determination of the bile acids was done by the addition of internal standards. Lithocholic acid (Supelco), which has a shorter retention time, and norcholic acid (Aldrich qual. Puriss.), which has a retention time placing it equally between chenodeoxycholic acid and cholic acid, were used as internal standards. The elution order of the mixture of components was: cholesterol, monohydroxylated acids (lithocholic acid), dihydroxylated acids (deoxycholic acid, chenodeoxycholic acid, hyodeoxycholic acid), norcholic acid and trihydroxylated acids (muricholic and cholic acids).

RESULTS

No significant difference was noticed between the control group and the experimental group with respect to growth rates. Table 1 shows the changes in liver weight, the choleresis and the level of serum lipids as a function of the treatments. A dose of 100 mg/kg/day for 1 week caused a significant hepatomegaly. This weight increase was more noticeable in female rats and reached 18% with tiadenol. The treatment caused a slight decrease in the bile flow in both sexes, which was more notable with clofibrate, without affecting the total lipids and serum cholesterol.

Given in 200 mg/kg/day doses, both drugs produced a very marked hepatomegaly (reaching 40% in both sexes) and a marked choleresis (reaching 30% with tiadenol and 40% with clofibrate). This dose of clofibrate caused a greater effect on lowering serum lipids level in female animals when compared to males, whereas tiadenol caused a greater effect in male animals than female animals.

It seems, therefore, that a short treatment with small doses of the drugs will cause hepatic disturbance even if it does not lower the level of lipids.

EFFECTS OF DRUGS ON THE COMPOSITION OF BILE

In female rats, treatment with 200 mg/kg/day of clofibrate reduced by 30% the concentration of biliary cholesterol without significantly changing the bile output of this sterol (Table 2). Both drugs reduced the bile output of cholesterol in males, though the concentration was only significantly lowered by 200 mg/kg doses. Clofibrate was always more potent than tiadenol.

In females, the only change observed was a reduction in the concentration of phospholipids induced by a treatment of 200 mg/kg/day of clofibrate. In males, both drugs in small doses

(100 mg/kg/day) increased the concentration without changing the bile output.

Neither drug affected the concentration of total bile acids in the bile of female rats compared to the control values. On the other hand, the output of bile salts was significantly increased by doses of 200 mg/kg/day (an increase of 30% with tiadenol and 40% with clofibrate). No significant change in the concentration or output of bile salts was observed in male rats with either treatment.

The solubilization power of bile, which is the molar ratio of bile salts + phospholipids to cholesterol, was altered only with high doses of the hypolipidemic drugs. Administered in 100 mg/kg/day doses for 7 days, neither clofibrate nor tiadenol had any effect on the cholesterol solubilization ratio. With a 200 mg/kg/day dose, both drugs significantly increased the solubilization ratio (+ 25% with tiadenol; + 45% with clofibrate) in both male and female rats.

EFFECTS OF DRUGS ON THE ELIMINATION OF PRINCIPAL BILE ACIDS IN BILE

In the bile from control rats, the concentration of deoxycholic acid was 0.61 ± 0.22 g/ℓ in females and 0.92 ± 0.18 g/ℓ in males. Doses of 100 mg/kg/day did not significantly affect the concentration of deoxycholic acid in the bile, although 200 mg/kg/day doses decreased the concentration in both sexes (33% with tiadenol and about 50% with clofibrate). In animals treated with 200 mg/kg/day of tiadenol, the concentration was 0.41 ± 0.13 g/ℓ for females, and 0.61 ± 0.19 g/ℓ for males. With clofibrate, the concentration was 0.33 ± 0.12 g/ℓ for females and 0.43 ± 0.21 g/ℓ for males. The bile output of deoxycholic acid was significantly reduced in males, even though treatment with 200 mg/kg/day of clofibrate caused choleresis. In females, the output of deoxycholic acid was not significantly affected as the increase of choleresis with 200 mg/kg/day doses was compensated by the decrease in concentration (Fig. 1).

Both drugs significantly reduced the output of chenodeoxycholic acid in the bile in both male and female rats (Fig. 1). In females, the concentration was reduced from 0.81 ± 0.19 g/ℓ to 0.65 ± 0.12 g/ℓ by 100 mg/kg/day of tiadenol and to 0.21 ± 0.13 with 200 mg/kg/day of clofibrate. The effect obtained was directly related to the dose since 100 mg/kg/day and 200 mg/kg/day doses caused, respectively, a decrease in concentration of about 20% and 60% with tiadenol and about 25% and

TABLE 1

Effect of a Short-Term Treatment (100 or 200 mg/kg/day for one week) with Clofibrate or Tiadenol on Body Weight, Hepatic Weight, Serum Lipids and Choleresis of Male and Female Rats

	Sex (Female, F; male, M)	Number	Body weight (g)	Hepatic weight (g/100 g BW)	Serum lipids			Bile flow (ml/hr/100 g BW)
					Cholesterol (g/ℓ)	Phospholipids (g/ℓ)	Total lipids (g/ℓ)	
Controls	F	21	225	2.41 ± 0.12	0.53 ± 0.16	1.07 ± 0.32	3.07 ± 0.62	0.24 ± 0.06
	M	17	253	2.82 ± 0.24	0.57 ± 0.12	0.99 ± 0.15	2.70 ± 0.54	0.25 ± 0.06
Clofibrate 100[a]	F	15	224	2.48 ± 0.08*	0.50 ± 0.05 NS	0.93 ± 0.12 NS	2.81 ± 0.17 NS	0.17 ± 0.03**
	M	15	244	2.55 ± 0.19*	0.54 ± 0.14 NS	0.87 ± 0.18 NS	2.71 ± 0.43 NS	0.18 ± 0.03*
Tiadenol 100[a]	F	16	227	2.88 ± 0.31**	0.47 ± 0.14 NS	0.94 ± 0.20 NS	2.72 ± 0.62 NS	0.20 ± 0.04*
	M	15	273	2.98 ± 0.20**	0.47 ± 0.08 NS	0.76 ± 0.14**	2.70 ± 0.50 NS	0.15 ± 0.02**
Clofibrate 200[b]	F	19	220	3.33 ± 0.17***	0.35 ± 0.09***	0.65 ± 0.07***	1.81 ± 0.23***	0.37 ± 0.08**
	M	17	259	3.92 ± 0.36***	0.44 ± 0.11**	0.84 ± 0.11**	2.47 ± 0.30 NS	0.35 ± 0.07***
Tiadenol 200[b]	F	18	218	3.47 ± 0.24***	0.41 ± 0.08**	0.74 ± 0.21**	2.45 ± 0.27**	0.32 ± 0.03**
	M	16	277	4.01 ± 0.38***	0.39 ± 0.10***	0.68 ± 0.19***	2.28 ± 0.65**	0.33 ± 0.13**

[a]Treatment with 100 mg/kg/day.
[b]Treatment with 200 mg/kg/day.
Significance of difference determined by Student's t-test: asterisks (*, ** or ***) indicate differences from the control group (respectively, $p<0.05$; $p<0.01$; $p<0.001$); NS: no significance.

TABLE 2

Effect of a Short-Term Treatment (100 or 200 mg/kg/day for one week) with Clofibrate or Tiadenol on the Biliary Lipids of the Basal Bile of Male and Female Rats

	Sex (Female, F; male, M)	Biliary cholesterol		Biliary phospholipids		Total bile acids		BS + PL/CH
		g/ℓ	µmol/hr/100 g	g/ℓ	µmol/hr/100 g	g/ℓ	µmol/hr/100 g	
Controls	F (20)	0.13 ± 0.02	0.084 ± 0.022	3.99 ± 0.45	1.29 ± 0.24	11.98 ± 1.56	6.85 ± 1.07	96.9 ± 8.2
	M (17)	0.15 ± 0.03	0.102 ± 0.028	3.67 ± 0.58	1.23 ± 0.24	11.99 ± 1.97	7.38 ± 1.37	84.4 ± 11.1
Clofibrate 100[a]	F (15)	0.12 ± 0.03	0.074 ± 0.012	3.36 ± 0.93	0.88 ± 0.26	11.39 ± 1.25	5.57 ± 1.21	87.1 ± 11.3
	M (15)	0.18 ± 0.04	0.070 ± 0.015*	5.52 ± 0.41**	1.07 ± 0.25	13.18 ± 1.81	6.10 ± 1.38	102.4 ± 13.2
Tiadenol 100[a]	F (16)	0.15 ± 0.02	0.086 ± 0.011	4.05 ± 0.85	1.08 ± 0.29	12.67 ± 1.19	6.18 ± 0.91	84.4 ± 12.6
	M (15)	0.17 ± 0.03	0.080 ± 0.023	5.88 ± 0.71**	1.29 ± 0.31	12.36 ± 1.13	6.13 ± 0.96	92.7 ± 9.7
Clofibrate 200[b]	F (19)	0.09 ± 0.02***	0.080 ± 0.015	3.04 ± 0.38**	1.43 ± 0.38	11.25 ± 1.07	9.81 ± 1.37**	140.5 ± 11.2***
	M (17)	0.082 ± 0.03***	0.067 ± 0.009**	2.92 ± 0.97	1.28 ± 0.22	8.78 ± 1.82	7.09 ± 1.18	124.9 ± 15.1***
Tiadenol 200[b]	F (18)	0.11 ± 0.016	0.086 ± 0.013	4.12 ± 0.67	1.51 ± 0.33	12.46 ± 1.83	8.92 ± 1.43*	121.3 ± 10.7**
	M (16)	0.12 ± 0.03**	0.066 ± 0.018**	4.13 ± 0.84	1.18 ± 0.28	11.48 ± 1.71	5.98 ± 1.85	106.5 ± 15.6**

[a]Treatment with 100 mg/kg/day.
[b]Treatment with 200 mg/kg/day.
Significance of difference determined by Student's t-test: asterisks (*, ** or ***) indicate differences from the control (respectively, $p<0.05$; $p<0.01$; $p<0.001$).

70% with clofibrate. Clofibrate is significantly more effective than tiadenol.

The concentration of hyodeoxycholic acid in the bile was only significantly lowered by 200 mg/kg/day doses of clofibrate. In male rats, it was lowered from 0.53 ± 0.14 g/ℓ to 0.30 ± 0.11 g/ℓ. On the other hand, the output was reduced significantly by 200 mg/kg/day of tiadenol and all doses of clofibrate. With clofibrate, the bile output of hyodeoxycholic acid was reduced in relation to the dose administered (less 20% for 100-mg doses and less 30% for 200-mg doses). In female rats, the bile output was only significantly lowered by 200 mg/kg/day doses of drugs (Fig. 1).

The concentration of cholic acid was 3.9 ± 0.7 g/ℓ and 4.6 ± 0.2 g/ℓ in male and female control rats, respectively. Both drugs greatly increased the concentration and bile output of cholic acid in rats of both sexes. With doses of 100 mg/kg/day, the concentrations of cholic acid was increased from 35% to 40%, whereas with 200-mg doses, they reached 70%. At high doses of the drugs, the outputs were doubled with tiadenol and tripled with clofibrate (Fig. 2).

The analysis of different bile acids clearly shows a decrease in the excretion of dihydroxylated acids together with an increase in the excretion of cholic acid. These changes cause a large increase in the ratio of trihydroxylated acids to dihydroxylated acids. This ratio was increased 2 or 3 times by 200 mg/kg/day doses (Fig. 3).

Therefore, we can conclude that clofibrate has an effect that is more important than tiadenol, when given in equivalent doses, and that the changes in male rats are more marked than in females.

DISCUSSION

Both drugs used in this study caused an increase in liver weight that was proportional to the doses administered. The increase was slightly more marked with tiadenol when given in larger doses (15). Contrary to certain other studies, we have observed no significant differences in effect between male or female rats. The considerable increase in liver weight is linked to a cellular hypertrophy caused by a large increase in the volume and concentration of peroxisomes with clofibrate (23) as well as with tiadenol (24). Recent studies (25,26) have shown a carcinogenic effect on several species of hypolipidemic substances, including clofibrate. The development of hepatic tumors seems to be linked to the proliferation of peroxisomes, and it would therefore seem desir-

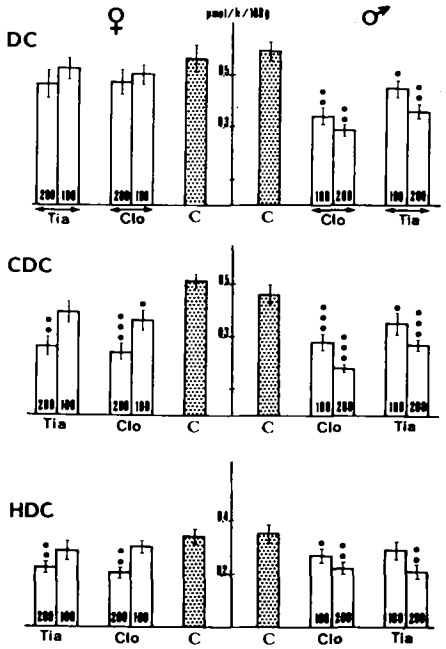

FIG. 1. Effect of a short-term treatment with clofibrate (Clo) or tiadenol (Tia) upon the dihydroxylated bile acids output in bile. C = control group; DC = deoxycholic acid; CDC = chenodeoxycholic acid; HDC = hyodeoxycholic acid. 100 = treatment with 100 mg/kg/day; 200 = treatment with 200 mg/kg/day. Asterisks (*, ** or ***) indicate differences from the control group (respectively, $p<0.05$; $p<0.01$; $p<0.001$).

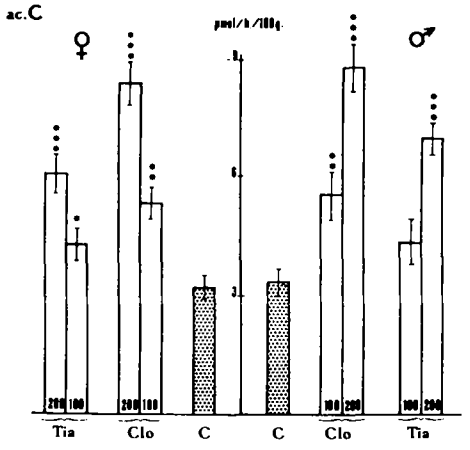

FIG. 2. Comparison of the cholic acid output in bile of control (C) rats with rats treated for a week with clofibrate (Clo) or tiadenol (Tia). 100 = treatment with 100 mg/kg/day; 200 = treatment with 200 mg/kg/day.

able to test tiadenol with this in mind, as even in small doses over a very short period it causes a significant hepatomegaly.

The comparative study of different groups of animals treated with hypolipidemic drugs indicates that hypercholeresis appears only after a sufficiently high daily dosage and is not proportional to the quantity taken. On the other hand, the stability of the total bile acid concentration of the bile leads us to agree with Debray et al. (16), that the increase of the bile flow is independent of the bile salts of bile but is related to a disturbance of the relative proportion of chloride and bicarbonate anions under the influence of both drugs.

A comparative study of the serum disturbances leads us to conclude that (a) tiadenol administered in small doses (100 mg/kg/day) to rats for a week is more effective than clofibrate; (b) the effect of clofibrate in different doses is always more marked in female than male rats (this is in accordance with a clinical study [27] which states that clofibrate produces a more marked decrease in serum phospholipids in women than in men); and (c) the hypotriglyceridemic effect of both drugs demands a larger dose than is needed to produce a hypocholesterolemic effect. Hypocholesterolemia is produced by small daily doses and increases with larger doses. This has already been demonstrated by Owen and Billimoria (28) with doses of clofibrate ranging from 0.25 to 200 mg/kg/day. On the other hand, hypotriglyceridemia is only produced by high daily doses, or with small doses over a long period (29). It seems difficult, therefore, to relate the hypocholesterolemic action with the decrease of the newly formed triglycerides. The decrease of fatty acids in the liver, followed by a decrease in the hepatic production of lipoproteins (30), is a phenomenon that appears later, and for the most part, independently of the decrease of cholesterolemia. The hypocholesterolemic effect is essentially due to a decrease of the synthesis of cholesterol to which can be added a change in the hepatic conversion of bile acids.

The determination of cholesterol and bile acids excreted by the bile of treated rats requires the following comments: (a) the most marked effect on the concentration and output of bile acids in the bile was obtained with 200 mg/kg/day doses of clofibrate or tiadenol. It seemed that clofibrate was more effective than tiadenol when given in this dosage. On the other hand, while we have observed a more marked effect of the drugs on the serum components in female rats, it appears that the changes in the excretion of acids and bile

cholesterol are more significant in male rats. (b) Clofibrate and tiadenol affect the formation of bile acids, especially the hepatic synthesis of primary acids. The capacity of total synthesis hardly varies at all with the 2 drugs, but the respective synthesis of the 2 primary acids is affected—the synthesis of cholic acid is increased, whereas the synthesis of chenodeoxycholic acid is reduced. This observation is in agreement with results obtained in vitro, which show a stimulation of the hepatic 12α-hydroxylase by clofibrate, without affecting the 7α-hydroxylase and the 26α-hydroxylase (31,32). The altered distribution of the 2 primary acids in rat bile makes it possible to contest the hypothesis that clofibrate and its derivatives work by way of thyroxine, i.e., by creating a "hepatic hyperthyroidy." In fact, rats which become hyperthyroid show an increased excretion of bile acids as well as a large increase in the concentration of the primary dihydroxylated acid (33), which is the opposite of what we have observed in hypolipidemic animals. Finally, (c) both drugs greatly increase the ratio of trihydroxylated acids to dihydroxylated acids. This perturbation is a direct consequence of the change in respective synthesis of the 2 primary bile acids. A similar observation has been noted by Einarsson et al. (34) in patients with type II hyperlipoproteinemia. These authors have shown a diminished ratio between the 2 primary acids in patients, related to the below-normal formation of cholic acid. During treatment with clofibrate, the total synthesis of the 2 acids remains unchanged but the ratio of cholic acid to chenodeoxycholic acid is increased. The increase of this ratio seems to provoke a change in the role of bile salts in the intestinal absorption of cholesterol. As Raicht et al. demonstrate in rats (35), taurocholate greatly increases the absorption of cholesterol whereas taurochenodeoxycholate has no effect. This increase of the intestinal absorption of cholesterol in rats, whose level of trihydroxylated acids is increased, is followed by an increase of plasma and hepatic cholesterol. Therefore, it appears that hypocholesterolemia induced by the drugs is not directly associated with an alteration of the cholesterol absorption; nevertheless, it is important to remember that the bile acids act directly on the hepatic metabolism of cholesterol and that the alteration of the quantity of each bile salt in the portal blood alters the synthesis and the degradation of hepatic cholesterol. On the other hand, the change of the intestinal reabsorption of cholesterol observed with an excess of trihydroxylated bile acids can also produce a disturbance of bile salts absorption. This fact would explain why, although the synthesis of bile acids is not disturbed by a treatment with hypolipidemic drugs, a decrease of acid steroids in the feces without modification of the neutral steroids excretion frequently has been observed.

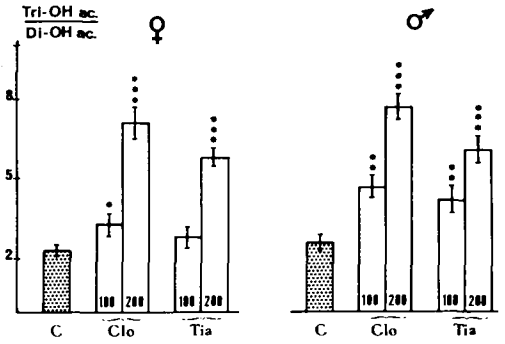

FIG. 3. Comparison of the influence of a short-term treatment with clofibrate (Clo) or tiadenol (Tia) upon the dihydroxylated acids to trihydroxylated acids ratio in the bile of the rat. 100 = treatment with 100 mg/kg/day; 200 = treatment with 200 mg/kg/day.

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Fatty Acid and Cholesterol Synthesis from Specifically Labeled Leucine by Isolated Rat Hepatocytes

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ABSTRACT

Hepatocytes isolated from female rats meal-fed a high-glucose diet were incubated in Krebs-Henseleit bicarbonate medium containing 16.5 mM glucose, ^3H_2O, and ^{14}C-labeled amino acids (-)-Hydroxycitrate depressed the incorporation of ^3H_2O and [^{14}C]alanine into fatty acids and cholesterol. Incorporation of [U-^{14}C]leucine into lipids was not affected but incorporation of ^3H_2O into lipids was decreased significantly by (-)-hydroxycitrate. (-)-Hydroxycitrate depressed the incorporation of radioactivity from [2-^{14}C]leucine into fatty acids and cholesterol by 61 and 38%, respectively, and stimulated the incorporation of radioactivity from [4,5-^3H]leucine 35 and 28%. As [2-^{14}C]leucine labels the acetyl-CoA pool and [4,5-^3H]leucine labels the acetoacetate pool, it was concluded that mitochondrial 3-hydroxy-3-methylglutaryl-CoA is not incorporated intact into cholesterol, and that acetoacetate can be activated effectively in the liver cytosol for support of cholesterol and fatty acid synthesis.

INTRODUCTION

It is generally accepted that there are separate cytoplasmic and mitochondrial generating systems for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) for cholesterogenesis and ketogenesis (1-6). During high carbohydrate feeding, the transport of carbon precursors for fatty acid synthesis from the mitochondria to the cytosol is primarily via citrate, which is subsequently cleaved to acetyl-CoA by ATP citrate lyase (7). Less is known about the transport of carbon for cholesterol synthesis, although it is known that the same citrate pathway is involved.

An additional source of carbon precursors for HMG-CoA for cholesterol synthesis could be available through the reutilization of free acetoacetate in hepatic cytosol. Stern found acetoacetyl-CoA synthetase activity in liver cytosol (8). Sauer (9), Chung and Dupont (10), and Rous (11) demonstrated that ^{14}C-labeled acetoacetate was incorporated into liver lipids. Pleasure et al. reported recently that the extramitochondrial pathway for acetoacetate metabolism served as a significant source of carbons for sterol synthesis by calf brain oligodendrocytes (12).

Leucine is metabolized to HMG-CoA in the mitochondrion; 2-labeled leucine will appear only in the mitochondrial acetyl-CoA pool and [U-^{14}C]labeled leucine will appear in both the acetyl-CoA and acetoacetate fractions. These labeling patterns are illustrated in Figure 1. A greater incorporation of radioactivity from [U-^{14}C]leucine than [2-^{14}C]leucine into fatty acids was observed in mouse liver and adipose tissue and rat adipose tissue, suggesting that these tissues have a reduced capacity to use citrate for lipogenesis in comparison to rat liver in which citrate is an important precursor of fatty acids (13). Patel and Owen (14) compared the incorporation of [U-^{14}C]leucine and [2-^{14}C]leucine into lipids by cortex slices of rat brain. They reduced the generation of acetyl-CoA from citrate by adding (-)-hydroxycitrate which inhibits ATP-citrate lyase and found that the incorporation of the 2-label was inhibited to a much greater extent than the U-label. These studies demonstrate that cytosolic acetoacetate derived from the mitochondrial metabolism of leucine could supply carbons for lipogenesis.

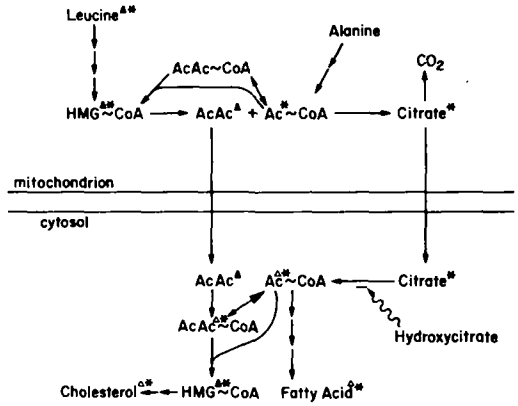

FIG. 1. Effect of (-)-hydroxycitrate on metabolic pathways of lipid synthesis from leucine and alanine. *Traces the pathway for 2-labeled leucine and Δ traces the pathway for 4,5-labeled leucine.

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The isolated hepatocyte system was employed in this study, as it is an excellent model for studying lipid synthesis, exhibiting normal physiological rates and profiles of lipid biosynthesis in a homogenous cell type. (-)-Hydroxycitrate was used to block the synthesis of acetyl-CoA from citrate because it is a potent competitive inhibitor of ATP citrate lyase (15,16), and inhibits hepatic fatty acid (17) and cholesterol synthesis (18,19). This report examines the hypothesis that the mitochondrial HMG-CoA pool and cytosolic use of acetoacetate are involved in lipid biosynthesis, by comparing the effect of (-)-hydroxycitrate on the incorporation of specifically labeled leucine into fatty acids and cholesterol.

EXPERIMENTAL PROCEDURES

Female rats were meal-fed a diet containing 70% glucose at least 1 week prior to the experiment. After a 3-hr meal, 2 or 3 rats were anesthetized and their livers were perfused in situ for 20 min with 80 ml calcium-free Hanks solution containing 16.5 mM glucose, 35 mg collagenase and 100 mg hyaluronidase. The liver was then minced and filtered through nylon mesh, and the hepatocytes were washed, pooled and suspended in Krebs-Henseleit bicarbonate medium. The final vol of the incubation medium was 2.1 ml and it contained 10-16 mg dry wt of hepatocytes, 16.5 mM glucose, radiolabeled substrates as indicated in the tables and 2 mCi ^3H_2O, in Krebs-Henseleit bicarbonate medium. [U-^{14}C] Alanine and ^3H_2O were supplied by Amersham/Searle, Arlington Heights, IL; [2-^{14}C] alanine by ICN, Irvine, CA, and specifically labeled leucine by Schwarz/Mann, Orangeburg, NY. When the effect of (-)-hydroxycitrate was studied, it was added as the trisodium salt at 2 mM. The pH remained at 7.4 to 7.6 throughout the incubation. Triplicate incubations were performed for 60 min at 37 C in a gas phase of 95% O_2/5% CO_2 in 25-ml Erlenmeyer flasks, fitted with high-walled glass center wells at 90 rotations/min. Incubation without hepatocytes were run as blanks.

Incubations were terminated by the addition of 0.4 ml of 3 M citric acid, and the $^{14}CO_2$ was trapped for 1 hr then counted (20) under dual label settings in order to discriminate against ^3H radioactivity due to vaporized water that was dissolved in the CO_2 trap. The incubation mixture was made basic by addition of 3.0 ml of 5 N NaOH, transferred to screw-capped tubes and saponified at 90 C overnight. Cholesterol digitonide was prepared from the petroleum ether extract of the nonsaponifiable fraction and free fatty acids were extracted after acidification. Liquid scintillation counting was performed with a Packard Tri-Carb Model 3390 equipped with an Absolute Activity Analyzer, Model 544, for efficiency and spill-over calculations. Data are expressed as nmol of ^3H_2O or radiolabeled amino acid converted to carbon dioxide, fatty acid or cholesterol/hr/mg dry wt of hepatocytes. Calculation is based on the sp act of exogenous substrate with no correction for known losses during metabolism.

The details of preparation of the rats, isolation and incubation of hepatocytes and determination of rates of fatty acid and cholesterol synthesis have been published (19).

RESULTS AND DISCUSSION

The effects of varying the concentration of alanine and leucine are shown in Table 1. The lower concentration of 0.5 mM is near the physiological range; the higher concentration of 10 mM is approaching the Km of aminotransferases (21,22). The ca. 7-fold increase in incorporation of alanine into carbon dioxide, fatty acid and cholesterol that occurred between these 2 concentrations was probably due to the kinetic properties of alanine aminotransferase. The slight increase in absolute rates of lipogenesis as measured by ^3H_2O was probably due to an increased source of carbons. The liver cells did not use 0.5 mM leucine to any significant rate in agreement with reports of others that branched-chain amino acids are primarily catabolized extrahepatically (22,23). An increased use of leucine occurred by increasing the leucine concentration, reflecting the kinetic properties of leucine aminotransferase. Incorporation of ^{14}C label into carbon dioxide and lipids continued to increase at 40 mM (data not shown), but this concentration approaches the maximal solubility for leucine. Omission of glucose from the incubation medium (data not shown) depressed fatty acid synthesis ca. 40-50% at all amino acid concentrations. Adding 2.5 mM 2-ketoglutarate (an amine acceptor for aminotransferases) stimulated use of 10 mM alanine 10-60%, but had no statistically significant effect at 2.5 mM alanine, nor did it affect leucine use at 2.5 mM or 10 mM (data not shown).

The conditions selected for testing the effect of (-)-hydroxycitrate were 2.5 mM amino acid, 16.5 mM glucose and omission of exogenous 2-ketoglutarate. The position of ^{14}C label in alanine was changed from [U-^{14}C] to [2-^{14}C] to eliminate any bias at the 2-keto acid decarboxylase step, and to make it analogous to [2-^{14}C] leucine. A composite of results from 4-6

TABLE 1

Utilization of Different Concentrations of ^{14}C-Labeled Alanine and Leucine by Isolated Rat Hepatocytes[a]

Exogenous carbon source (mM)	[U-^{14}C]Amino acid			^3H_2O	
	Carbon dioxide	Fatty acid	Cholesterol	Fatty acid	Cholesterol
	(nmol/hr/mg dry wt)			(nmol/hr/mr dry wt)	
Alanine					
0.5	13.9 ± 0.1[b]	1.83 ± 0.05	0.17 ± 0.01	36.9 ± 3.6	2.64 ± 0.07
2.5	46.9 ± 0.8	7.31 ± 0.36	0.62 ± 0.03	41.1 ± 0.6	3.79 ± 0.20
10.0	83.5 ± 4.1	14.74 ± 1.00	1.16 ± 0.07	49.3 ± 2.6	4.21 ± 0.22
Leucine					
0.5	0.39 ± 0.01	0.13 ± 0.04	0.031 ± 0.002	24.9 ± 1.5	1.78 ± 0.12
2.5	1.14 ± 0.07	0.42 ± 0.01	0.082 ± 0.003	29.7 ± 0.8	1.90 ± 0.01
10.0	2.83 ± 0.52	1.35 ± 0.3	0.222 ± 0.016	36.5 ± 0.5	2.16 ± 0.05

[a]Incubation was for 60 min at 37 C in 2.1 ml Krebs-Henseleit bicarbonate medium containing 0.7 μCi [U-^{14}C]alanine or 2.6 μCi [U-^{14}C]leucine; 1.8 mCi ^3H_2O; 16.5 mM glucose and 10 mg dry wt of hepatocytes.

[b]Mean and standard error of the mean calculated from triplicate incubations.

experiments is shown in Table 2. Addition of (-)-hydroxycitrate significantly depressed incorporation of [2-^{14}C]alanine, [2-^{14}C]leucine, and ^3H_2O into fatty acids and cholesterol. and had no effect on $^{14}CO_2$ production. These results are consistent with the known inhibition of ATP citrate lyase by this compound. In the presence of (-)-hydroxycitrate, incorporation of [U-^{14}C]leucine into cholesterol and fatty acids was not altered, whereas the absolute rate of lipogenesis determined from ^3H_2O was depressed ca. 37%. These results are similar to those obtained by Rous and Favarger who studied mouse liver (13) and Patel and Owen who studied rat brain (14). Pleasure et al. reported a stimulation of the incorporation of [3-^{14}C]acetoacetate carbons and a reduction of ^3H_2O incorporation for sterol synthesis in the presence of (-)-hydroxycitrate in oligodendrocytes (12). These studies suggest that, under conditions of low use of citrate for lipid synthesis, acetoacetate could be an important alternate carbon source of hepatic cytosolic acetyl-CoA units. The contribution to these results of a diminution in the size of the pool of cytosolic acetyl-CoA from citrate and activation of acetyl-CoA carboxylase caused by (-)-hydroxycitrate must also be considered.

As [U-^{14}C]leucine labels both the acetyl-CoA and acetoacetate pools, a more definitive test of the acetoacetate pathway was done by following radioactivity from [4,5-^{3}H]leucine; positions 4, 5, and 6 of leucine yield acetoacetate (Fig. 1). (-)-Hydroxycitrate stimulated (Table 2) the incorporation of [4,5-^{3}H]leucine into fatty acids and cholesterol by 35 and 28%, respectively, thus accounting for the difference between its effect on [U-^{14}C]- and [2-^{14}C]-leucine. These observations can best be explained as follows: When the use of citrate for acetyl-CoA synthesis is depressed, there can be an increase in the use of acetoacetate in the cytosol for lipid synthesis. The different effects of (-)-hydroxycitrate on 2-^{14}C and 4,5-^{3}H label from leucine support the view that mitochondrial HMG-CoA is not incorporated intact into cholesterol (1-6). The ratio of incorporation of [2-^{14}C]alanine, [2-^{14}C]leucine and ^3H_2O into cholesterol vs fatty acid was between 0.11 and 0.18. The ratio for [U-^{14}C]leucine was 0.22 and for [4,5-^{3}H]leucine, 0.39. The greater propensity to label cholesterol by the latter 2 substrates would be predicted from the direct reutilization of acetoacetate in the cytosol as shown in Figure 1 and the unfavorable equilibrium of acetoacetyl-CoA thiolase (2,5). Webber and Edmond (24) reported ratios in rat brain of 0.22 for glucose and 0.48 for ketone bodies and used an identical logic for interpreting the results.

Further support for the transport of acetyl-CoA units from mitochondria via acetoacetate was obtained by testing for dilution of label from [2-^{14}C]leucine and [4,5-^{3}H]leucine by addition of 1 mM unlabeled acetoacetate (Table 3). If the presence of an unlabeled acetoacetate pool does dilute the incorporation of leucine into lipids, this would suggest that acetoacetate derived from mitochondrial HMG-CoA is transported to the cytosol and subsequently incorporated into fatty acids and cholesterol. The absolute rates of lipogenesis determined

TABLE 2

Effect of (-)-Hydroxycitrate on Utilization of Specifically Labeled Amino Acids by Isolated Rat Hepatocytes[a]

Labeled substrate	(-)-Hydroxycitrate (2 mM)	[^{14}C] Precursor			[^{3}H] Precursor	
		Carbon dioxide	Fatty acid (nmol/hr/mg dry wt)	Cholesterol	Fatty acid (nmol/hr/mg dry wt)	Cholesterol
[2-^{14}C] Alanine and $^{3}H_2O$	–	40.6 ± 8.8[b]	8.36 ± 4.46	1.14 ± 0.64	35.9 ± 11.8	4.36 ± 1.57
	+	36.9 ± 5.7	2.70 ± 1.65[c]	0.42 ± 0.20[c]	24.2 ± 6.5	2.61 ± 0.83[c]
[U-^{14}C] Leucine and $^{3}H_2O$	–	2.31 ± 1.25	0.60 ± 0.23	0.13 ± 0.05	35.1 ± 13.0	3.72 ± 1.27
	+	2.15 ± 0.99	0.62 ± 0.36	0.15 ± 0.08	20.7 ± 6.2[c]	2.44 ± 1.31[c]
[2-^{14}C]; [4,5-^{3}H] Leucine	–	5.07 ± 1.00	1.81 ± 0.61	0.21 ± 0.07	0.46 ± 0.34	0.18 ± 0.11
	+	5.35 ± 0.76	0.71 ± 0.18[c]	0.13 ± 0.07[c]	0.62 ± 0.46	0.23 ± 0.14

[a]The final concentration of amino acid was 2.5 mM. Media contained 1.3 μCi [2-^{14}C] alanine, 2.7 μCi [U-^{14}C] leucine, 4.5 μCi [2-^{14}C] leucine and/or 15 μCi [4,5-^{3}H] leucine. Further details about incubation conditions are given in Experimental Procedures.

[b]Mean and standard deviation calculated from 4 to 6 experiments, using the average of triplicate incubations.

[c]Effect of (-)-hydroxycitrate significant at $p<0.05$ using a paired Student's t-test.

TABLE 3

Effect of Acetoacetate on Utilization of Specifically Labeled Leucine by Isolated Rat Hepatocytes[a]

Acetoacetate (1 mM)	[2-^{14}C] Leucine utilization			[4,5-^{3}H] Leucine utilization		$^{3}H_2O$[b]	
	Carbon dioxide	Fatty acid	Cholesterol	Fatty acid (nmol/hr/mg dry wt)	Cholesterol	Fatty acid	Cholesterol
–	3.85 ± 0.6[c]	2.99 ± 0.05	0.167 ± 0.001	1.06 ± 0.01	0.355 ± 0.006	100.6 ± 3.2	3.78 ± .06
+	3.83 ± 0.8	1.85 ± 0.22	0.089 ± 0.002	0.32 ± 0.01	0.038 ± 0.003	83.6 ± 1.7	2.88 ± .30

[a]Incubation was for 60 min at 37 C in 2.1 ml Krebs-Henseleit bicarbonate medium containing 1.4 μCi [2-^{14}C] leucine, 10 μCi [4,5-^{3}H] leucine, 2.5 mM leucine, 16.5 mM glucose and 15 mg dry wt of hepatocytes.

[b]Tested in separate flasks, only radioactive label was 2.5 mCi $^{3}H_2O$.

[c]Mean and standard error of the mean calculated for triplicate incubations.

from ^3H_2O were depressed about 20% by addition of acetoacetate. This decrease is probably due to a change in subcellular redox potential. The incorporation of 2-^{14}C label into $^{14}CO_2$ was not affected but incorporation into fatty acids and cholesterol was depressed 38 and 47%, respectively. This decrease is partially due to the reason already given and partially due to dilution of the cytosolic acetyl-CoA pool. A marked decrease of 70 and 89%, respectively, was observed when incorporation of [4,5-^{3}H]leucine into lipids was studied and is additional support for the idea that acetoacetate plays a unique role in lipogenesis. The findings reported in Tables 2 and 3 have been confirmed at 10 mM and 40 mM leucine, and in the presence of 2.5 mM 2-ketoglutarate.

The liver is considered to be primarily a producer of acetoacetate, whereas extrahepatic tissues consume acetoacetate for energy and lipid synthesis. Brain adipose tissue and muscle have been shown to incorporate ^{14}C-labeled acetoacetate into lipids (11,14,24-27). [U-^{14}C]-Leucine was incorporated effectively into lipids by extrahepatic tissues and thus it has been suggested to be a major source of cholesterol (28-31). The use of substrates such as [1-^{14}C]-acetate, [U-^{14}C]pyruvate, or [1-^{14}C]octanoate does not distinguish between citrate and acetoacetate as precursors of fatty acids and cholesterol, whereas specifically labeled leucine does.

The rate of acetoacetate activation reported by Stern (8), Rous (11,26) and Buckley and Williamson (32) was ca. 3 to 4 nmol/hr/mg liver. If one assumes a dry-to-wet weight correction factor of 3.7 (33), rates of fatty acid and cholesterol synthesis reported here were 12 and 1.5 nmol acetyl-CoA units/hr/mg liver, respectively. Thus, the acetoacetate pathway can support cholesterol synthesis and has the added advantage of circumventing the unfavorable equilibrium of acetoacetyl-CoA thiolase (2,5). The activity of acetoacetyl-CoA synthetase is decreased by fasting (8,26,32) and thus, should be considered in the regulation of cholesterol synthesis.

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Metabolic Discrimination between Cholesterol and β-Amyrin by *Phytophthora cactorum*[1]

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ABSTRACT

When cultures of *Phytophthora cactorum* were incubated on solid medium for 3 weeks in the dark at 20 C with [^{14}C]cholesterol or [^{14}C]β-amyrin, the [^{14}C]cholesterol was assimilated into the advancing mycelium to a greater extent than the [^{14}C]β-amyrin. Examination of the mycelium and medium for radioactive metabolites showed significant differences in the metabolism of the 2 labeled compounds. Cholesterol was converted mainly to esters and to some extent to glycosides, whereas β-amyrin was only slightly converted to esters and not at all to glycosides.

INTRODUCTION

Although species of *Phytophthora* and *Phythium* (Pythiaceae) do not require sterols for growth, they assimilate sterols from the culture medium into their membranes (1,2). This results in altered membrane permeability (2,3), increased glucose utilization (4), growth stimulation in terms of both dry weight and hyphal extension (5,6), and in beneficial morphological changes (7). Recently, it was found that the structural requirements for polycyclic isopentenoid molecules that stimulate mycelial growth in *Phytophthora cactorum* are rather broad (8). Not only certain sterols, but also certain pentacyclic triterpenoids, e.g., tetrahymanol, are capable of increasing the rate at which this fungus grows in vitro on a synthetic medium. However, the structural requirements for the induction of sexual reproduction by polycyclic isopentenoids are very specific (9). Only sterols with certain spatial features of the ring and side chain are capable of inducing oospore production (9,10). This indicates that the mechanisms whereby polycyclic isopentenoids induce growth responses are different from those involved in sexual reproduction.

MATERIALS AND METHODS

For thin layer chromatography (TLC), glass plates precoated with Silica Gel G (Analtech) were used with the following solvent systems: system I, benzene/ether (9:1); system II, chloroform/methanol/acetic acid/water (90:8:1:1). The hR_f values in systems I and II were, respectively: cholesterol, 29,75; β-amyrin, 50,82; cholesteryl and β-amyrin esters, 96,99; cholesteryl glycoside, 0,33; acylated cholesteryl glycoside, 0,42. Reference steroids were purchased from Supelco; β-amyrin was a gift from W.R. Nes. For high pressure liquid chromatography (HPLC) (11), an Altex Model 110A pump (Beckman) was connected through a Model 7125 (Rheodyne) injector to a 250 x 4 mm id stainless steel chromatography tube (Beckman), packed with either Zorbax BP-SIL (7-8 μm; Du Pont) or with Zorbax BP-ODS (7-8 μm; Du Pont). The columns were packed in our laboratory. The eluent for adsorption chromatography was 4% ethanol in hexane and for reversed-phase chromatography it was 96% aq. methanol. The flow-rate was 2 ml/min in both chromatographic systems. The detector was a Hitachi variable-wavelength spectrometer (Altex Model 155), set to 202 nm. Gas liquid chromatography (GLC) was performed at 235 C on XE-60. Electron-impact mass spectra were obtained on a Micromass-70/70 F instrument (VG-Organic Ltd.) by direct probe at 190 C ion source temperature.

Radioactive Cholesterol and β-Amyrin

[4-^{14}C]Cholesterol (sp act 58.4 mCi/mmol> 98%) pure was purchased from Amersham. Approximately 500 μg of [^{14}C]labeled β-amyrin (10.5 mCi/mmol) was prepared biosynthetically by germinating 2 batches of 20 *Pisum sativum* seeds in the presence of 0.05 and 0.025 mCi, respectively, of [2-^{14}C]mevalonic acid (New England Nuclear) (sp act 47 mCi/mmol) (12). The product was purified by reversed-phase HPLC, (>99% pure). [^{14}C]-Cholesterol and [^{14}C]β-amyrin were quantitated by triangulation of their HPLC peaks.

P. cactorum, strain 51-22, obtained from the algal and fungal collection of the University of California, Berkeley, was grown as previously described (6) for 6-8 days. Then, 50-mm Petri dishes containing ca. 5 ml of the defined agar medium were each inoculated with a 5-mm

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disc, cut from the edge of that agar culture. For each experiment, cultures in 5 identical agar plates were incubated for 21 days at 20 ± 1 C in the dark.

Labeled [^{14}C]cholesterol and [^{14}C]β-amyrin in ethanol were added separately at a level of 10 ppm to the culture medium as the agar was cooling after autoclaving. In 2 replicate experiments, 250 μg of labeled cholesterol or β-amyrin was added to 25 ml of culture medium and equally distributed among 5 Petri dishes. The uptake by the mycelium was determined by triangulation of the GLC peak of cholesterol and β-amyrin. Comparable cultures with non-radioactive compounds were also done.

After 21 days, the solid medium from the 5 Petri dishes was combined and placed into a 250-ml beaker, 100 ml of distilled water was added, and the mixture was boiled for 10 min. The boiling liquid, decanted from the mycelium, and two 50-ml water washes of the mycelium with boiling water were put into a separatory funnel and sequentially extracted with three 150-ml portions of each of the following organic solvents: Skellysolve B, ethyl acetate and 1-butanol. To obtain maximal yields, it was necessary for the agar to be in the liquid state during extraction. The radioactivity in each of the 3 extracts was determined by counting an aliquot in a Packard Tricarb Scintillation Counter (Model 3255).

Sequential extraction of the mycelium was carried out with acetone and ethyl acetate by a modification of the procedure of Elliott and Knights (13). The radioactivity in both extracts was determined. Pyrogallol saponification of the extracted mycelium was then carried out as described by Adams and Parks (14). The radioactivity in the pyrogallol extract was also determined.

Each extract of the mycelium and medium was analyzed by TLC and radioscanning. The radioactivity of each zone was determined by eluting the silica gel with ether, followed by scintillation counting.

Cellulase (2 mg) (Calbiochem) was added to 5 ml phosphate buffer, pH 4.6, containing the radioactive cholesteryl glycoside, isolated by TLC (solvent system II) and incubated for 36 hr in the dark at 35 ± 1 C. The product was worked up with ether and water. A sample of the extract was fractionated by TLC with either solvent system I alone or with system I followed by system II. Radioactive zones corresponding to cholesterol and cholesteryl glycoside were eluted from the plate and their radioactivity was determined.

RESULTS AND DISCUSSION

Both [^{14}C]labeled cholesterol and β-amyrin were assimilated by mycelia of *P. cactorum* (Table 1). During a 21-day period, cholesterol was taken up more readily than β-amyrin. The

TABLE I

Recovery of Radioactive Material from the Mycelium and Medium of *P. cactorum* after 21 Days' Growth on Agar Containing [^{14}C]Cholesterol or [^{14}C]β-Amyrin

	Total radioactivity, in 10^6 cpm, in cultures containing:				
	[^{14}C]cholesterol		[^{14}C]β-amyrin		[^{14}C]cholesterol and β-amyrin
Extracts	Expt. I	Expt. II	Expt. I	Expt. II	
Acetone extract	7.89	24.7	2.23	2.15	7.75
Ethyl acetate extract	0.285	1.34	.253	0.147	0.035
Pyrogallol extract	0.802	3.22	.254	0.127	1.32
Total radioactivity recovered	8.97	29.3	2.74	2.42	9.07
Radioactivity administered	12.9	40.5	11.6	5.05	12.8
Recovery of supplied radioactivity (%)	69.5	72.3	23.6	47.9	70.9
Skellysolve B extract	0.249	0.250	0.150	0.247	0.201
Ethyl acetate extract	0.167	0.390	0.448	0.599	2.04
1-Butanol extract	0.205	3.10	0.460	0.272	1.55
Total radioactivity recovered	0.621	3.74	1.06	1.12	3.79
Radioactivity administered	12.9	40.5	11.6	5.05	12.8
Recovery of supplied radioactivity (%)	4.8	9.2	9.1	22.1	29.6
Total radioactivity recovered (%)	74.3	81.5	32.7	70.0	100.5

TABLE 2

Proportions of Radioactivity in the 3 Radioactive TLC Zones from Cultures Containing [^{14}C]Cholesterol, [^{14}C]β-Amyrin, and [^{14}C]Cholesterol with Nonradioactive β-Amyrin

Treatment	Total radioactivity (%) in: Acetone extract of mycelium			Skellysolve B extract of medium		
	FP[a]	GP	EP	FP	GP	EP
[^{14}C]β-Amyrin	92	0	8	92	0	8
[^{14}C]Cholesterol	72	2	26	85	4	11
[^{14}C]Cholesterol and β-amyrin	50	6	44	63	3	34

[a]FP, GP and EP correspond to polycyclic isopentenoids (either cholesterol or β-amyrin) isolated in the free state, as the glycoside, or as the esters, respectively, by TLC with solvent system I.

addition of β-amyrin (10 ppm) to the cholesterol-containing (10 ppm) culture medium did not significantly influence the uptake of [^{14}C]cholesterol by the mycelium. Our extraction methods, except for expt. 1 with β-amyrin, allowed adequate recovery of the radioactive materials in the mycelium. Inability to recover all the radioactivity from the medium may be due to problems with the solidification of the medium during extraction with organic solvents. In a separate experiment, [^{14}C]cholesterol was added to mycelia cultured in liquid medium. Both glycosylated and esterified cholesterol were present in the mycelium and medium. When the mycelium was boiled for 10 min in 30 ml of distilled water, less than 2% of the supplied radioactivity was leached out. Thus, the glycosylated and esterified products found in the medium are not the product of the boiling treatment; rather, they represent excretion products of the fungus.

Isolation of Cholesterol and β-Amyrin from Mycelia

The acetone extract of the mycelium and the Skellysolve B extract of the medium were analyzed by TLC for metabolites of radioactive cholesterol or β-amyrin added to the culture medium. An aliquot of the neutral lipids gave 4 principal radioactive zones when chromatographed in solvent system I: hR_f 0 (polar metabolites), hR_f 29 (cholesterol) hR_f 50 (free β-amyrin), and hR_f 96 (cholesterol and β-amyrin esters). Each of the zones was eluted from the plate, and the radioactivity determined. The proportions of the radioactivity in the zones eluted from the plate for expt. 1 (cf. Table 1) with cultures containing [^{14}C]-cholesterol and [^{14}C]β-amyrin are shown in Table 2. The amount of radioactivity in the ester fraction, but not the glycoside fraction, varied from 2 (mycelium) to 10% (medium) when the experiment was repeated.

When *P. cactorum* was supplied with nonradioactive cholesterol or β-amyrin, equal amounts of cholesterol and β-amyrin or neither compound, the dry weights of the mycelium of 21-day-old cultures were (mg): 151, 140, 200 and 74, respectively. On a dry weight basis, the amount of total sterol and triterpenoid in the mycelium was (%): 0.01, 0.005, 0.015 and <0.0001. The ratio of cholesterol to β-amyrin in the treatment containing equal amounts of cholesterol and β-amyrin was ca. 3:1.

The acetone extract of mycelia incubated with [^{14}C]cholesterol, separated by TLC (solvent system I), showed a radioactive zone corresponding to free cholesterol, which was eluted from the plate and subjected to HPLC. A peak, corresponding to cholesterol (retention time, ca. 24 min), was collected and analyzed by mass spectrometry, m/e 386 (M^+, 20%), 371 (M^+-CH_3, 9%), 368 (M^+-H_2O, 10%), 273 (M^+-side chain, 10%), 255 (M^+-side chain-H_2O, 10%), 213 (M^+-side chain-H_2O-C_3H_6, 11%) (15).

The same chromatographic methods were used to isolate [^{14}C]β-amyrin from mycelia incubated with this compound. The principal radioactive peak was found to be free [^{14}C]β-amyrin, m/e 426 (M^+, 6%), 411 (M^+-CH_3, 2%) and a structurally diagnostic fragmentation pattern due to a reverse Diels-Alder rearrangement, giving rise to a base peak of 218 (M^+, 100%) with subsequent loss of CH_3 at 203 (M^+, 23%) (16).

The radioactive material at the chromatographic origin from [^{14}C]cholesterol incubates was found to consist principally of 2 polar derivatives. They were separated by redeveloping the plate with solvent system II (Fig. 1) and corresponded chromatographically to acylated cholesteryl glycoside and cholesteryl glycoside. After elution from the plate with methanol, the zone corresponding to acylated cholesteryl glycoside was partially saponified with 10% methanolic KOH. Following hydroly-

sis, the radioactive material was chromatographed sequentially with solvent systems I and II. Scanning revealed 3 radioactive zones, corresponding in R_f values to cholesteryl glycoside, acylated cholesteryl glycoside and free cholesterol. The zone corresponding to cholesteryl glycoside was eluted with MeOH and gave a radioactive peak (retention time, ca. 13 min, reversed-phase HPLC), corresponding to reference steryl glycoside. This material was hydrolyzed with cellulase and then subjected to TLC with solvent system I. The radioscan showed that 86% of the applied radioactivity had been converted to free cholesterol.

The material from the cholesteryl glycoside zone (Fig. 1) was also hydrolyzed with cellulase and subjected to TLC with solvent system I. This gave 3 radioactive zones, with hR_f 0.0, 29, and 90, respectively. They were separately eluted from the plate and aliquots of the eluates were analyzed by scintillation counting. The eluate corresponding to cholesterol (hR_f 29) was diluted with 50 μg of carrier cholesterol and subjected to reversed-phase HPLC. Most of the radioactivity was eluted with the carrier cholesterol.

When *P. cactorum* was incubated with 10 ppm of commercial steryl glycoside, radial growth was stimulated by 20%, as in the cholesterol-treated cultures (8). Moreover, steryl glycoside induced approximately the same number of oospores as cholesterol. Examination of the mycelial extract for metabolites of steryl glycoside by TLC with solvent systems I and II showed bands corresponding to free sterol, steryl glycoside and acylated steryl glycoside.

Our results show that cholesterol is converted by *P. cactorum* to cholesteryl glycoside, acylated cholesteryl glycosides and cholesteryl esters, whereas β-amyrin is converted only to esters. These products occur both in the mycelium and medium of cultures grown on solid and liquid media (17).

The contribution made by steryl esters and glycosides to mycelial growth and reproduction is not clear. Apparently, *P. cactorum* can modify sterols in the same way as tracheophytes. The esterification of cholesterol and hydrolysis of cholesteryl esters as well as the formation of cholesteryl glycoside and its hydrolysis to cholesterol are reversible reactions which occur in *Phytophthora*. Cholesterol previously has been shown to be converted to esters in *P. cactorum* (17), and acylated steryl glycosides have been isolated from *Pythium* and characterized by chemical methods (18). Also, steryl glycosides are formed in an in vitro system in *Phytophthora infestans* (19). Thus,

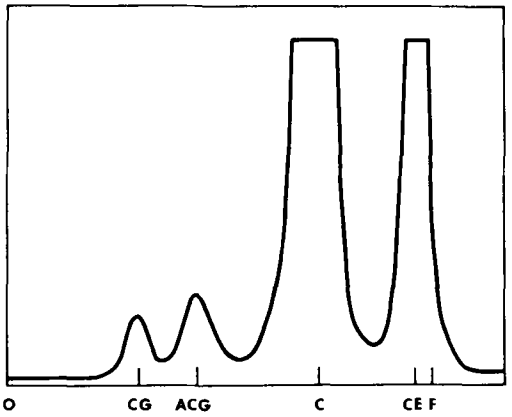

FIG. 1. TLC radiochromatogram of mycelial extract containing ca. 7.8 × 10^5 cpm [^{14}C]cholesterol, developed sequentially with solvent system I and then II. C = cholesterol, CG = cholesteryl glycoside, ACG = acylated cholesteryl glycosides, CE = cholesteryl esters, O = origin, F = solvent front.

the ability of *P. cactorum* to acylate steryl glycosides indicates that the sequence of steps, cholesterol → cholesteryl glycoside → acylated cholesteryl glycosides, which operates in this fungus and *Pythium*, is similar to that in other plants (20).

The inability of *P. cactorum* to glycosylate β-amyrin demonstrates the specificity of the glycosyl transferase in this organism. Since esters of β-amyrin were formed, it seems likely that β-amyrin entered the cells but was not recognized by the glycosyl transferase. Recently we have shown that cholesterol and β-amyrin stimulate vegetative growth, e.g., as measured by dry weight and radial diameter (8), but only cholesterol induced a significant number of oospores (9). Thus, *P. cactorum* is capable of discriminating between some sterols and pentacyclic triterpenoids in terms of their metabolism, uptake, and effect on growth and reproduction.

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Aqueous Lipid Phases of Relevance to Intestinal Fat Digestion and Absorption

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ABSTRACT

The phase behavior of monoglyceride/water systems, with oleic and linoleic acid as the dominating fatty acid residues, was investigated. Increased solubilization of triglycerides (oil) or oleic acid in the cubic liquid-crystalline phase formed by monoglyceride and water resulted in the formation of a reversed hexagonal liquid-crystalline phase followed by an L2-phase. The liquid-crystalline phases have different dispersion properties compared to each other in dilute micellar bile salt solutions. The cubic phase is found to be easily dispersed. The relevance of aqueous lipid phases other than micellar is discussed in relation to intestinal lipid digestion and absorption.

INTRODUCTION

Dietary glycerides are partially hydrolyzed by pharyngeal lipase in the stomach (1). They reach the duodenum as tri- and diglycerides, where they are mixed with the pancreatic and biliary secretions. By the action of pancreatic lipase and its coenzyme colipase, the glycerides are further hydrolyzed to free fatty acids and 2-monoglycerides. These lipolytic products constitute the dominating lipid mass in the luminal contents after a fat-containing meal. Other lipid classes present are phospholipids, cholesterol and its esters, and bile salts. The total lipid system in the intestinal lumen is thus a complex mixture of compounds with different behavior in water.

Hofmann and Borgström (2,3) showed that, after feeding a test meal, it was possible to separate human intestinal contents by ultracentrifugation into an oil phase and an aqueous mixed micellar phase. Triglycerides and diglycerides were found to be the major constituents of the oil phase, which also contained minor amounts of fatty acids. The micelles were composed of conjugated bile salts, 2-monoglycerides, and dissociated fatty acids. The micellar phase of human intestinal contents was further characterized by the work of Porter and Saunders (4) and of Mansbach et al. (5).

New aspects of the physicochemical nature of lipid digestion originate from observations by Patton and Carey (6). In a polarizing microscopy study of triolein hydrolysis under conditions resembling the intestinal environment, they could initially observe the formation of a birefringent phase. Thereafter, a viscous isotropic phase was formed. The birefringent phase could be separated from the aqueous phase by sedimentation. Precipitating phases previously have been observed in ultracentrifuged human intestinal contents (7). These results indicate that, besides the oil and the mixed micellar phase, other lipid phases could occur which might be of importance for lipid absorption.

The solubility of monoglycerides and fatty acids in bile salt solutions was investigated by Hofmann (8,9) and Small (10). The existence of liquid crystalline phases is also evident from the investigations by Hofmann (11) of the monoolein/oleic acid/sodium oleate/sodium taurodeoxycholate system in water. The phase behavior of various monoglyceride/water systems was examined earlier (12-14); it is known that different types of phases can be formed, depending on temperature, the structure of the hydrocarbon chain, and the amount of water.

This work investigated different possible lipid/water phases likely to occur during intestinal fat digestion and absorption. Also reported is the effect of diluted micellar bile salt solutions on these phases.

MATERIALS AND METHODS

We used oleic acid that was more than 99% pure, according to the manufacturer (Nu-Chek-Prep. Inc., Elysian, MN). 1-Monoolein was prepared in the laboratory according to Fischer et al. (15); its purity was checked by TLC. Monolaurin and sunflower oil monoglycerides were obtained as gifts from A/S Grindstedvaerket (Brabrand, Denmark). The fatty acid composition of the sunflower oil monoglycerides is reported elsewhere (16). Soybean oil was supplied by AB Karlshamns Oljefabriker (Karlshamn, Sweden) and the fatty acid pattern has been reported (17). Sodium taurodeoxycholate was synthesized in the laboratory

according to Hofmann (18). Sodium cholate was prepared by neutralization of cholic acid (Fluka AG, W. Germany) with sodium ethoxide in ethanol. Sodium caseinate, a spray-blend product from DMV, Holland, is completely soluble in distilled water at pH 7. Hexadecane of practical grade was obtained from Eastman Kodak, New York.

Buffers used were 50 mM citrate-HCl, pH 3.0, or 50 mM tris maleate, pH 6.5, both containing 150 mM NaCl and 0.02% sodium azide to prevent microbial growth.

Phase equilibria were studied according to standard methodology (19) including polarizing microscopy (Ortholux 2, Zeiss, W. Germany) and X-ray diffraction technique using either a point focus camera (according to Kiessig) with the sample in a glass capillary, or a line focus camera (according to Luzzati) with the sample kept in a closed cell between mica sheets.

RESULTS

Ternary Monoglyceride/Triglyceride/Water Model System

Figure 1 shows the phase diagram of the studied ternary system sunflower oil monoglycerides/soybean oil triglycerides/water. There are 3 regions consisting of liquid-crystalline phases: one lamellar, one cubic, and one reversed hexagonal. The region of liquid crystalline phases with excess of water consists mainly of the hexagonal liquid-crystalline phase, as the cubic phase can only solubilize a few percent of triglycerides. At still higher amounts of triglycerides, an L2-phase is formed. According to Ekwall (20), an L2-phase is an isotropic solution in organic solvents of micelles of the inverse type. They have a core composed of polar groups and water, which is surrounded by a layer of hydrocarbon chains of the micelle-forming substance. The organic solvent acts as an intermicellar liquid; in this case, the triglyceride constitutes the organic solvent. Most of the phase diagram contains a 3-phase region consisting of water, triglyceride oil, and an L2-phase with about 15% (w/w) water. The triglyceride oil phase should also be classified as an L2-phase according to Ekwall's nomenclature.

Monoolein/Oleic Acid/Aqueous Buffer System

Figure 2 shows different phases formed by various mixtures of monoolein and oleic acid at a constant aqueous buffer content of 99% (w/w). The lipid content was held constant at 1% (w/w). Monoolein interacts with buffer at pH 3.0 to form a cubic liquid crystalline phase; oleic acid forms an L2 phase (Fig. 2A). Increasing the proportions of oleic acid to monoolein

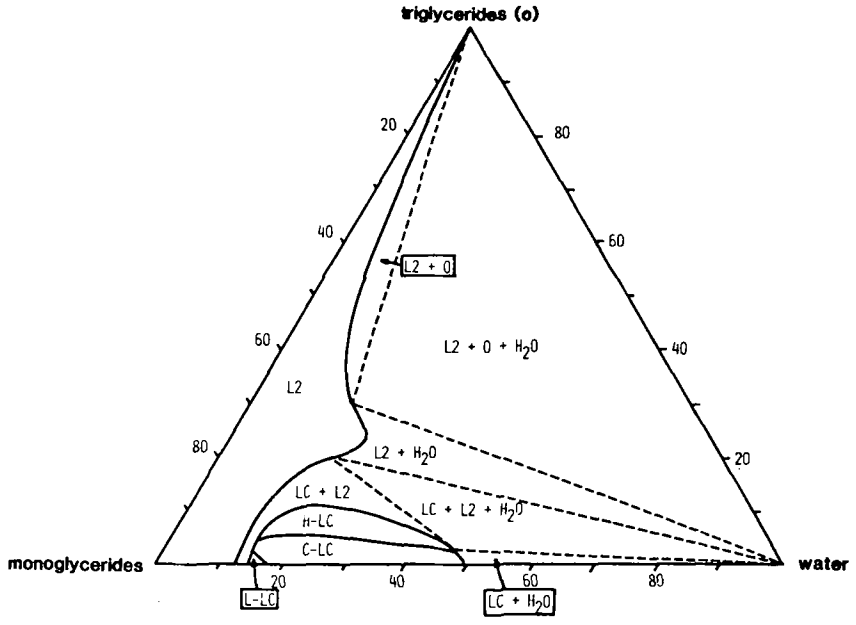

FIG. 1. Phase diagram of the ternary system sunflower oil monoglycerides/soybean oil/water at 40 C. The liquid-crystalline (LC) phases are lamellar (L-LC), cubic (C-LC), and reversed hexagonal (H-LC).

transfers the cubic phase to a reversed hexagonal liquid crystalline phase, and then, via another viscous isotropic phase, into an L2-phase. The structure of the viscous isotropic phase between the reversed hexagonal and L2-phase has not yet been determined. When the pH is changed to 6.5 (Fig. 2B), the same phases are observed, although the phase boundaries are shifted. At higher pH, the dissociation of oleic acid is increased, and soap micelles in water are formed.

It should be pointed out that these systems are not 3-component systems as they include sodium and chloride ions and also citrate or tris-maleate ions; therefore, they can contain regions with more than 3 phases.

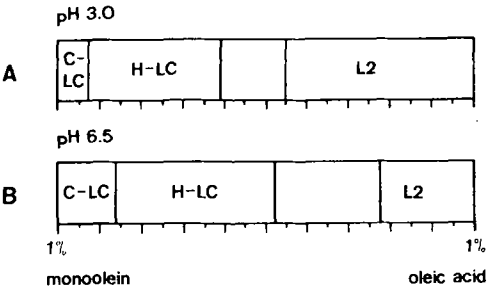

FIG. 2. Lipid phases formed by different proportions of monoolein and oleic acid coexisting with excess of aqueous buffer phase at pH 3.0 (A) and pH 6.5 (B). The total amount of lipid was held constant at 1% (w/w). Observed phases are cubic (C-LC), reversed hexagonal (H-LC), and an L2-phase. Between the hexagonal and L2-phase, there is a viscous isotropic phase of unknown structure.

Effect of Aqueous Bile Salt Solutions on the 3 Liquid Crystalline Phases Formed in Monoglyceride/Water Systems

At 37 C, monolaurin can form a lamellar liquid-crystalline phase containing up to about 40% (w/w) water. This phase is easily dispersed in an excess of water, forming a liposomal dispersion. Ionic amphiphiles are known to increase the water swelling of the lamellar phase, and bile salts have been seen to give this effect in monoglyceride/water systems (21). Small amounts of sodium cholate (0.25% w/w) were observed to transform a liposomal dispersion of monolaurin (10% w/w) in water to an optically clear phase, which exhibited birefringence when viewed in polarized light. When the monolaurin content was lowered to 5% (w/w), very weak or no birefringence was noted. It is thus probable that the lamellar phase can exist at these water concentrations.

Monoglycerides from sunflower oil form a cubic, viscous, isotropic phase at 37 C with a maximum of 50% (w/w) water. This liquid-crystalline phase can coexist with water and is rather insensitive to ultrasonic treatment. The cubic phase is very rigid in the binary monoglyceride/water systems. The addition of sodium cholate, however, will drastically lower its viscosity. At a monoglyceride content of 5% (w/w) with 0.25% (w/w) sodium cholate, a dispersion of the cubic phase can be obtained by hand shaking. The dispersion is quite stable with a typical particle size of ca. 10 μm. The particles are shaped like polyhedra with well-defined edges, characteristic of the cubic phase.

We have found that the cubic phase of monoolein is as easily dispersed by conjugated bile salts. Other surface active biomolecules, such as sodium caseinate, are able to disperse this phase, although not as readily as bile salts.

The dispersed cubic phase has different solubilization properties compared to the micellar phase, which can be illustrated by interaction with paraffins. The limit of hexadecane solubilization into the cubic monoglyceride-water phase is difficult to determine with precision. If water is added to a liquid phase of sunflower oil monoglyceride and hexadecane (95:5, w/w) a transparent cubic phase is obtained. Higher concentrations of hexadecane yield separation of hexadecane from the cubic phase. If sodium cholate is added to the water dispersion of the cubic phase (about 90% of water and 10% w/w of monoglyceride-hexadecane 95:5 w/w) until a micellar solution is formed, it can be seen that part of the hexadecane separates at the top of this solution just as the micellar solubility is reached.

Monoglycerides from sunflower oil with an excess of water at 70 C form a reversed hexagonal phase, with a water content of about 40% (w/w) (16). This phase is rather unaffected by a diluted bile salt solution compared to the cubic phase.

Effect of Bile Salts on the Monoolein/Oleic Acid/Water System

A mixture of monoolein (35% w/w) and a 10 mM solution of sodium taurodeoxycholate in water (65% w/w) was analyzed by X-ray. The diffraction pattern shown in Figure 3A corresponds to the cubic monoolein-water phase (22). The cubic phase can thus exist in an aqueous phase containing bile salts above the critical micellar concentration.

A monoolein-oleic acid mixture with the molar ratio 1:2 at pH 6.5, the composition found in intestinal contents, forms an inverse micellar phase, L2-phase, when swelled in water, according to Figure 2. The X-ray scatter-

ing pattern (Fig. 3B) of this phase, after dispersion by sodium taurodeoxycholate and concentration of the dispersed particles by centrifugation, gives a picture similar to an L2-phase (23).

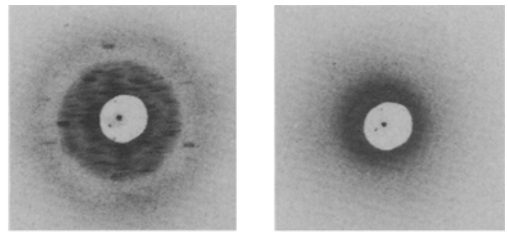

FIG. 3. X-ray diffraction patterns obtained with the point focus camera. (A) The system monoolein/10 mM sodium taurodeoxycholate in water. Composition 35:65% (w/w). (B) The system of monoolein/oleic acid (2.5% w/w; molar ratio 1:2) dispersed in 50 mM Tris-Maleate pH 6.5, 150 mM NaCl, 0.02% NaN_3, and 10 mM NaTDC. The dispersed lipid phase was concentrated by centrifugation prior to X-ray diffraction analysis. Composition of the system before centrifugation was 3% lipids and 97% aqueous buffer (w/w).

DISCUSSION

Most earlier physicochemical studies on fat digestion and absorption have focused on a mixed micellar phase and an oil phase.

Among the glycerides present during fat digestion, only the monoglycerides form liquid crystalline phases. From phase equilibria studies of monoglyceride/water systems (12, 13), short-chain members such as monolaurin are known to form a lamellar phase at 37 C, and an increase in chain length and degree of unsaturation of the monoglycerides favors the transitions cubic → hexagonal (of reversed type) → L2-phase (16).

At 37 C, monoolein forms a cubic liquid crystalline phase with an excess of water (12). As shown in Figures 1 and 2, a cubic phase can, by increased solubilization of triglycerides or fatty acids, undergo the same transitions. Addition of diglycerides to monoglycerides has been reported to have a similar effect (13).

The dispersion effects of the bile salt micelles on the cubic phase might be explained from the proposed structure: a continuous lipid bilayer separating 2 water channel systems (22). Figure 4A illustrates this structure. The bile salts could reduce the surface energy by adsorbing on the edges where bilayers are broken during the mechanical dispersion.

The reversed hexagonal phase (Fig. 4B) is not dispersed by dilute bile salt solutions as easily as the cubic phases. The lamellar phase can be dispersed even in the absence of bile salts into liposomes.

From the phase diagrams presented, it is also evident that the optically isotropic L2-phase might be one of the dominating phases together with the ordinary micellar phase. This L2-phase is also of interest with regard to interaction with other phases. As it consists of a continuous hydrocarbon chain matrix (17), its surface tension properties resemble those of the oil. This L2-phase might therefore act to help disperse the oil and therefore increase the velocity of the lipolysis. A structure of the L2-phase in the binary system monoglyceride water has recently been proposed (23) with, as shown in Figure 4C, water forming inverse

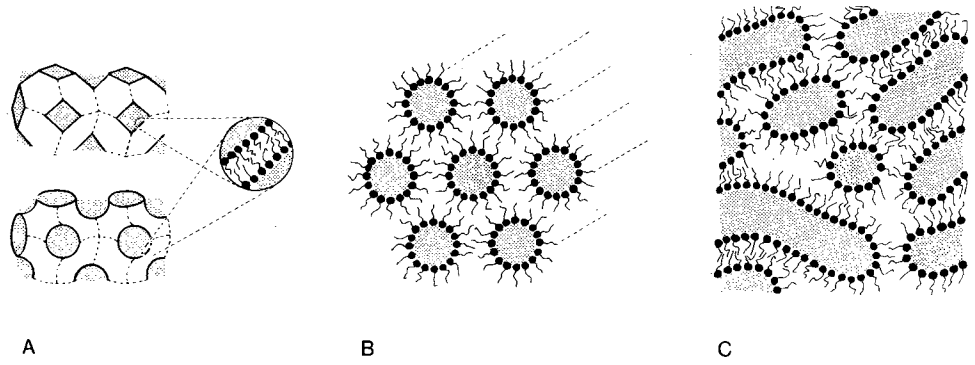

FIG. 4. (A) Proposed model for the cubic phase. Hexagon bilayer units are connected into polyhedra, which are connected along the cubic cell axis resulting in a continuous lipid bilayer separating 2 continuous water regions (gray). If the bilayer is curved, another possible structural arrangement with the same general characteristics is obtained. (B) Structure of the reversed hexagonal phase which shows birefringence in the polarizing microscope. Infinite water channels (gray) arranged parallel in a continuous lipid matrix. (C) Proposed structure of the L2-phase. Units of water layers (gray) are separated by lipid bilayers.

micelles in a lipid bilayer matrix.

It should be mentioned that fat containing predominantly C_{12} chain length fatty acids (like coconut fat) forms monoglycerides which only give a lamellar liquid crystalline phase (12), and the addition of triglyceride oil to this phase directly gives an L2-phase (17). Thus, there are lipid systems which would not be expected to give cubic or reversed hexagonal phases.

The lamellar phase has been reported to exist in other systems of dietary lipids. It is present in the ternary system of oleic acid/sodium oleate/water (24) and in the monoglyceride/cholesterol/water system (25).

Cholesterol and phospholipids constitute only a smaller part of the total lipid mass in intestinal contents during digestion of a fatty meal. Thus, these lipids are expected to be of less importance for the phase equilibria.

Lipolysis is a rapid process. It is almost completed at the transition between duodenum and jejunum, where the mucosal absorption of lipids begins (26). Thus, the amount of lipolytic products in the jejunum will be high compared to the solubilizing capacity of the bile salt micellar phase. Analysis of intestinal contents after a fatty meal has shown that the bile salt concentration is around 10 mM in normal subjects whereas the total fatty acid concentration is at least twice as high (5,7). This will probably result in the formation of liquid crystalline and/or inverse micellar (L2) phases in addition to the mixed micellar phase.

Borgström (27) has shown that absorption of a paraffin, octadecane, is related to the presence of dietary triglycerides, and that relatively large quantities can be absorbed. Dispersion of lipolytic product phases, with better solubilization properties for nonpolar lipids compared to mixed micelles as just shown for hexadecane, might provide an additional route for uptake of nonpolar lipids. The observation that latex particles can be absorbed in the small intestine (28) indicates that absorption of colloidal particles larger than micelles might be possible.

Porter et al. (29) found that patients with an external biliary fistula will absorb most of their dietary fat. This indicates that bile salt micelles are not obligatory. Aqueous dispersions of the different phases discussed might suffice for absorption to take place.

It must be pointed out that the action of lipase is necessary; consequently, direct uptake of oil emulsion particles, as suggested by Frazer (30), is not proposed by us.

Our general conclusion is that the different aqueous lipid phases just described, cubic, reversed hexagonal, lamellar, and L2-phase, can occur in human intestinal contents as well as mixed micelles. Their significance must depend on their relative amount and dispersion properties.

Whether or not the lipid/water phases we have discussed are directly involved in the mucosal uptake of lipids is an open question.

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METHODS

Synthesis of $\Delta^{5,22}$-Cholestadien-3β-ol from $\Delta^{5,7,22}$-Cholestatrien-3β-ol by a Liver Enzyme

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ABSTRACT

The rat liver enzyme system, which catalyzes reduction of $\Delta^{5,7,24}$-cholestatrien-3β-ol to cholesterol (Δ^{5}-cholesten-3β-ol), converted radiolabeled $\Delta^{5,7,22}$-cholestatrien-3β-ol to $\Delta^{5,22}$-cholestadien-3β-ol, but not to cholesterol. This enzyme system thus contains membrane-bound Δ^{7}- and Δ^{24}-reductases and no Δ^{22}-reductase. Kinetic and competition studies showed that the enzyme system contains a single $\Delta^{5,7}$-sterol Δ^{7}-reductase, which is not influenced by unsaturation at the Δ^{22}-position of the sterol side chain. The identity of $\Delta^{5,22}$-cholestadienol was established by chromatographic, spectral and chemical analyses. Use of the enzyme system and readily available $\Delta^{5,7,22}$-cholestatrienol provides a facile procedure for specific production of $\Delta^{5,22}$-cholestadien-3β-ol in quantity.

Biosynthesis of cholesterol requires the reduction of double bonds at the 7,8- and 24,25-positions of $\Delta^{5,7,24}$-cholestatrienol. The occurrence of membrane-bound $\Delta^{5,7}$-sterol Δ^{7}-reductase and Δ^{24}-sterol Δ^{24}-reductase in rat liver homogenates has been demonstrated and the characteristics of these enzymic reactions defined (1). The present study shows that an unusual substrate, $\Delta^{5,7,22}$-cholestatrienol, is saturated at the Δ^{7}-position at the same rate and under identical reaction conditions as the *normal* substrates, i.e., $\Delta^{5,7}$-cholestadienol and $\Delta^{5,7,24}$-cholestatrienol. The absence of Δ^{22}-reductase activity in the liver enzyme system results in accumulation of $\Delta^{5,22}$-cholestadienol. Thus, this communication provides new information on the specificities of membrane-bound Δ^{7}- and Δ^{24}-reductases and describes a facile method to obtain milligram quantities of $\Delta^{5,22}$-cholestadienol, which is available in only trace amounts from a few natural sources (2-5).

PROCEDURES

Cholesterol (Sigma Chem. Co., St. Louis, MO) was purified by passage through the dibromide (6). Δ^{7}-Cholestenol, $\Delta^{5,7}$-cholestadienol, $\Delta^{5,24}$-cholestadienol and $\Delta^{5,7,24}$-cholestatrienol (Schwartz/Mann Inc., Orangeburg, NY) were purified by repeated recrystallization. [^{3}H]Δ^{7}-cholestenol was a gift of I. Frantz (University of Minnesota, Minneapolis, MN). Authentic $\Delta^{5,22}$-cholestadienol was kindly provided for comparison purposes by R.L. Conner (Bryn Mawr College, Bryn Mawr, PA). [26-^{14}C]$\Delta^{5,7,22}$-Cholestatrienol (Δ^{22}-*trans* isomer) was isolated from cultures of the protozoan *Tetrahymena pyriformis* W grown in a peptone-based fluid supplemented with [26-^{14}C]cholesterol (Schwartz/Mann Inc.) and was purified by the procedure of Conner et al. (5). Each sterol showed a single peak when subjected to gas liquid chromatography (GLC). The rat liver microsomal enzyme system and its soluble protein activator which together catalyze the reduction of $\Delta^{5,7,24}$-cholestatrienol to cholesterol were prepared by the method of Dempsey (1). Protein content was determined by the biuret procedure (7). NAD, NADP, their reduced forms and GSH were purchased from Calbiochem Co., La Jolla, CA; AY-9944 (*trans*-1,4-*bis*-(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride) was a gift from D. Dvornik, Ayerst, McKenna and Harrison Ltd., Montreal, Canada. All organic solvents were distilled prior to use. All other chemicals were reagent or analytical grade.

[26-^{14}C]$\Delta^{5,7,22}$-Cholestatrienol was dissolved in propylene glycol (18.4 μg/0.1 ml; 0.38 μCi/μmol) and incubated with 20 mg enzyme system protein (1), 5 mM GSH and appropriate cofactors and/or inhibitors in 100 mM potassium phosphate buffer (pH 7.3) at a final vol of 2.1 ml. The reaction progressed under

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anaerobic conditions with gentle shaking in the dark for 30 min and was terminated by the addition of 2.1 ml 95% ethanol. Parallel assays with equivalent substrate concentrations of labeled $\Delta^{5,7,24}$-cholestatrienol or $\Delta^{5,7}$-cholestadienol served as positive controls for Δ^{24}-sterol Δ^{24}-reductase and $\Delta^{5,7}$-sterol Δ^{7}-reductase activities, respectively (1).

For separation and identification of [26-^{14}C]$\Delta^{5,22}$-cholestadienol, sterols from pooled incubation mixtures were extracted (6), dried under N_2, suspended in benzene and where appropriate, mixed with pure sterol standards. They were then subjected to silicic acid-super-Cel (silicic acid, 100 mesh: Mallinckrodt Corp., St. Louis, MO; super-Cel, Johns-Manville Corp., Denver, CO) (8) or argentation chromatography (9). The $\Delta^{5,7}$-sterol content of column fractions was determined from the specific ultraviolet absorption spectrum in cyclohexane using a Cary Model 15 recording spectrophotometer (1). Sterols were also quantitated by the rate of color formation in the Liebermann-Burchard assay (10) and/or by liquid scintillation spectrometry (Packard Tri-Carb Model 314-EX) of aliquots dissolved in 0.3% 2,5-diphenyloxazole (DPO) in toluene. For chemical analyses and structural identification of [26-^{14}C]$\Delta^{5,22}$-cholestadienol, multiple incubations with the labeled trienol were performed as above and the sterols separated by argentation chromatography. The pool of the fractions containing the radiolabeled derivative of $\Delta^{5,7,22}$-cholestatrienol was subjected to gas liquid chromatography (GLC) at 235 C (F&M Model 400, 6-ft glass column packed with 80-100 mesh Gas Chrom S coated with 0.75% SE-52 silicone gum rubber, Hewlett-Packard Co., Avondale, PA), to periodate-permanganate oxidation (11) and to partial hydrogenation with a palladium catalyst.

RESULTS AND DISCUSSION

The data presented in Figure 1 support the conclusion that enzymes present in rat liver catalyze the reduction of [26-^{14}C]$\Delta^{5,7,22}$-cholestatrienol to [26-^{14}C]$\Delta^{5,22}$-cholestadienol. Identification of $\Delta^{5,22}$-sterol as the only catabolite of this reaction is based on the following criteria. During silicic acid chromatography (Fig. 1A), the peak of the [^{14}C]-labeled product does not coincide with that of cholesterol but migrates to a position analogous to cholesta-5,7-dien-3β-ol. The behavior of the [^{14}C]-labeled material in this system is also distinct from that of Δ^{7}-cholestenol, Δ^{8}-cholestenol which migrates with the Δ^{7} isomer (12), and the $\Delta^{7,24}$- and $\Delta^{8,24}$-cholestadienols (13). Argentation chromatography further

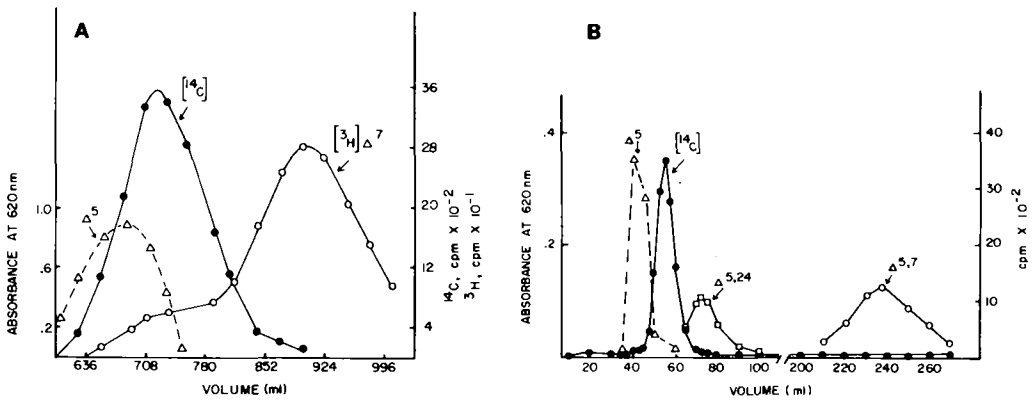

FIG. 1. Chromatographic migration relative to known sterols of the sterol formed from [26-^{14}C]$\Delta^{5,7,22}$-cholestatrienol by liver enzymes. (A) [26-^{14}C]$\Delta^{5,7,22}$-cholestatrienol was incubated with the liver enzyme system and excess NADPH and GSH, as described in Procedures. The labeled, nonsaponifiable sterol was extracted and mixed with cholesterol (Δ^{5}) (2 mg) and [^{3}H]Δ^{7}-cholestenol (Δ^{7}) (15,000 dpm). The mixture was separated on a 2 cm X 1 m column of silicic acid-super Cel developed with 1 % benzene (8). (B) [26-^{14}C]$\Delta^{5,7,22}$-cholestatrienol was incubated with the liver enzyme system as described for part A. The labeled sterols were extracted and mixed with cholesterol, $\Delta^{5,7}$- and $\Delta^{5,24}$-cholestadienol. The mixture was separated on a 1.3 X 30 cm column of silicic acid-super Cel/$AgNO_3$ using 300 ml benzene as eluent (9). Aliquots of alternate 2.5-ml fractions were dissolved in 0.3% DPO and analyzed by liquid scintillation spectrometry. The remaining fractions were assayed by the Liebermann-Burchard reaction (10). The identities of the sterols used as standards were verified by their retention relative to cholestane on GLC. •—•, ^{14}C-labeled sterol; Δ---Δ, cholesterol (Δ^{5}); □—□, $\Delta^{5,24}$-cholestadienol ($\Delta^{5,24}$); ○—○, $\Delta^{5,7}$-cholestadienol ($\Delta^{5,7}$). The position of the more polar radioactive $\Delta^{5,7,22}$-trienol substrate is not shown.

resolved the ^{14}C-labeled sterol from cholesterol, $\Delta^{5,7}$- and $\Delta^{5,24}$-cholestadiens added as internal standards (Fig. 1B). The labeled sterol purified by argentation chromatography also migrated on GLC with authentic $\Delta^{5,22}$-cholestadienol. Periodate-permanganate oxidation of the reaction product yielded isovaleric acid which demonstrates the existence of a double bond in the 22-23-position (11). The newly formed sterol did not exhibit the spectral characteristics of a $\Delta^{5,7}$-dienol (14), and was "slow-acting" in the Liebermann-Burchard reaction, indicating the absence of an unsaturation of the 7,8-position (10). For final structural identification, the pristine-labeled sterol was subjected to partial hydrogenation (Pd in EtOH) in the presence of unlabeled cholesterol until ∿50% of the starting cholesterol was reduced to cholestanol. The reaction mixture was resolved into its component sterols on a silicic acid-super-Cel/$AgNO_3$ column. The cholesterol fraction was purified by passage through the dibromide derivative and contained the expected level of labeled cholesterol. The latter finding is strong evidence for the occurrence of the Δ^5-bond in the unreduced labeled sterol.

Previous work with the liver enzyme system showed that the endogenous substrates $\Delta^{5,7,24}$-cholestatrienol and $\Delta^{5,7}$-cholestadienol are reduced to $\Delta^{5,24}$- and Δ^5-sterols, respectively, by the same sterol Δ^7-reductase (1 and 12). Further information on the specificity of this membrane-bound enzyme is provided by the studies described here. The rates of reduction of the Δ^7-bond of the $\Delta^{5,7,22}$-trienol and $\Delta^{5,7}$-dienol to their respective products with $\Delta^{5,22}$- and Δ^5-unsaturations are optimal and equivalent when studied under identical incubation conditions. NADPH is a required cofactor for both substrates and cannot be replaced by NADH (Table 1 and Ref. 1). Product formation in each case is inhibited by 0.1 μM AY-9944, an inhibitor of Δ^7-reductase activity (Table 2 and ref. 15). These 2 sterol substrates have the same K_m value, i.e., 12 μM (data not shown). Further, both unlabeled $\Delta^{5,7}$-dienol and $\Delta^{5,7,22}$-trienol, when added in equivalent concentrations, inhibit the conversion of [^{14}C]$\Delta^{5,7,22}$-sterol to the same extent. Thus, it appears that the enzyme system contains a single $\Delta^{5,7}$-sterol Δ^7-reductase and that $\Delta^{5,7,24}$-trienol, $\Delta^{5,7}$-dienol and $\Delta^{5,7,22}$-trienol all serve as equivalent substrates. This conclusion also implies that the activity of the Δ^7-reductase is not influenced by side chain unsaturation at either the Δ^{22}- or Δ^{24}-positions of the sterol side chain. With regard to specificity of the Δ^{24}-reductase which is also known to be present in the enzyme system, it is now clear from the data of Figure 1 that this enzyme will not act on the Δ^{22}-bond of either $\Delta^{5,7,22}$-cholestatrienol or $\Delta^{5,22}$-cholestadienol. Finally, the procedures described here offer a convenient method for the preparation of milligram quantities of $\Delta^{5,22}$-cholestadienol, the sole product of $\Delta^{5,7,22}$-cholestatrienol reduction. This naturally occurring (2-5) but uncommon dienol should serve as a probe for further analyses of membrane-bound enzyme specificity and mechanism of action.

TABLE 1

Cofactor Requirements for Formation of $\Delta^{5,22}$-Cholestadienol from $\Delta^{5,7,22}$-Cholestatrienol[a]

Cofactor	% Conversion
None	23
NAD	23
NADH	30
NADP	38
NADPH	76

[a]Each experimental flask contained 1 mM of the specified cofactor, 18.4 μg [26-^{14}C]$\Delta^{5,7,22}$-cholestatrienol (0.38 μCi/μmol), the liver enzyme system, 20 mg protein (fractions "A" and "C" in a ratio of 18:1 as in ref. 1) and 5 mM GSH adjusted to 2.1 ml with 0.1 M $KHPO_4$ buffer, pH 7.3. The reaction conditions were as described in Procedures. The sterols were extracted from the mixture and separated by argentation chromatography (1.5 X 9 cm columns) with benzene and ethyl acetate. The column was stripped with methanol to collect the cholestatrienol. The radioactivity was determined by liquid scintillation spectrometry. The % conversion is the percentage of total counts recovered in the $\Delta^{5,22}$-diene fraction.

TABLE 2

Effects of the Δ^7-Reductase Inhibitor AY-9944 on the Conversion of $\Delta^{5,7,22}$-Cholestatrienol to $\Delta^{5,22}$-Cholestadienol[a]

AY-9944 (M)	% Conversion
0	75
1.13×10^{-7}	5
1.13×10^{-8}	68
1.13×10^{-9}	74

[a]Each experimental flask contained 1 mM NADPH. All other conditions of the experiment were identical to those on Table 1 except for the presence of the inhibitor.

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A Nondestructive Spray Reagent for the Detection of Prostaglandins and Other Lipids on Thin Layer Chromatograms

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ABSTRACT

The spray reagent 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (10 mg/100 ml methanol) is extremely sensitive for locating prostaglandins on thin layer chromatograms. This reagent does not alter the PG, nor interfere with liquid scintillation counting.

INTRODUCTION

Thin layer chromatography (TLC) has been extensively applied for the isolation and identification of prostaglandins (PG) and related compounds (1-3). Most of the spray reagents used for the detection of PG following TLC separation, e.g., concentrated sulfuric acid, phosphomolybdic acid, 2,4-dinitrophenylhydrazine (4), anisaldehyde/sulfuric acid (5,6), acidic ceric sulfate, vanillin/phosphoric acid/ethanol (1) spray, are destructive. Nondestructive methods for detection include the use of iodine vapor spray (2) which requires the presence of microgram quantities of the compound on TLC plates. In addition, the iodine reacts with the unsaturated bonds and interferes with subsequent analyses. The use of a nondestructive spray reagent would facilitate the study of the biosynthesis and metabolism of PG because they could be separated by TLC and recovered for analysis and identification. This paper reports such a nondestructive spray reagent which is quite sensitive at the submicrogram level.

The compound 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt has been used in the past for the detection of bile acids on thin layer chromatograms (7). This reagent has the advantage that it remains firmly bound to the silica gel during the elution of the separated lipid materials with acetone (7). We have successfully used this spray reagent for the detection of PG subsequent to TLC.

MATERIALS AND METHODS

Prostaglandin standards were either purchased from Sigma Chemical Co., St. Louis, MO, or were gifts from Dr. John E. Pike, Upjohn Company, Kalamazoo, MI. Radioactive PG and thromboxane were purchased from New England Nuclear, Boston, MA. Silica Gel G plates were either purchased from VWR Scientific, Rochester, NY, or made in our laboratory. All solvents are of analytical grade.

In practice, 10 mg of 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (Eastman Organic Chemicals, Rochester, NY) was dissolved in 100 ml methanol. Following TLC with the solvent system chloroform/isopropyl alcohol/ethanol/formic acid (45:5:0.5:0.3) (3), the chromatogram containing different PG was sprayed with the reagent, dried by cold air and inspected under shortwave UV light.

For determination of the recovery of PG from TLC plates, known amounts of ^3H PG and thromboxane were spotted on plates and then eluted with acetone. The acetone extract was transferred to scintillation vials, evaporated to dryness and the radioactivity measured after the addition of Liquiscint (National Diagnostics, Somerville, NJ).

RESULTS AND DISCUSSION

The PG (as low as 200 ng) show up as light greenish spots on a dark background. The spots can also be viewed under longwave UV light. The recovery of PG and thromboxane from TLC plates by acetone was 92-95% as monitored by radioactive $PGF_{2\alpha}$, PGE_2 and thromboxane B_2.

Occasionally, a small fluorescence was detected when the eluted PG samples were respotted. To remove any spray reagent, the eluted PG samples were evaporated to dryness, resuspended in 0.05 N KCl, pH 4.5, and then extracted with ethyl acetate.

If extracted with chloroform/methanol (1:1), some of the reagent eluted with the solvent but it does not interfere with gas chromatographic analysis. When radioactive PG are scraped from TLC plates, the silica gel containing the spray reagent does not cause quenching during radioactivity measurement in a liquid scintillation counter using Liquiscint.

This reagent can also be used for the sensitive detection of phospholipids, fatty acids,

triglycerides, cholesterol and cholesteryl esters, all of which give a purple fluorescent spot in UV light and is much more sensitive than the 2,7-dichlorofluorescein generally used for this purpose. The fluorescent color of these compounds intensify with time whereas the PG colors tend to gradually fade upon standing at room temperature. However, the TLC plates can be resprayed for locating the spots. Hence, this unique reagent can be used for the nondestructive detection of lipids in general.

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High Pressure Liquid Chromatographic Separation of Molecular Species of Phosphatidic Acid Dimethyl Esters Derived from Phosphatidylcholine

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ABSTRACT

A majority of the individual molecular species of phosphatidic acid dimethyl esters derived from multispecies egg yolk and soybean phosphatidylcholines have been separated by reverse-phase high pressure liquid chromatography. Two Partisil-10 ODS columns connected in tandem and the eluents acetonitrile or methanol/water (95:5) were used for molecular species resolution, based on total fatty acyl carbon number and degree of unsaturation.

INTRODUCTION

Recently we have reported (1-4) a simple and general liquid chromatographic method for the separation and identification of multiple molecular species of phosphatidic acid dimethyl esters (PAME) and dibenzyl esters (PABE) derived from egg yolk phosphatidylcholines (PC). An objective has been to assess this methodology for the quantitative determination of glycerophospholipid clinical profiles, which would enable more meaningful investigations of theoretical and practical interest. Incomplete separations of molecular species (4) limited the prospect of quantitation, which prompted improvement of resolution of PAME reported herein. The PAME, dervied from egg yolk and soybean PC, can be considered as representative in part of physiologic molecular species of human glycerophospholipids differing in fatty acyl composition and positional distribution.

MATERIALS AND METHODS

Egg yolk PC was isolated from fresh eggs and purified by adapting published methods (5); soybean PC was obtained from Sigma Chemical Co. (St. Louis, MO). PAME derived from egg and soy PC and standards were synthesized as described previously (1,4). Separations were performed with a Waters Associates (Milford, MA) ALC-202 liquid chromatograph (differential refractometer detector) on either 2 Whatman (Clifton, NJ) Partisil-10 ODS columns connected in tandem or on a Partisil-10 ODS-2 column. Acetonitrile and methanol were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI).

RESULTS AND DISCUSSION

Baseline separation of 8 of 9 egg-PC-derived PAME molecular species on 2 Partisil-10 ODS columns in tandem is shown in Figure 1. Under the same chromatographic conditions, acetonitrile provided better resolution than several percentage combinations of acetonitrile/water, methanol/water, ethanol/water, 1- or 2-propanol/water, or tetrahydrofuran/water; although many gradient elution solvent programs also were tried, no further improvement in resolution was achieved.

The retention times of the PAME molecules were longer with increasing chain length, but shorter with increasing unsaturation. "Adding" one olefinic unsaturation and 2 carbons in the *sn*-1 fatty chain decreased the relative retention time of the PAME molecules (Fig. 1); e.g.,

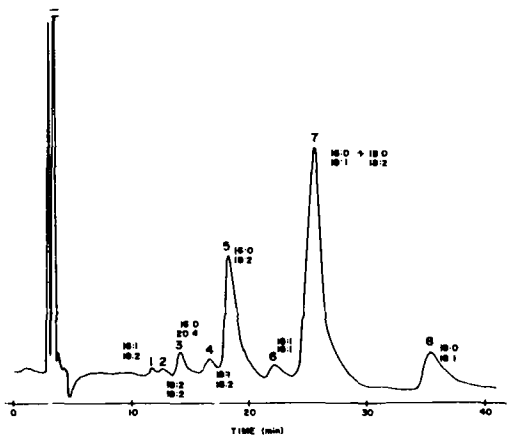

FIG. 1. HPLC separation of phosphatidic acid dimethyl esters derived from egg yolk PC. Columns: 2 Partisil-10 ODS in tandem. Mobile phase: acetonitrile. Flow rate: 2.5 ml/min @ 800 psig. Detection: RI X 16. The designation "16:1, 18:2" means a PAME molecule esterified at the *sn*-1- and *sn*-2-positions with the respective fatty acids.

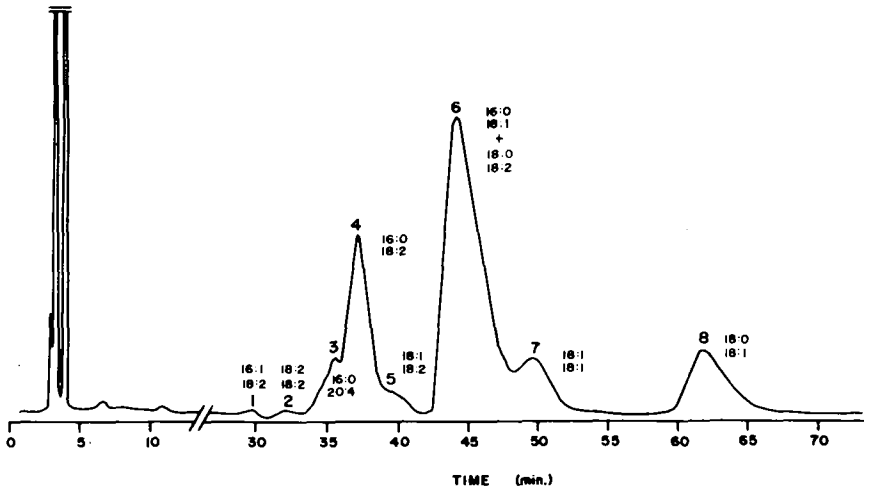

FIG. 2. HPLC separation of PAME derived from egg yolk PC. Column: Partisil-10 ODS-2. Temperature: 55 C. Mobile phase: methanol/water (95:5, by vol). Flow rate: 0.9 ml/min at 1,050 psig. Detection: RI X 16.

18:1/18:2-PAME (peak #4) vs 16:0/18:2-PAME (peak #5); and 18:1/18:1-PAME (peak #6) vs 16:0/18:1-PAME (peak #7). A similar separation was achieved (2) when UV-absorbing PAME derived from egg yolk PC were chromatographed on a μ-Bondapak C-18 column (mobile phase = CH_3OH:H_2O/95:5, λ = 254 nm). Egg yolk PC itself (i.e., nonderivatized) has been only partially separated by reverse-phase high pressure liquid chromatography (HPLC); Porter et al. (6) resolved 4 major molecular species (16:0/18:2-, 16:0/18:1-, 18:0/18:2-, and 18:0/18:1-PC), which have been similarly identified by reverse-phase HPLC of PAME (3).

Figure 2 shows an HPLC chromatogram of egg yolk PC-derived PAME obtained using different chromatographic conditions: Partisil-10-ODS-2 column; methanol/water (95:5); 55 C. The chromatograms (Figs. 1 and 2) show similar separation characteristics, except that "increasing" one unsaturation and 2 carbons in the *sn*-1 chain inverted the retention times of certain PAME molecules: i.e., 18:1/18:1-PAME (peak #7, Fig. 2) eluted after 16:0/18:1-PAME (peak #6, Fig. 2), and 18:1/18:2-PAME (peak #5, Fig. 2) after 16:0/18:2-PAME (peak #4, Fig. 2) in contrast to separations under conditions described in Figure 1 of respective molecular species. Parallel resolution patterns have been observed in independent HPLC studies with fatty acid methyl esters on C-18 reverse-phase by Scholfield (7), Warthen (8), and Hsieh (1), in that elution of methyl oleate (18:1) was just after methyl palmitate (16:0) using methanol/water solvent systems, whereas methyl oleate eluted just before methyl palmitate with acetonitrile.

The success in separating PAME molecules derived from egg yolk warranted a further investigation of those of soybean, which have a high percentage of esterified polyunsaturated fatty acids. An HPLC baseline separation of soy-PAME using acetonitrile and 2 Partisil-10 ODS columns is shown in Figure 3. The elution orders of these molecular species were in close agreement with those of egg-PAME molecules

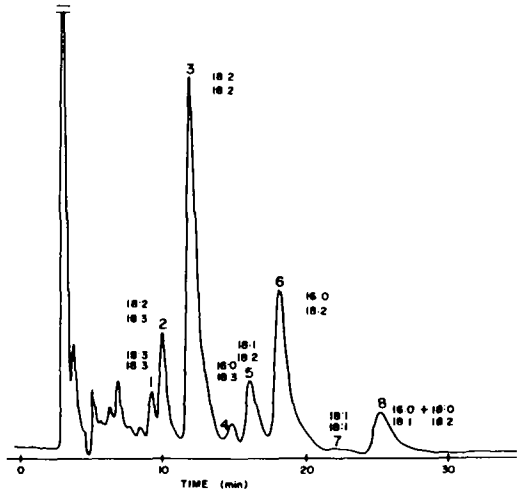

FIG. 3. HPLC separation of PAME derived from soybean PC. Chromatographic conditions: same as in Fig. 1.

(Fig. 1). As in the case with HPLC of egg yolk PC (6), better resolution of molecular species of "soy-PC" can be demonstrated by a comparison of the HPLC chromatograms obtained for soy-PC-derived PAME on 2 Partisil-10 ODS columns in tandem (Fig. 3) and for soy-PC on reverse phase (μ-Bondapak C-18), as described recently by Crawford et al. (9). By applying the HPLC (derivatization, PAME) method, we have analyzed (10) a clinically used pharmaceutical, "essential phospholipids (EPL)," which has soybean PC (60%) as its major ingredient; a qualitatively identical separation of PAME molecules derived from EPL and soybean (Fig. 3) was obtained (10).

The improved reverse-phase HPLC-phosphatidic acid derivative method, besides its rapidity, good resolution, and nondestructiveness (phosphatidic acid moiety), has several additional advantages: excellent reproducibility–the same columns under the described chromatographic conditions were used repeatedly for more than 18 months without any loss of reproducibility; no interfering chromatographic contamination with possible decomposition products–free fatty acids and/or glycerolysophospholipids elute just before the first PAME peak; potential for universality and enhancement of sensitivity–either "chromophoric" (2) or "electrochemical-active" (11) derivatives of phosphatidic acids can be synthesized.

This type of methodology should be investigated further for its utility in analytical studies on glycerophospholipid profiles and function in areas such as molecular biology and normophysiologic and pathophysiologic states of interest in medicine.

ACKNOWLEDGMENTS

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A 2-Phase Liquid Scintillation Assay for Glycolipid Synthetases

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ABSTRACT

Glycolipid synthetases can be assayed conveniently by incubating the lipid substrate with the radiosugar-labeled nucleotide in a small plastic scintillation vial. At the end of the incubation period, water and perchloric acid are added, then *n*-butanol, then a toluene-based scintillation cocktail. The radioactive lipid partitions into the scintillation fluid, leaving excess sugar nucleotide in the aqueous phase. Only a small fraction of the total radioactivity in the aqueous layer is detectable. This method is illustrated for ceramide:UDP-glucose glucosyltransferase. The approach should be applicable to other lipid synthetases that can be assayed with a radioactive hydrophilic substrate.

In the many widely used radiometric enzyme assay techniques, it is necessary to separate the radioactive product of enzyme action from the radioactive substrate. In the case of lipids, where the labeled substrate may be nonlipoidal, solvent partitioning is commonly done, typically with chloroform/methanol/water. The chloroform layer must be evaporated to dryness (after multiple washings) before the lipid can be counted by liquid scintillation, since chloroform is a quenching agent.

Potter (1) used a partition method to separate the labeled acetate formed by acetylcholinesterase, in which the solvent–toluene/isoamyl alcohol–was not a quencher and could then be added to a scintillation fluid directly. This approach was improved by Sankaran and Pogell (2), who simply incubated the assay mixture in a scintillation vial, partitioned the labeled product directly into a scintillation fluid, and counted the entire assembly in the normal way. Because the water in the lower phase absorbed most of the β-radiation coming from the unused tritiated substrate, the observed background activity in the scintillation fluid was not too high. In the case of ^{14}C, as opposed to ^{3}H, there was enough penetration of the water layer to raise the background to an unpleasant level and the lower layer had to be removed. A few additional examples of the scintillation partitioning method have been published since then (3-7) but none have involved lipids, which ought to be particularly suited to the approach. In our initial attempts to use the approach, however, we found excessive differences between duplicate samples and variable drift of observed activities as a function of time. These were alleviated by centrifuging, which presumably brought down small amounts of the lower phase that were adhering to the walls of the vial in contact with scintillation fluid. Additional improvement was obtained by denaturing the proteins with perchloric acid.

Another problem was the size of the boiled-enzyme blank, which resulted in part from radiation entering the scintillation fluid from the lower phase. This was reduced by lowering the specific activity (sp act) of the lower phase with added water.

MATERIALS AND METHOD

The assay medium for ceramide:UDP-GLC glucosyltransferase contained liposomes made from N-octanoyl sphingosine, lecithin, and cerebroside sulfate (8) as well as uridine diphospho[^{3}H]glucose (190,000 cpm), Tris-Cl pH 7.4, dithioerythritol, EDTA, $MgCl_2$ and ATP. The enzyme source was liver from Harlan ICR mice, homogenized in 4 vol of water and diluted further with water to contain 50 mg/ml of tissue (9). The total incubation vol was 0.2 ml and the mixture was incubated for 1 hr at 37 C in a 7-ml polyethylene scintillation vial ("Mini-Vial").

At the end of the incubation period, the vials were placed in ice and 0.2 ml of 5.6% perchloric acid was added to the first vial. This was vortexed briefly and left for a few minutes while the other vials were processed similarly. Now 0.4 ml of water and 0.4 ml of *n*-butanol were added to the first vial, which was vortexed for 30 sec. After the other vials were processed the same way, 3.6 ml of scintillation liquid (18 mg of PPO and 1.1 mg of dimethyl POPOP in toluene) was added and the vials were vortexed for 30 sec again.

The vials were then centrifuged in an angle-head rotor (GSA, DuPont/Sorvall) for 10 min at 10,000 rpm (8,500 × g–max). Rubber stoppers were placed in the rotor cups to keep the vials high enough for easy retrieval. The vials were then inserted into glass holders, using plastic collars, and loaded into a scintillation counter.

RESULTS AND DISCUSSION

In our previously described assay procedure (8), the system was incubated in a glass test tube and biosynthesized cerebroside was extracted with chloroform/methanol. Residual radioactive precursor was removed by several partitioning steps, most of the chloroform-rich layer was then evaporated to dryness in a scintillation vial and the cerebroside was dissolved in a scintillation fluid containing water and Beckman BBS-3. Comparison of [^{3}H]cerebroside (10) in this counting system and in toluene/butanol (90:10, v/v) showed that the latter yielded 12% higher readings. Toluene alone, however, dissolved only part of the labeled cerebroside and low activities were obtained.

When the toluene/butanol mixture was added to incubation vials, however, erratic activities were seen which tended to increase fairly rapidly. Various attempts at stabilization were made based on the idea that variable amounts of lower phase were clinging to the vial walls. Centrifugation did not help, possibly because sufficient speed could not be achieved with the thin-walled vials. The addition at the end of the incubation period of 100 mg of silica gel, which could have made the droplets of liquid denser, proved effective in stabilizing the activities, but this method was dropped because of the work involved in weighing out the powder. Also effective was the addition of aqueous perchloric acid to yield 2.8% acid, the concentration recommended for protein precipitation. Alumina (100 mg) and 0.2 ml of 1 N NaOH were ineffective. Centrifugation improved the degree of replication, apparently because variable amounts of precipitated protein and aqueous phase adhered to the vial walls. However, fairly high centrifugal force was required. The observed activities were now stable for 3 days (but toluene evaporates through the plastic vial upon prolonged storage).

The perchloric acid not only stabilized the counts, but it also increased them, probably because denaturing the proteins improved the extraction of the radioactive lipid from the membranes in which it had been formed. The extra water was added (0.4 ml) simply to reduce the amount of radiation entering the upper phase. Adding more would reduce the blank activity even further, but we felt that vortexing too full a vial might produce losses in the threads of the vial cap.

The butanol was added before adding the toluene because we thought this might help extract the radioactive lipid from the precipitated membranes. Conceivably, adequate extraction could be obtained even when the butanol is premixed with the toluene by lengthening the vortexing step.

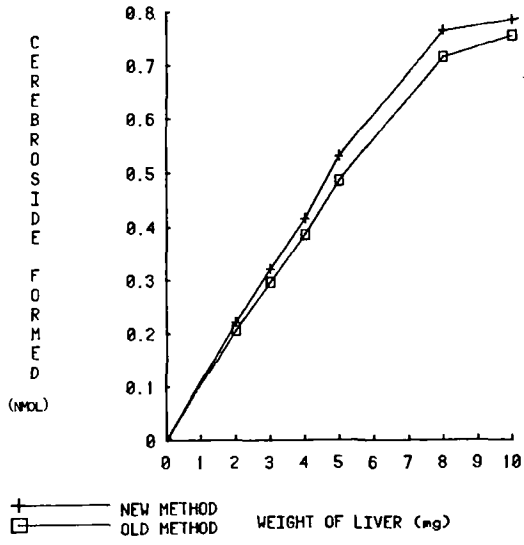

FIG. 1. Amount of glucocerebroside formed by various amounts of liver homogenate from a 26-g male mouse in 0.2 ml of incubation medium. The upper curve shows the data obtained with the new 2-phase counting system and the lower curve shows the data obtained with the earlier glass incubation system.

It is possible that the method could be made more convenient by adding the 0.4 ml of water together with the perchloric acid (i.e., 0.6 ml of 3.7% acid).

As in our previous study (8), the activity of the transferase was found to be proportional to the weight of liver up to about 7 mg (Fig. 1). An unexpected finding is that the samples incubated in the plastic vial gave higher enzyme activities (nmol product). Examination of the incubation tubes showed that most of the homogenate membranes had aggregated on the walls of the glass tube in the form of a ring. This did not happen in the plastic vials. It seems likely that the better suspension in the plastic vials yielded more efficient reactivity of the substrates with the enzyme. The data in Figure 1 also show that the relatively large amount of protein in the lower layer or in the liquid/liquid interface did not interfere with the extraction of the lipid into the upper phase.

A comparison of the 2 assay methods with 5 mg of liver (in triplicate) showed that the observed activities with the chloroform/methanol method were 65 ± 11 cpm for the zero-time blank and 2807 ± 25 cpm for the incubated sample. With the new method, the

activities were 295 ± 6 cpm for the blank and 3439 ± 12 cpm for the incubated sample (corrected for the blank activity). The improvement in variability was typically observed. The blank activity with the new method was distinctly higher (8.6% of the observed activity vs 2.3% with the older method) but this is partially offset by the higher observed activity with the new method. The higher activity results not only from the use of a better counting solvent and higher enzyme activity, but also from the fact that some of the radioactive lipid must be discarded in the chloroform/methanol partitioning method to avoid transfer of upper layer. The blank value of 295 cpm constitutes 0.15% of the total incubated tritium.

Attempts were made to lower the blank by floating a disk of polyethylene at the interface between the scintillation and aqueous layers, but these were ineffective.

The method described in this paper minimizes the exposure of personnel to radiochemicals and organic solvents, reduces the time required for processing by over 40%, and reduces variability and the danger of spillage during transfer. All of the radioactivity used in the incubation remains in the original tube, making disposal easier.

The assay method has also been used in this laboratory for ceramide:UDP-gal galactosyltransferase. It should be suitable for other lipid synthetases, such as the enzymes that make lecithin (labeled CDP-choline or S-adenosyl methionine), fatty acids (labeled acetyl-CoA or malonyl-CoA), or phosphatidylethanolamine (labeled CDP-ethanolamine). Hydrolase assays, using a lipid labeled in its polar moiety (e.g., glucose-labeled cerebroside for glucosidase), could also be handled by this method but a lower precision must be expected because the calculations would require subtracting one large number from another. Still, such reactions usually go relatively well and an appreciable degree of hydrolysis can be obtained even while maintaining substrate saturation.

ACKNOWLEDGMENT

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COMMUNICATIONS

Desaturation of Endogenous and Exogenous Palmitate in Lung Tissue in vitro[1]

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ABSTRACT

Lung slices from rats fed a fat-free diet supplemented with safflower oil (control) or tripalmitoylglycerol (essential fatty acid [EFA]-deficient) were incubated with [^{14}C] acetate, [^{14}C] palmitate, or [^{14}C] stearate. Of the ^{14}C recovered in phospholipids after incubation with [^{14}C] acetate, more than 87% was in 16-carbon fatty acids. Desaturation, as assayed by the percentage of radioactivity in monoenoates in phospholipid fatty acids, was generally double in EFA-deficient slices compared to control slices, regardless of substrate. Desaturation was significantly greater in slices incubated with acetate or octanoate compared to palmitate, indicating that endogenously synthesized palmitate was desaturated more actively than that derived from an exogenous source.

ABBREVIATIONS

PL–phospholipid; TL–Total lipid; EFA–essential fatty acid; PLFA–phospholipid fatty acid.

INTRODUCTION

It is well established that lung tissue is capable of synthesizing long-chain fatty acids, predominantly palmitate, from acetate (1-5). Lung tissue has been shown to be able to desaturate palmitic and stearic acids (6,7). We have shown that lung slices from (EFA)-deficient rats have increased Δ9-desaturase activity as compared to controls (7). The experiments reported here were, therefore, undertaken to examine the desaturation of both endogenously synthesized and exogenous fatty acids in lung slices in vitro using [1-^{14}C]-acetate, [1-^{14}C] octanoate, [1-^{14}C] palmitate, or [1-^{14}C] stearate as tracers.

MATERIALS AND METHODS

Isotopes and Chemicals

[1-^{14}C] Acetate (48 μCi/μmol), [1-^{14}C]-octanoate (30 μCi/μmol), [1-^{14}C] palmitate (58.5 μCi/μmol), and [1-^{14}C] stearate (56.6 μCi/μmol), purchased from Amersham Corp., Arlington Heights, IL, were stated to be 99% pure. Purity of the palmitate, octanoate and stearate was confirmed by thin layer chromatography (TLC) and gas liquid chromatography (GLC). Palmitic acid of greater than 99% purity and albumin (bovine fraction V) were purchased from Sigma Chemical Co., St. Louis, MO.

Animals

Weanling male Sprague-Dawley rats were obtained from Blue Spruce Farms, Altamont, NY, and were fed fat-free diets (U.S. Biochemical Corp., Cleveland, OH, standard fat-free test diet) containing 58% by weight sucrose, 20% casein, salt mixture and vitamins. Control rats were fed this diet supplemented with 4% by weight of safflower oil for 14-18 weeks, whereas the EFA-deficient animals received this diet supplemented with 4% by weight of tripalmitoylglycerol for a similar period. Rats were housed in pairs at constant temperature and humidity, and allowed free access to food and water.

Preparation of Tissue Slices and Incubations

Lungs obtained from nonfasting animals, killed by exsanguination during ether anesthesia between 9-10 a.m., were perfused in situ with 18.0 ml 0.15 M NaCl solution via the right ventricle. Lung slices (500 μm thick) were prepared using a Stadie-Riggs microtome and incubations were performed in a modified Krebs-Ringer bicarbonate buffer (pH 7.4) containing albumin, amino acids and glucose (8) for 1 hr, as previously described (9). Each

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incubation contained in 3 ml, 400-500 mg tissue, and either 1.0 mM [1-^{14}C]acetate (~2 μCi), 0.25 mM [1-^{14}C]octanoate (~6.5 μCi), 1.0 mM [1-^{14}C]palmitate (0.7-1.0 μCi), or 1.0 mM [1-^{14}C]stearate (2 μCi). Octanoate, palmitate, and stearate were albumin-bound (7). At least 2 or 3 rats from each dietary group were used to provide enough tissue for at least 3-5 incubations for each experiment. At the end of all incubations, slices were carefully rinsed prior to extraction as previously described (7).

Analytical Procedures

Tissues were extracted in chloroform/methanol (9). Total PL were separated from other lipids by TLC on Silica Gel H plates (500 μm thick) using a solvent system of hexane/ethyl ether/acetic acid (40:10:1, v/v/v). Spots were detected with iodine vapor and eluted from the gel using methanol/acetic acid/H_2O (94:1:5, v/v/v). Methyl esters of fatty acids of PL were prepared using boron trifluoride (9), and separated into saturated, monounsaturated and polyunsaturated species using argentation chromatography as described earlier (7). Fatty acid composition of the methyl esters was confirmed by GLC (7). A stream splitter was used to separate the specific fatty acids in order to determine their ^{14}C content (7). Radioactivity of the various lipid fractions was determined using a Beckman LS-250 instrument as previously described (7,9). The extent of desaturation of palmitic or stearic acid was calculated as the dpm of ^{14}C in monounsaturated fatty acids expressed as a percentage of dpm of ^{14}C in the total phospholipid methyl esters. Statistical significance of differences between groups was determined using Student's t-test for unpaired variables. Data are presented as the mean ± SEM.

RESULTS

As in previous studies (7,10), EFA-deficient animals were 15-20% below controls in weight at the time of experimentation despite comparable food intakes. EFA status of both controls and EFA-deficient rats, as assessed by GLC analysis of liver lipid fatty acid composition, was similar to that previously reported (10).

We examined the extent of Δ9-desaturation of palmitic acid provided exogenously or synthesized from acetate or octanoate in slices from control and EFA-deficient rats, and compared these findings to those seen with exogenous stearate. As can be seen from Figure 1A, slices from EFA-deficient animals desaturated palmitic acid almost twice as actively as controls, in agreement with our previous observations (7). Thus, 5.6% of ^{14}C in PLFA was in monoenoic FA after incubation of control slices with acetate or octanoate compared to an average of 11% in slices from EFA-deficient rats ($p<.001$). After incubation with palmitic acid, the respective values were 2.9 and 5.6% ($p<.001$). Stearate was a better substrate for this desaturation than palmitate with 12.4 and 27.3% of ^{14}C in monoenoic species in controls and EFA-deficient slices, respectively. As can be seen in Figure 1B in slices from both controls and from EFA-deficient animals, endogenously synthesized palmitate (whether from acetate or octanoate) was desaturated to palmitoleate about twice as actively as exogenous palmitate.

In 8 different incubations using [1-^{14}C]-palmitate as substrate, the saturated methyl esters of PL fatty acids were analyzed by GLC using an effluent stream splitter, and 84-93% of ^{14}C was recovered as palmitic acid. In 12 different incubations using [1-^{14}C]acetate, similar analyses revealed that 87-93% of ^{14}C in saturated fatty acids was again in palmitic acid. In both cases, the remaining ^{14}C in saturated fatty acids was recovered as stearic acid. Similar analysis of the monounsaturated species of methyl esters from slices incubated with either [1-^{14}C]palmitate or [1-^{14}C]acetate yielded significant counts only in the palmitoleic acid area. Only traces of ^{14}C were recovered in oleic acid. These data confirmed the observations of others that little chain elongation of palmitic acid occurs in lung tissue, and that the end-product of fatty acid synthesis from acetate in lung is palmitic acid (3-5).

DISCUSSION

The results of these in vitro studies are consistent with the findings of others in showing that palmitic acid accounts for 80-90% of the products of fatty acid synthesis in whole lung and Type II cells (5,11), and that chain elongation by microsomes and mitochondria makes only a minor contribution (5,11).

Octanoate was used in these experiments as a means of evaluating possible differences between the metabolism of exogenous and endogenous acetate. Of the ^{14}C incorporated into PL from octanoate, more than 80% was in the form of palmitate or palmitoleate which was comparable to the results with [1-^{14}C]-acetate. In a preliminary study, we also examined the production of $^{14}CO_2$ by trapping the $^{14}CO_2$ in hyamine. While 21 to 23% of [1-^{14}C]-

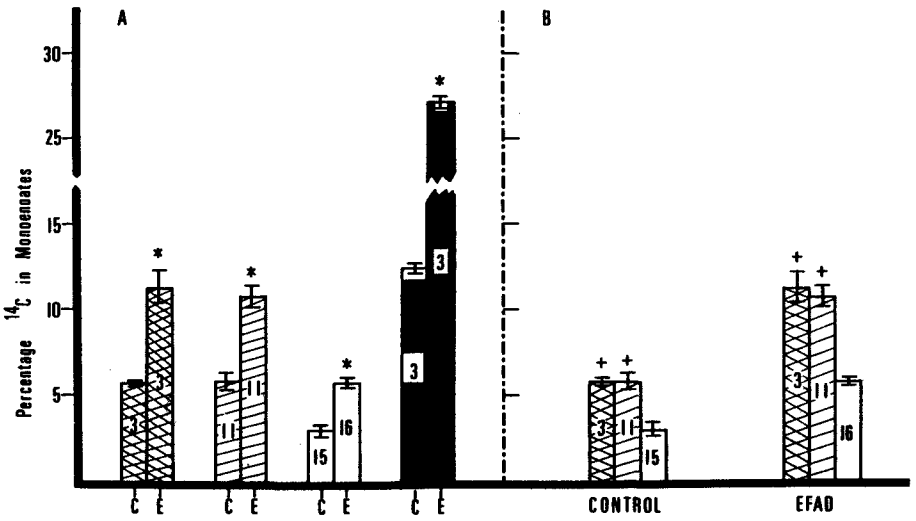

FIG. 1. Desaturation of endogenous and exogenous palmitate (*mean* ± SEM) in phospholipids of control and EFA-deficient rats. Substrates used: [1-^{14}C] octanoate–cross hatched bars, [1-^{14}C] acetate–slashed bars, [1-^{14}C] palmitate–open bars, [1-^{14}C] stearate–solid bars. Panel A compares desaturation between control (C) and EFA-deficient (E) rats. *Indicates $p<.001$ compared to corresponding control. Panel B compares desaturation of endogenously synthesized and exogenously provided palmitate. $^{+}$Indicates $p<.001$ compared to the exogenous palmitate.

acetate and 26-34% of [1-^{14}C] octanoate were oxidized to $^{14}CO_2$ in 1 hr, only 3-4% of palmitate was so oxidized.

Jobe suggested that his in vivo results indicating preferential incorporation of endogenously synthesized palmitate into disaturated phosphatidylcholine could be due to the existence of separate pools of palmitate within lung tissue (12). Our data provide some support for the existence of separate pools of palmitate in lung tissue since, as shown in Figure 1, we have demonstrated that newly synthesized palmitate is desaturated to palmitoleate twice as actively as exogenous palmitate. A possible explanation for this apparently greater desaturation of newly synthesized palmitate could have been that chain elongation to stearate occurred, since we (7) and Montgomery (6) have shown earlier, and have confirmed in the present report that stearate is a better substrate than palmitate for Δ9 desaturase. This possibility, however, is ruled out by the finding that 80-90% of ^{14}C from acetate or octanoate was, in fact, found in palmitate, in agreement with the results of others (5,11). Thus, it may be that palmitate, newly synthesized in the tissue from acetate, enters the acyl CoA pool more rapidly than palmitate taken up from the external medium. Further support for the contention that palmitate synthesized de novo in lung is metabolized differently from exogenous palmitate, at least in vitro, is provided by the recent observations of Nakamura et al. (13). These workers showed that when lung slices from EFA-deficient rats were incubated with [1-^{14}C] palmitate, the addition of linoleate to the incubation medium enhanced the incorporation of ^{14}C into disaturated phosphatidylcholine, whereas no such increase in incorporation occurred when [1-^{14}C] acetate was the substrate. Regardless of the precursor used, the extent of desaturation of palmitate by lung tissue from EFA-deficient rats was almost twice as great as from controls, thus confirming our own earlier observations (7).

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Zinc Deficiency Increases the Rate of Δ^6 Desaturation of Linoleic Acid in Rat Mammary Tissue

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ABSTRACT

The effect of zinc deficiency on the Δ^6-desaturation of [1-^{14}C] linoleic acid was studied in mammary tissue microsomes from lactating rats. The rats were maintained on zinc-adequate (20 ppm zinc) or zinc-deficient (10 ppm zinc changing to 0.5 ppm zinc during last trimester) diets throughout gestation and for the first 3 days of lactation. Mammary tissue microsomes were incubated with [1-^{14}C] linoleic acid and other samples of mammary tissue, mammary milk and the milk in the stomachs of the pups were analyzed for total fatty acid composition. In mammary microsomes from zinc-deficient rats, Δ^6-desaturation of linoleic acid was 3.4 times greater than in microsomes from zinc-adequate rats. This change in metabolism of linoleic acid was reflected by comparable changes in the relative tissue and milk composition of linoleic and arachidonic acids and in the ratios of palmitic to palmitoleic acid, stearic to oleic acid and linoleic and arachidonic acid.

Zinc deficiency in the rat has previously been shown to alter tissue essential fatty acid (EFA) composition. Specifically, arachidonic acid (20:4[n-6]) has been shown to increase in livers (1), skin (2) and testes (3) in zinc deficiency and linoleic acid (18:2[n-6]) in various tissues increases abnormally in zinc-deficient, EFA-supplemented rats (1). EFA-rich oils such as safflower oil (75% linoleic acid), corn oil (45% linoleic acid) and evening primrose oil (72% linoleic acid and 9% γ-linolenic acid, 18:3[n-6]) have been shown to ameliorate partially the gross lesions of zinc deficiency (2,4). These studies have provided evidence that zinc may be a cofactor in EFA metabolism. This possibility has been investigated by assaying the Δ^6-desaturase activity of mammary microsomes from lactating zinc-deficient and control rats. We present further evidence that zinc modulates linoleic acid metabolism and fatty acid composition in mammary tissue and milk.

MATERIALS AND METHODS

Second parity, female, Hooded Lister rats (Rowett strain) were kept individually in stainless steel/polypropylene cages under "barrier-maintained" conditions. After successful mating with males of the same strain, they were fed a semisynthetic diet which was either zinc-adequate (20 ppm zinc) or zinc-deficient (10 ppm zinc for 2 weeks and then 0.5 ppm zinc for the final week of gestation). The diet and deionized water were both available ad libitum. The composition of the diet which was based on egg albumin, sucrose, corn oil, inorganic salts and a mineral and vitamin mix has been fully described previously (5).

On day 3 of lactation, the mothers were killed by decapitation and mammary tissue excised and homogenized in Tris-buffer (pH 7.4) at 4 C. Subcellular fractions were prepared by differential centrifugation at 4 C (6). The microsomes were resuspended in buffer to a final protein concentration of 1-30 ng/ml. Portions of mammary tissue, mammary milk and the milk in the stomachs of the pups were taken for analysis of total lipid fatty acid composition. The method used for the desaturase assay of mammary tissue microsomes has been described in detail elsewhere (6). Two to three mg microsomal protein was incubated with 0.1 μCi [1-^{14}C] linoleic acid (Amersham, sp act 200 μCi/mg) for 30 min at 37 C. Carrier acids (linoleic and γ-linolenic) were then added, and the samples were acidified, extracted and methylated with diazomethane. Argentation thin layer chromatography (TLC) was used for fatty acid separation using the following solvent: chloroform/ethanol/acetic acid (96:4:2, v/v/v).

The tissue and milk samples were saponified with ethanolic KOH and the nonsaponifiable lipids were removed by extraction into diethyl ether. The solution of fatty acid soaps was acidified and the fatty acids were extracted with ether. The methyl esters of fatty acids were prepared with diazomethane and analyzed by gas liquid chromatography (GLC) on a Perkin Elmer Model F17 gas chromatograph at 185 C. The glass column used (6 m × 4 mm id) was packed with 3% E.G.S. P-Z on 100-120 mesh Gas Chrom Q (Phase Separations, Clwyd, Wales).

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Statistical analysis was done using Student's t-test.

RESULTS

The rate of Δ^6-desaturation of linoleic acid in mammary tissue microsomes from zinc-deficient rats was 3.4 times greater than that found in similar preparations from control rats (Fig. 1).

In mammary tissue from zinc-deficient rats, palmitoleic (16:1[n-7]) and oleic acids (18:1[n-9]) were increased whereas palmitic (16:0) and stearic acids (18:0) were decreased when compared to tissue from control animals (Table 1). Similarly, the ratios of palmitic to palmitoleic acid and stearic to oleic acids were both decreased in zinc-deficient compared to control animals (Table 1). The proportions of palmitic and arachidonic acids were increased in the milk obtained from both mammary gland and stomach of the pups in the zinc-deficient group (Table 2). Linoleic acid was decreased in the milk from stomachs of zinc-deficient pups and the linoleic-to-arachidonic acid ratio was also decreased in both stomach milk and mammary milk in the zinc-deficient compared to control animals.

DISCUSSION

Our findings of an enhanced rate of Δ^6-desaturation of linoleic acid by mammary

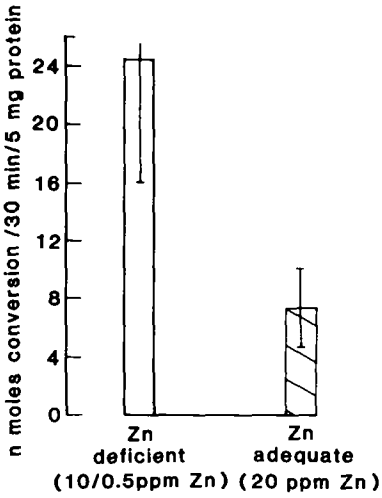

FIG. 1. The effect of zinc deficiency on Δ^6-desaturase activity in mammary tissue microsomes from lactating rats. Each column represents the mean desaturase activity ± SEM for 5 rats as determined by percentage conversion of [^{14}C]linoleic acid. The difference between the 2 groups is significant at $p < 0.05$ (Student's t-test).

TABLE 1

Zinc Deficiency and Percentage Fatty Acid Composition of the Lipids in Mammary Tissue

	Fatty acid composition								Fatty acid ratios		
	16:0	16:1(n-7)	18:0	18:1(n-9)	18:2(n-6)	18:3[a]	20:3[b]	20:4(n-6)	16:0/ 16:1(n-7)	18:0/ 18:1(n-9)	18:2(n-6)/ 20:4(n-6)
Zinc-deficient	15.7 ± 1.1[c]	13.2 ± 0.9	4.1 ± 0.7	27.5 ± 2.3	28.7 ± 1.1	4.9 ± 0.7	TR[d]	5.1 ± 1.4	1.21 ± 0.17	0.15 ± 0.01	6.99 ± 1.47
Zinc-adequate	17.8 ± 0.4*	11.5 ± 0.1*	5.6 ± 0.6	24.8 ± 1.3*	27.8 ± 1.5	5.4 ± 0.5	TR	5.3 ± 0.6	1.54 ± 0.03†	0.23 ± 0.03*	5.39 ± 0.60

[a](n-6) and (n-3) acids.
[b](n-6) and (n-9) acids.
[c]% of total, mean ± SEM, n = 5 for all samples.
[d]TR = trace amounts (≤0.5%).
*$p < 0.05$.
†$p < 0.01$.

TABLE 2

Zinc Deficiency and Percentage Fatty Acid Composition of Milk Lipids in Mammary Milk and Stomach Contents of Pups

	Fatty acid composition								Fatty acid ratios		
	16:0	16:1(n-7)	18:0	18:1(n-9)	18:2(n-6)	18:3[a]	20:3[b]	20:4(n-6)	16:0/ 16:1(n-7)	18:0/ 18:1(n-9)	18:2(n-6)/ 20:4(n-6)
Mammary milk											
Zinc-deficient	24.1 ± 0.5[c]	11.0 ± 2.1	3.9 ± 0.1	32.7 ± 0.1	24.4 ± 1.9	2.4 ± 0.3	0.3 ± 0.0	3.8 ± 0.3	2.78 ± 0.65	0.12 ± 0.00	6.71 ± 1.32
Zinc-adequate	19.1 ± 1.8*	10.6 ± 0.4	4.2 ± 0.4	32.9 ± 2.4	25.4 ± 1.3	2.9 ± 0.2	TR[d]	2.2 ± 0.3†	1.80 ± 0.12*	0.13 ± 0.00	10.78 ± 1.66†
Stomach milk											
Zinc-deficient	31.9 ± 3.3	13.1 ± 2.2	3.0 ± 0.2	29.4 ± 0.2	19.2 ± 0.8	1.4 ± 0.2	1.2 ± 0.3	4.9 ± 0.4	2.51 ± 0.28	0.10 ± 0.01	3.95 ± 0.40
Zinc adequate	24.4 ± 1.8*	11.4 ± 1.6	3.0 ± 0.3	31.7 ± 1.6	21.7 ± 1.1*	1.6 ± 0.2	1.2 ± 0.3	3.0 ± 0.5†	2.18 ± 0.16	0.10 ± 0.01	7.51 ± 0.92†

[a](n-6) and (n-3) acids.
[b](n-6) and (n-9) acids.
[c]% of total, mean ± SEM, n = 5 for all samples.
[d]TR = trace amounts (≤0.5%).
*$p<0.05$.
†$p<0.01$.

tissue microsomes from zinc-deficient compared to control rats is, to our knowledge, the first report directly implicating the involvement of zinc in the rate-limiting step of EFA metabolism. The enhanced Δ^6-desaturase activity of mammary microsomes is also reflected in a decreased ratio of linoleic to arachidonic acid in mammary milk and stomach milk of the pups of the zinc-deficient rats. However, the linoleic acid/arachidonic acid ratio was unchanged in the mammary tissue of the zinc-deficient rats. A possible explanation for this apparent discrepancy is that, if one assumes that the function of the Δ^6-desaturase in the mammary tissue is to provide adequate amounts of arachidonic and dihomo-γ-linolenic acids mainly for neonatal requirements, then one would *not* necessarily expect the *tissue* fatty acid composition to change in zinc deficiency, but rather the composition of the secreted milk would change.

It has been suggested elsewhere that the ratios of stearic to oleic acids and linoleic to arachidonic acids in tissue lipids are good indicators of the relative activities of the Δ^9- and Δ^6-desaturase enzymes, respectively (7,8). The data in Table 1, therefore, indicate that although some transfer of palmitic acid from mammary tissue to milk can account for its increase in the milk, the rate of Δ^9-desaturation of long-chain fatty acids may also be increased in zinc deficiency. The lack of a significant change in the 20:3(n-9)/20:4(n-6) ratio in tissue lipids of zinc-deficient rats (1) also supports this suggestion that Δ^9-desaturase activity is enhanced in zinc deficiency since the synthesis of these fatty acids depends on the activity of the Δ^9- and Δ^6-desaturase enzymes, respectively.

A consistent feature of the zinc-deficient state in rats is an enhanced proportion of arachidonic acid in the total lipids of various tissues when compared to controls (1-3) which is probably due, in part, to increased metabolism of linoleic acid by way of the Δ^6-desaturase reaction, as shown by our present findings.

In dietary EFA deficiency, both Δ^6- and Δ^9-desaturation of long-chain fatty acids are also increased (9), as shown here for zinc deficiency. This further supports the suggestion that zinc deficiency may induce a physiological EFA deficiency (1); e.g., even in the presence of adequate linoleic acid intake, biochemical and pathological symptoms of EFA deficiency may be present due to a disruption in metabolism of linoleic acid.

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Occurrence of 3-Keto Triterpenes in the Unsaponifiable Matter of Sal (*Shorea robusta*) Seed Fat

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ABSTRACT

The unsaponifiable matter from sal (*Shorea robusta*) seed fat contains, in addition to the usual sterols and triterpenes, 12% of another fraction mainly consisting of 3-keto triterpenes. The major constituents of the keto triterpene fraction have been identified as β-amyrenone, cycloartenone and lupenone. The structural assignments were based on physical and spectral characteristics (infrared, ^{1}H- and ^{13}C-nuclear magnetic resonance, and gas chromatography-mass spectroscopy) of the alcohol acetates obtained by reduction, acetylation and fractionation of individual components by argentation thin layer chromatography. This appears to be the first recorded instance of the occurrence of 3-keto triterpenes in vegetable fats.

INTRODUCTION

The nuts of sal tree (*Shorea robusta*) yield about 14% of a greenish yellow fat containing 55-65% symmetrical triglycerides that are useful in the formulation of cocoa butter substitutes (1,2). The theoretical potential of sal fat in India is estimated to be 688,000 tons, but the present collection is about 10,000-20,000 tons per year, much of which is exported. The occurrence of *cis*-9,10-epoxystearic acid and *threo*-9,10-dihydroxystearic acid in small proportions in sal fat has been reported previously (1). Jeong et al. (3,4) have analyzed the unsaponifiable matter of sal. The sterol fraction consists of sitosterol, campesterol and stigmasterol and the triterpene fraction, mainly cycloartenol, β-amyrin and 24-methylenecycloartenol. We re-examined the unsaponifiable matter of sal fat and preliminary results demonstrating the occurrence of 3-keto triterpenes are now reported.

EXPERIMENTAL PROCEDURE

The unsaponifiable matter (0.6%) of sal fat was obtained by saponification of a commercial sample of refined fat (1980 crop) followed by petroleum ether (bp 40-60 C) extraction. The separation of major fractions was achieved by column chromatography on silica gel (1:15) using hexane as eluent. The keto fraction was reduced with sodium borohydride using ether/methanol (1:1, v/v) as solvent and the alcohols thus obtained were purified on 20 x 20 cm thin layer chromatography (TLC) plates coated with 1 mm silica gel; sample load was 80 mg/plate.

Developing solvent was 15% methylene chloride in carbon tetrachloride (v/v) and bands were visualized under ultraviolet (UV) light after spraying with 0.1% methanolic dibromofluorescein. The major band corresponding to triterpene alcohols was eluted with 25% methanol in ether (v/v). Further fractionation of these triterpene alcohols was carried out using their acetates on 20 x 20 cm TLC plates coated with 1 mm of silica gel impregnated with 20% silver nitrate. The eluting solvent was 30% methylene chloride in carbon tetrachloride (v/v), and different zones were visualized under UV after spraying with 0.1% methanolic dibromofluorescein. The bands were extracted with 25% methanol in ether (v/v).

Infrared (IR) spectra (nujol) were recorded on a Perkin Elmer Model 197 spectrophotometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) data were recorded in deuterchloroform on Bruker WP 80 (80 MHz) spectrometer using tetramethylsilane as internal standard. Gas chromatography-mass spectrometry (GC-MS) analyses were performed on an LKB Model 2091, electron energy was 70 eV, trap current 50 μA, ion source temperature 290 C and accelerating voltage 3.5 kV. The samples were introduced through the GC inlet using 2 m x 2 mm id glass column packed with 1% OV-1 on 100-120 mesh Supelcoport. The column temperature was programmed at 220-280 C at 5 C/min. Optical rotations were measured in chloroform with a Jobin Vyon micropolarimeter using a 1-mm cell.

RESULTS AND DISCUSSION

The unsaponifiable matter from vegetable oils usually consists of Δ^5 sterols, 4-α-methylsterols and triterpene alcohols which can be easily separated on TLC. Tocopherols and hydrocarbons are normally encountered at higher R_f values.

Preliminary analysis of sal fat unsaponifiable matter indicated the presence of a fraction with

a higher R_f (0.55) than the triterpene fraction. This fraction, upon gas liquid chromatography (GLC) analysis, showed 3 major and 4 minor components. The IR spectra of this fraction had the characteristic carbonyl absorption at 1725 cm^{-1} and hydroxyl absorption was absent. The major components all had a molecular ion at m/e 424 (2 mass units less than the corresponding alcohols). This absorption, coupled with the absence of mass fragments m/e 406 ($M-H_2O$) and 391 ($M-H_2O-CH_3$), suggested these to be ketones. The total ketone fraction, isolated by column chromatography (12% of total unsaponifiable matter) gave a major fraction (90%) having the same R_f on TLC as triterpene alcohols (after sodium borohydride reduction). The reduced alcohols were purified by preparative TLC (silica gel), acetylated with acetic anhydride/pyridine and subsequently fractionated on silver-nitrate-impregnated silica gel plates. Three major bands having R_f 0.85, 0.75 and 0.65 were isolated for further characterization.

Compound 1 (20%) with R_f 0.85 had mp of 240-241 C and $[\alpha]_D^{25}$ + 77° (c 0.1). Its mass spectrum showed the molecular ion at m/e 468 and base peak at m/e 218 which is highly characteristic of Δ^{12} urs- or olean-compounds (retro Diels-Alder fragmentation). Other important fragments were at m/e 453 ($M-CH_3$), 408 (M-60), 393 (M-60-15), 203 (218-15), 189 (loss of C, D and E rings along with one hydrogen and acetic acid) (5). The position of 2 olefinic carbons at δ 145.49 and δ 122.03 and other carbons in ^{13}C-NMR correspond to the reported values for β-amyrin acetate (6). The free alcohol obtained by hydrolysis of the acetate had mp 197 C and $[\alpha]_D^{25}$ + 97° (c 0.1). The physical and spectral characteristics of both acetate and alcohol clearly indicate that compound 1 is β-amyrin acetate. Thus, the structure of the parent keto compound is established as β-amyrenone.

Compound 2 (50%) with R_f 0.65 had mp of 123-124 C and $[\alpha]_D^{25}$ + 61° (c 0.1). The mass spectrum showed a molecular ion at m/e 468, base peak at m/e 69, and a fragment at m/e 286 (loss of A ring + 1 H) characteristic of a cycloartenol skeleton (7,8). Other important fragments were at m/e 453 (M-15), 408 (M-60), 393 (M-60-15), 365 (M-60-15-28), 339 (M-60-69), 203, and 175 (loss of A ring and side chain). ^{1}H-NMR showed 2 doublets at δ 0.33 and δ 0.59 (J = 4 Hz) which are characteristic of the 9,19-cyclopropane methylene of cycloartenol (9). On the basis of the physical and spectral evidence of the acetate, the structure of corresponding parent ketone is established as cycloartenone.

Compound 3 with R_f 0.75 (10%), had mp of 214-215 C and $[\alpha]_D^{25}$ + 45° (c 0.02). The mass spectrum showed a molecular ion at m/e 468 and base peak at m/e 189. Other principal fragments were observed at m/e 408, 393, 365, 249, 229, and 218. ^{1}H-NMR (10) gave signals at δ 0.83, 0.88, 0.91, 1.04, 1.69 (CH_2 = C-CH_3) 2.02 (-$OCOCH_3$) 4.51 and 4.6 (2 singlets C = CH_2) and 5.33 (triplet, J = 7.2 Hz, > CHOAc) ppm. The IR spectra showed absorptions at 3070, 1640, and 880 cm^{-1} due to terminal olefinic methylene. The spectral characteristics are in full agreement with those reported for lupeol acetate (3,10,11) and hence, the structure of the parent ketone is assigned as lupenone.

The triterpene alcohol fraction (15%) of sal unsaponifiable matter obtained by preparative TLC, consists mainly of cycloartenol, β-amyrin and lupeol, in conformity with the findings of Jeong et al. (4). The structural assignments were based on physical and spectral characteristics of the individual alcohols and their acetates.

The occurrence of 3-keto triterpenes in sal fat is unique. The observation that they correspond to oxidized versions of the major triterpene alcohols present in the unsaponifiable fraction indicate the presence of a specific dehydrogenase enzyme in sal seeds.

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On the Occurrence of α-Tocopherolquinone in Rat Tissue

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ABSTRACT

The amount of α-tocopherolquinone in rat liver has been reinvestigated comparing (a) a conventional procedure including saponification and thin layer chromatography followed by high peformance liquid chromatography (HPLC), with (b) the direct HPLC analysis of a total lipid extract. Recovery of added α-tocopherolquinone was quantitative with both procedures. In contrast to a recent report of 124 nmol/g in rat liver, we found no more than 1-4 nmol/g by procedure a and less than 1 nmol/g by procedure b.

Many investigators have been concerned with metabolites of α-tocopherol and several oxidation products, including α-tocopherolquinone, have been identified (1). This product has been reported to be present in rat liver in amounts varying from traces (2) to 2-12 nmol/g fresh tissue (3). Relative to the amount of α-tocopherol in liver (15-55 nmol/g), α-tocopherolquinone has been reported to be barely detectable (2). In view of this background, it was remarkable that Hughes and Tove (4) recently reported that rat liver contained 124 nmol/g of α-tocopherolquinone and an additional 90 nmol/g of α-tocopherolhydroquinone. They attributed these high values to their use of special anaerobic conditions during analysis and the failure of earlier workers to prevent losses due to polymerization by light and oxidation by air. Although tocopherolhydroquinone is well known to oxidize readily to the quinone when exposed to air, tocopherolquinone is generally considered to be a relatively stable compound. Because these observations of Hughes and Tove are at variance from the experience of many workers, we decided to reinvestigate the occurrence of α-tocopherolquinone in rat liver using the sensitive technique of high performance liquid chromatography (HPLC).

METHODS

Livers from male Sprague-Dawley rats were frozen on Dry Ice, stored at -20 C and analyzed within 1-4 weeks. The dietary history of the rats is indicated in the tables. Two analytical procedures were compared. In the "classical" procedure, 0.5 g liver was finely chopped and placed into a screw-capped test tube with 1.5 ml 2% ethanolic pyrogallol and 0.5 ml 11 N KOH. After saponification and extraction as described previously (5), the extract was separated by 2-dimensional thin layer chromatography (TLC) on plates of Silica Gel G containing sodium fluorescein (5). The quenching spots corresponding to α-tocopherol and α-tocopherolquinone, visualized under UV light, were scraped off, eluted with ethanol and transferred into hexane. The hexane was evaporated under nitrogen, the lipid dissolved in ether/methanol and an aliquot injected into the HPLC apparatus, as described previously (6). The HPLC system was a Waters instrument with a C-18 reverse phase column developed with water-methanol, 2.5:97.5, at 2.5 ml/min, with detection at 280 nm. This system separates α-tocopherolquinone cleanly from both α- and γ-tocopherols (6). Standard solutions of α-tocopherol and α-tocopherolquinone were injected after the liver samples for quantification from peak heights. For recovery experiments, α-tocopherolquinone in ethanol was added to the liver samples prior to saponification. In the second procedure, without saponification and TLC, 0.3 g liver was extracted with 7 ml acetone using an Ultraturrax homogenizer, an aliquot (0.3-0.6 ml) was evaporated under N_2, and the lipid was analyzed directly by HPLC. α-Tocopherolquinone from Kodak Chemicals, Rochester, NY, was purified by HPLC and the UV spectrum verified in a Cary Model 15 spectrophotometer.

RESULTS

The recovery of α-tocopherolquinone added to livers having a wide range of α-tocopherol content and analyzed after saponification and TLC is shown in Table 1. The apparent endogenous α-tocopherolquinone of these saponified livers was very low, less than 4 nmol/g, and was not related to their tocopherol content. Recovery of α-tocopherolquinone was essentially complete for all 3 experiments.

Analyses of livers from 2 groups of rats fed a

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TABLE 1

Recovery of α-Tocopherolquinone from Rat Liver[a]

Rat[b]	Endogenous T (nmol/0.5 g liver)	Endogenous TQ (nmol/0.5 g liver)	TQ added (nmol)	Total TQ found (nmol)	Recovery (%)
1	3.0	0.4	19.9	20.5	101.0
2	22.3	1.7	25.7	28.8	105.4
3	13.2	1.8	19.9	20.2	92.4

[a]Duplicate 0.5 g samples of liver, with or without added α-tocopherolquinone (TQ), were analyzed by TLC and HPLC after saponification. T = α-tocopherol.

[b]Rat 1 was fed a vitamin-E-deficient diet for 12 weeks; rat 2 was fed a purified diet with dl-α-tocopheryl acetate, 50 mg/kg, for 17 weeks; rat 3 was fed NIH-07 stock diet for 8 weeks.

low or high intake of α-tocopherol, using the same detailed procedure, are shown in Table 2. Although the contents of α-tocopherol varied 4-fold between the groups, the apparent α-tocopherolquinone contents were the same and ranged from 1-4 nmol/g. When saponification and TLC were avoided and the lipid from an acetone extract of liver was injected directly into the HPLC, there was an α-tocopherol peak but no α-tocopherolquinone peak (4 separate livers). This procedure would have detected 1.0 nmol of the quinone/g tissue. When 2 nmol of α-tocopherolquinone was added to 0.35 g of liver prior to homogenization, recovery was 100%.

DISCUSSION

Many investigators have reported finding α-tocopherolquinone in animal tissues, usually in trace amounts relative to the tocopherol or ubiquinone (2). Contrary to the statement of Hughes and Tove (4) that procedures used in the past destroy tocopherolquinone, we have found no such lability of the compound. Chow et al. (7) also had no problems with losses of tocopherolquinone. Tocopherolhydroquinone, on the other hand, is well known to spontaneously oxidize to the quinone during conventional procedures (7). Thus, most data in the literature would overestimate the α-tocopherolquinone content of tissues if significant amounts of hydroquinone had been present in the initial tissue. Hughes and Tove used, successively, lipid extraction, saponification, TLC and UV determination of the α-tocopherolquinone, all under an atmosphere of N_2/H_2.

TABLE 2

Tocopherol and Apparent Tocopherolquinone in Liver of Rats Fed Low or High Dietary α-Tocopherol

Diet[a]	T (nmol/g)	TQ (nmol/g)
Low	10.5 (5.1-14.5)	2.4 (0.9-3.7)
High	55.4 (43.2-67.2)	2.5 (1.1-4.3)

[a]Rats fed a purified diet with a low or high content of dl-α-tocopheryl acetate. Values are means of 5 rats; ranges are in parentheses. Samples were saponified prior to TLC and HPLC analysis.

In contrast to the 124 nmol/g of tocopherolquinone found by Hughes and Tove (4) from the analysis of only one rat liver, we find only 0.9-4.1 nmol/g. These workers also found 90 nmol of tocopherolhydroquinone, which in our procedures would have been oxidized to the quinone. It would appear that the high contents of tocopherolquinone, and also tocopherolhydroquinone, in rat liver reported by Hughes and Tove are either erroneous or highly atypical.

The true amount of α-tocopherolquinone in tissues has been controversial due to the in vitro oxidation of tocopherol during tissue work-up resulting in the artifactual production of the quinone (1,2). We are not certain that the values for α-tocopherolquinone shown in Table 2 may not be higher than the actual amounts in the livers. During the analyses by saponification and TLC, we occasionally obtained values for the same liver that varied as much as 3-fold. The high values were assumed to be artifactual and the analyses were repeated. We showed earlier, using radioactive α-tocopherol, that the amount of oxidation products obtained from rat liver varied with different extraction solvents and also with different TLC procedures (8). Also, administering α-tocopherol by intraperitoneal injection gave more polar compounds recoverable from liver than when

the dose was given orally. Based on our analyses of total lipid extracts injected directly into the HPLC, the apparent α-tocopherolquinone content of liver from rats fed a purified or stock diet does not exceed 1 nmol/g.

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Metabolism of Epoxidized Phosphatidylcholine by Phospholipase A_2 and Epoxide Hydrolase

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ABSTRACT

The isolation and measurement of phospholipid epoxides as major peroxidation products in biomembrane preparations prompted an investigation of enzymatic mechanisms which may be responsible for their elimination. Analysis of microsomal epoxide hydrolase and phospholipase A_2 activity against a phospholipid epoxide commonly encountered in tissues indicated it to be a poor substrate for epoxide hydrolase, but rapidly hydrolyzed by phospholipase A_2. Microsomal and purified phospholipase A_2 preparations hydrolyzed the phospholipid epoxide at rates 2-fold greater than were observed with a monoenoic phospholipid from which the epoxide would be derived. The product fatty acid epoxide, *cis*-9,10-epoxystearic acid, was rapidly hydrated by microsomal and cytosolic epoxide hydrolase. On the basis of earlier reports demonstrating increased phospholipase activity against oxidized phospholipids, and on the results of the present study, a model for the metabolism of oxidized membrane phospholipids is proposed.

The peroxidation of unsaturated lipids in vivo is believed to proceed through a free radical propagation mechanism (1) with the formation of hydroperoxides and their degradation products. Although the presence of such products, as determined directly or by measurement of conjugated dienes or malonaldehyde, has been documented in numerous in vitro and in vivo studies (2), its utility as a measure of lipid peroxidation in tissues or whole animals has been equivocal (3). Another method useful in determining the extent of in vivo lipid peroxidation involves measuring fluorescent products resulting from reaction of malonaldehyde with the amine group of phospholipids (2,4).

It is widely accepted that the major cellular sites for peroxidizable lipids are membranes, primarily those of the endoplasmic reticulum and mitochondria. Phospholipids are the major lipid components in these structures and, along with cholesterol, constitute the bulk of the peroxidizable lipids. Previous studies in our laboratory have demonstrated that peroxidation in biomembranes, or model structures resembling biomembranes, proceeds via a free radical oxidation process. Among the major products formed are epoxides and hydroxy-epoxy lipids derived from the corresponding unsaturated lipids (5,6). The relative stability of many of these compounds, or their hydration products, has enabled their isolation from biomembrane systems (6), or intact tissues (7) as well as quantitative estimates of their formation relative to the degree of lipid peroxidation. The relative ease of their isolation may be distinguished from the attempts to measure lipid hydroperoxides which, for numerous reasons, are rapidly degraded or eliminated by protective systems (8).

Characterization of the major isolable lipid epoxides from rat tissues revealed that most of this material existed in either triacylglycerols, phospholipids or sterols (7). In addition, the amount of epoxide was found to be greater in the lung than in any other organ examined. On the assumption that lipid peroxidation would take place predominantly in biomembranes, one would expect phospholipid fractions to possess the greatest proportions of epoxides, as well as other peroxidation products. Examination of the data has not supported this assumption. The triacylglycerols, instead, have been the fraction possessing the largest proportion of epoxides (7).

Speculation has persisted for a number of years that membrane phospholipases may participate in the selective elimination of specific fatty acyl moieties in phospholipids, particularly those which have been damaged by peroxidation reactions. This possibility was suggested several years ago by van Deenan (9), who noted that the selective replacement of deteriorated molecules could be accomplished more economically by replacement of the fatty acyl groups without the need of replacing the entire phospholipid. Increased phospholipase A_2 activity was found in rat liver mitochondria subjected to lipid peroxidation in the presence

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of ferrous ion and ascorbate (10). Tappel (4) has explored this effect in the context that membrane-bound hydroperoxides were found to be resistant to reduction by glutathione peroxidases (11). Evidence of a phospholipase that rapidly hydrolyzed fatty acid hydroperoxides from phospholipids suggested that these enzymes could be part of a membrane repair or protective system which could make hydroperoxides available for reduction.

We examined the possibility that phospholipases are involved in a repair process following peroxidation by using a phospholipid epoxide which is commonly isolated from biomembranes (7). This epoxide proved to be useful in terms of its stability and utility as a substrate in enzyme studies, and its resistance to microsomal epoxide hydrolase. Our attention was limited to phospholipase A_2 (E.C. 3.1.1.4) since this is primarily a membrane-bound enzyme (12) for which biochemical properties have been well described, and for operationally practical purposes since the substrates to be examined contained the critical fatty acids in the *sn*-2 position of phosphatidylcholine.

EXPERIMENTAL PROCEDURES

Preparation of Epoxide Substrates

L-α-1-Stearoyl-2-oleoyl phosphatidylcholine was purchased from Applied Sciences, Inc. (State College, PA) as well as L-α-1 palmitoyl-2-[1,C^{14}]oleoyl phosphatidylcholine (sp act 20 mCi/mMol). Both the radiolabeled and unlabeled phospholipids were mixed in a ratio of 1:10, respectively, and dissolved in chloroform at a concentration of 1 mg/ml. To this mixture was added a 2 M excess of *m*-chloroperoxybenzoic acid and the reaction was allowed to proceed for 24 hr at room temperature in a sealed vial. During this period, aliquots were removed in order to assess the extent of reaction. The conversion of the oleoyl moiety to the corresponding *cis*-9,10 epoxide was measured by hydrolysis of the phospholipid in a 2-phase system consisting of ether and 20% KOH in methanol as described previously (7). The fatty acid methyl esters obtained were analyzed by gas chromatography using a 6-ft, 3% OV 101 packed column at 210 C. The extent of the reaction was determined by the disappearance of me oleate and accumulation of me 9,10-epoxystearate. The reaction was found to be complete by 24 hr, established by the total disappearance of oleate. The reaction mixture was washed with an equal vol of 0.1 M K_2CO_3 and the chloroform phase was saved. The chloroform extract (~1 ml) was applied to a silicic acid column, 1 g, (Biorad, BioSil HA-325 mesh) and eluted with 25 ml chloroform, and this eluate dicarded. The column was subsequently eluted with 25 ml methanol and the eluate collected and evaporated to dryness. The residue was dissolved in benzene and phospholipid content and radioactivity were determined. The product after methanolysis, as already described, was found by gas chromatographic analysis to be 95% 1-palmitoyl/stearoyl-2-epoxystearoyl phosphatidylcholine, sp act 0.90 mCi/mMol.

Radiolabeled me-*cis*-epoxystearate, epoxystearic acid and cholesterol, 5α,6α-epoxide were prepared as described previously (13).

Preparation of Microsomal and Soluble Fractions

Male Sprague-Dawley rats (200-250 g) obtained from a specific pathogen-free colony (Hilltop Labs, MA) were sacrificed and the lungs and livers excised. After trimming away major vessels and connective tissues, the organs were immersed in 0.1 M phosphate-buffered normal saline, pH 7.4, containing 0.25 M EDTA, minced, and homogenized using a Polytron homogenizer. The use of buffer and pH was based on whether samples for phospholipase A_2 or epoxide hydrolase analysis were prepared. Phosphate buffer was used primarily for epoxide hydrolase whereas 0.1 M Tris buffer (pH 8.0) was used for phospholipase A_2 preparations. The homogenates were centrifuged for 10 min at 500 g to remove intact cells and debris. The supernatant was recentrifuged at 18,500 x g for 15 min and the supernatant fraction was collected and recentrifuged for 1 hr at 100,000 x g. The resulting pellet was resuspended in fresh media by sonication, 50w for 10 sec, while the supernatant (spnt. 1) was passed through 8 layers of surgical gauze and the filtrate collected. Both fractions were recentrifuged at 100,000 x g for 1 hr. The resulting pellet was resuspended to a final protein concentration of 0.15 mg/ml in fresh media (without EDTA) by sonication as just described. This represented the microsomal fraction to be used for study. The supernatant obtained by recentrifugation of spnt. 1 was collected and also adjusted to a final concentration of 0.15 mg/ml and represented the cytosolic fraction. The pH profile of either phospholipase A_2 or epoxide hydrolase was analyzed using either phosphate or Tris buffers adjusted to the desired pH.

Epoxide Hydrolase Measurements

The determination of epoxide hydrolase activity against lipid epoxide substrates was performed by preparing either microsomal or

cytosolic fractions in 0.1 M phosphate buffer, pH 7.4, at a final concentration of 0.15 mg protein/ml. The desired substrates (e.g., phosphatidylcholine epoxide) were dissolved in acetone at concentrations where the addition of 1 μl of acetone solution delivered 3 nmol of substrate to 100 μl of buffer at 37 C in 4 ml siliconized borosilicate tubes. Reactions were initiated in this manner. The conditions and methods for the assay were otherwise as described previously (13). Under these circumstances, ca. 8,000 cpm of radiolabeled phosphatidylcholine epoxide was delivered per tube. In those instances where inhibition of either epoxide hydrolase or phospholipase A_2 activities was desired, 3 mM cyclohexene oxide or 1 mM *p*-bromophenacyl bromide, respectively, was added to the sample. Conversion of phosphatidylcholine epoxide to the corresponding diol (1-palmitoyl/stearoyl-2-dihydroxystearoylphosphatidylcholine) was determined after incubation by hydrolyzing the product with phospholipase A_2 from snake venom (*Crotalus ademanteus*). This involved addition of 1 unit of enzyme in 50 μl of buffer containing 0.035 M $CaCl_2$, followed by 3 ml diethyl ether. After mixing for 1 hr using 1 x 5 mm magnetic mixing bars, the tubes were centrifuged briefly and the ether layer was removed. The volume of ether was reduced by evaporation under a stream of nitrogen and then applied to Whatman LK5D plates. Under these conditions, all of the radioactivity was recovered into the ether extract. The plates were developed in a solvent system consisting of petroleum ether/diethyl ether/acetic acid (70:30:1). The lipids were visualized by exposure to iodine vapor or by a B-camera (Berthold LB 2760) and the areas corresponding to authentic epoxystearic acid and dihydroxystearic acid were recovered and transferred to scintillation vials for measurement of radioactivity. The extent of epoxide hydrolysis was based on the conversion of epoxide to diol, assuming that the sp act of the original phospholipid substrate and product were equal. The extent of nonenzymatic hydrolysis was determined in blank preparation containing microsomal or cytosolic preparations which were boiled for 5 min. Values obtained from these samples were subtracted from the corresponding experimental samples to obtain final enzymatic rates for epoxide hydrolase.

Phospholipase A_2 Measurements

Phospholipase A_2 activity aginst phosphatidylcholine epoxide or the parent compound (1-palmitoyl/stearoyl-2-oleoylphosphatidylcholine) was determined in both microsomal and cytosolic preparations from lung and liver. Reactions were carried out at 37 C with 0.1 M Tris buffer, pH 8.0, containing 0.0125 M $CaCl_2$, and with one exception, were identical to those described for epoxide hydrolase. The exception was that final substrate concentrations were 20 μM and were added to the reaction system maintained at 0-4 C. The samples were transferred to a water bath at room temperature for 2 min and then to a water bath at 37 C. This preincubation procedure was used to facilitate interaction and binding of substrate to microsomes while minimizing enzymatic activity. Initiation of the reaction, or time-zero, was considered as the time at which samples were immersed into the 37 C water bath. The samples were then incubated for intervals up to 20 min. Reactions were stopped by removing all or aliquots of each sample using a repipetor and applying them to the absorbent zone of Whatman LK5D plates. The plates were developed in a solvent system consisting of chloroform/methanol/water (65:25:4). Regions corresponding to free fatty acids (or fatty acid epoxides), phosphatidylcholine epoxide and lysophosphatidylcholine were recovered into scintillation vials for determination of radioactivity. The basis of computation was similar to that described for epoxide hydrolase measurements. Measurement of lysophosphatidylcholine radioactivity provided an estimate of phospholipase A_1 activity; however, this measurement was not directly involved in the computations for phospholipase A_2. The extent of reaction was also determined in samples containing either EDTA plus calcium-free media, *p*-bromophenacyl bromide (1 mM), a combination of EDTA and *p*-bromophenacyl bromide, or in the presence of the epoxide hydrolase inhibitor, cyclohexene oxide, 3 mM.

Analysis of phospholipase A_2 activity was also performed in a purified enzyme preparation from snake venom, *C. ademanteus* (Sigma, St. Louis, MO). The conditions and methods for analysis were similar to those described by Misiorowski and Wells (14) (method II). Accordingly, 2.0 ml ether/methanol (95:5, v/v) containing 30 μM substrate (final concentration) was added to 40 μl of 0.1 M Tris buffer, pH 8.0, containing 100 μM $CaCl_2$. After mixing for 15 sec, 5 μl of Tris buffer containing 5 μg of enzyme (1.0 unit) and 100 μM $CaCl_2$ was added to initiate the reaction. After mixing continuously for 10 min at 22 C, 100 μl 95% ethanol was added to each tube to stop the reaction and the sample transferred to Whatman LK5D plates which were treated as described previously.

Radioactivity was measured in an aquasol based mixture (New England Nuclear) in a liquid scintillation counter (Beckman LS 8100). Protein content was determined by the method of Gornall et al. (15). Lipid phosphorus was determined by the method of Morrison (16). Measurements for each substrate under varying conditions or durations of incubation were made in triplicate, and are expressed as means ± 1 SD. The reported data are the results from 3 separate experiments.

RESULTS

The metabolism of phosphatidylcholine epoxide was examined under the same conditions as those of me epoxystearate and cholesterol epoxide in previous studies (13). Since epoxide hydrolase and phospholipase A_2 activities were likely to be competing reactions, an examination of the activity for each enzyme on phosphatidylcholine epoxide was undertaken. Figure 1 shows the effect of pH on the activity of lung microsomal phospholipase A_2 and epoxide hydrolase on phosphatidylcholine epoxide. The concentration of epoxide used was 20 μM and, aside from differences in buffer used (dependent on the pH examined), all other conditions were similar. The optimal pH for phospholipase A_2 was 8.0-8.4 whereas optimal range for epoxide hydrolase was 7.2-7.6. In a similar manner, the pH optimum for 1-palmitoyl/stearoyl-2-oleoyl phosphatidylcholine hydrolysis by phospholipase A_2 was found to be between 8.4 and 8.8 (not shown in figure). Similar results were found for liver microsomes, although the activity of each enzyme was greater than in the lung on the basis of protein content. Both enzymes displayed linear rates of reaction for up to 20 min, therefore, 10- and 15-min incubations were routinely used for phospholipase A_2 and epoxide hydrolase measurements, respectively.

Figure 2 shows the results for lung microsomal phospholipase A_2 with phosphatidylcholine and phosphatidylcholine epoxide as measured over a 20-min interval. Under these conditions, it was determined that nearly 90% of the added radioactivity could be recovered bound to microsomes following the preincubation interval. This was based on the recovery of counts associated with microsomes obtained by filtering the sample through a 0.22-μ Millipore (Millipore Corp.). There was an appreciable degree of enzymatic hydrolysis for both phospholipid substrates using this preincubation protocol. However, the rates of hydrolysis were linear over the subsequent 20 min of incubation.

We have previously reported the properties and activity of lipid epoxide hydrolase prepared from lung microsomal and cytosolic fractions

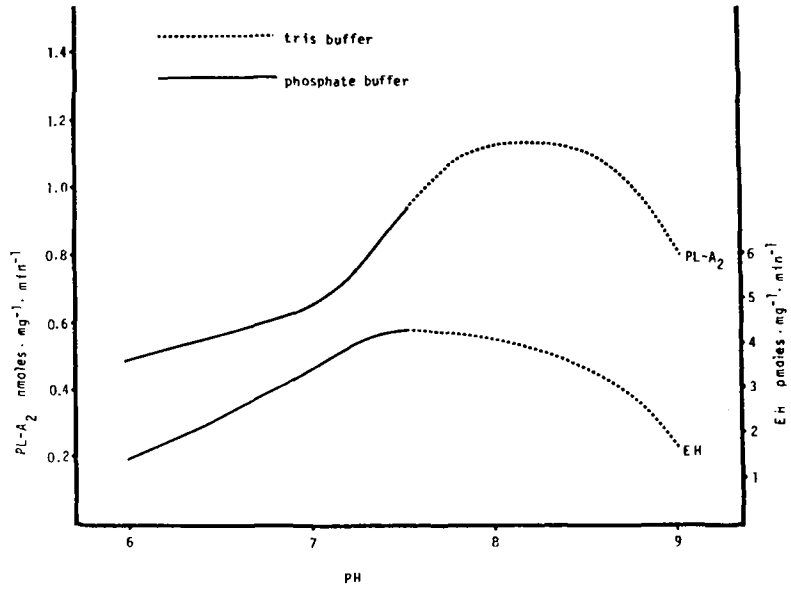

FIG. 1. The effect of pH on phospholipase A_2 ($PL\text{-}A_2$) and epoxide hydrolase (EH) activities against 1-palmitoyl/stearoyl-2-epoxystearoyl phosphatidylcholine (20 μM). Reactions were carried out in either 0.1M phosphate or Tris buffers using lung microsomes at a concentration of 0.15 mg protein/ml.

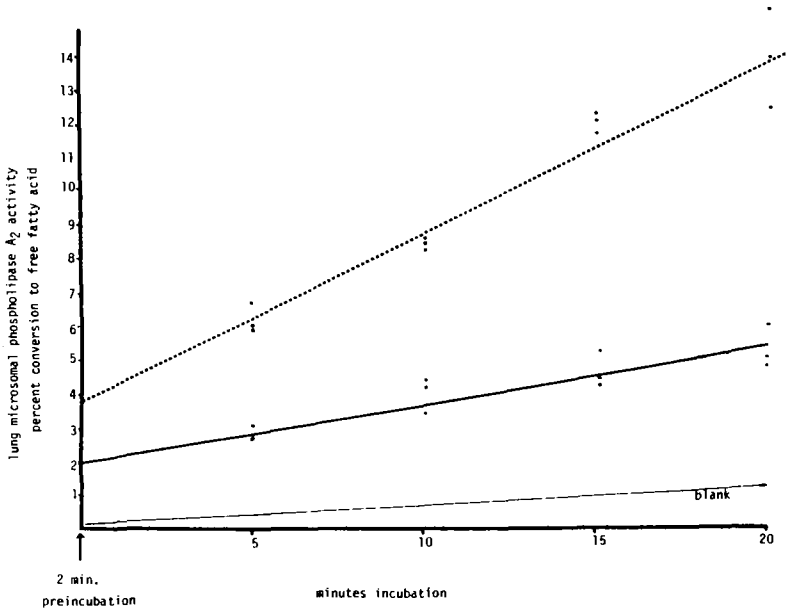

FIG. 2. Time course of lung microsomal phospholipase A_2 activity against 20 μM 1-palmitoyl/stearoyl-2-oleoyl phosphatidylcholine (——) or 20 μM 1-palmitoyl/stearoyl-2-epoxystearoyl phosphatidylcholine (– – –). Rates are expressed as percentage of total substrate hydrolyzed to free fatty acids over a 20-min interval. Blank determinations are represented using microsomes boiled for 5 min. Details for methods are given in the text.

(13). In these studies, the hydration rates were obtained for cholesterol epoxide and methyl epoxystearate. In the present study, we expanded the list of lipid epoxides and examined the rates for microsomal and cytosolic epoxide hydrolase isolated from rat lung and liver. We used a phosphate-buffered system, pH 7.4, as in previous studies and the concentrations of substrates used are indicated in Table 1. It is evident that, in most cases, microsomal epoxide hydrolase activity is greater than that of the cytosolic enzyme. The exception to this is the greater cytosolic activity with epoxystearic acid and phosphatidylcholine epoxide (lung). It is likely that activity against styrene oxide resides in microsomes (17).

Among the substrates examined, the highest activity is found with styrene oxide, obtained from Aldrich, Inc. and analyzed by the methods of Jerina et al. (18). Sytrene oxide is a well known and actively metabolized xenobiotic substrate. Methyl epoxystearate and epoxystearic acid are remarkably good lipid epoxide substrates. In liver preparations, it

TABLE 1

Epoxide Hydrolase Activity in Rat Lung and Liver Microsomes and Cytosol (expressed as pmol/mg protein/min)

Substrate	Lung microsomes	Cytosol	Liver microsomes	Cytosol
Methylepoxystearate (20 μM)	159 ± 16.7	90 ± 10.4	1301 ± 30.2	700 ± 16.2
Epoxystearic acid (20 μM)	180 ± 12.9	220 ± 12.4	–	–
Cholesterol epoxide (20 μM)	10 ± 2.3	4 ± 0.1	183 ± 17.4	64 ± 15.7
Styrene oxide (50 μM)	200 ± 7.6	–	1700 ± 19.9	–
Phosphatidylcholine epoxide (30 μM)	4 ± 0.8	17 ± 1.8	16 ± 2.1	13 ± 0.7
Phosphatidylcholine epoxide + pBPB[a] (1 mM)	15 ± 2.4	–	28 ± 4.2	–

[a]*p*-Bromophenacyl bromide.

appears that this fatty acid epoxide is hydrated at rates comparable to sytrene oxide. In contrast to this, cholesterol epoxide and phosphatidylcholine epoxide are poorly metabolized in all enzyme preparations, although cholesterol epoxide is hydrated to an appreciable extent in liver preparations. In the latter case, our findings agree with those of Aringer and Eneroth (19). In all instances, liver epoxide hydrolase sp act exceeds that of the lung (on a mg protein basis).

It is a reasonable assumption that most epoxide-containing fatty acids in membranes would be associated with phospholipids, and it is likely that they originate in membranes. Therefore, one would expect that a means for eliminating these products would be through membrane-associated epoxide hydrolase. The observation that phosphatidylcholine epoxide is a poor substrate for microsomal, as well as cytosolic, epoxide hydrolase suggests that an alternate means may exist for elimination.

Table 2 presents the results for phospholipase A_2 activity as measured with phosphatidylcholine and phosphatidylcholine epoxide. The data indicate that phosphatidylcholine epoxide is hydrolyzed at rates approximately twice that of phosphatidylcholine at pH 8.0. Although measurements at pH 8.0 favor activity against the epoxide, increasing the pH to 8.5 produces a 30% increase in the rate of hydrolysis for phosphatidylcholine but no rate difference for the epoxide. This effect is most pronounced in lung preparations where the levels of epoxide hydrolysis approximate those of the liver. Activity against either substrate is nearly abolished in the presence of EDTA, indicating the requirement for calcium or other divalent cations. The phospholipase A_2 inhibitor *p*-bromophenacyl bromide also reduces enzyme activity but to a lesser degree than EDTA. This is particularly evident with phosphatidylcholine epoxide, suggesting that an altered mode or degree of enzyme-substrate interaction may be involved in the hydrolysis of the epoxidized fatty acid. Inhibition of epoxide hydrolase using cyclohexene oxide has no apparent effect on phospholipase A_2 activity.

Reexamination of these substrates using a reversed micelle system containing purified phospholipase A_2 produced similar results, as shown in Table 3. As with microsomal preparations, the rate of hydrolysis against phosphatidylcholine epoxide was twice that for phosphatidylcholine. However, it is difficult to explain the finding that EDTA and *p*-bromophenacyl bromide were relatively ineffective in abolishing enzyme activity.

Reexpressing the data for phospholipase A_2 and epoxide hydrolase, as shown in Table 4, reveals that phospholipase A_2 plays a dominant

TABLE 2

Microsomal Phospholipase A_2 Activity against Phosphatidylcholine and Phosphatidylcholine Epoxide

	Lung (pmol/mg/min)		Liver (pmol/mg/min)	
	PC	PC epoxide	PC	PC epoxide
20 μM microsomes	490 ± 56	1150 ± 88	800 ± 47	1430 ± 107
+ EDTA	7.0 ± 0.9	8.8 ± 1.0	41.1 ± 8.1	60.5 ± 7.6
+ 1 mM pBPB[a]	40 ± 1.9	200 ± 15.9	–	–
+ EDTA + pBPB[a]	2.9 ± 1.1	9.8 ± 2.1	4.0 ± 0.7	31.1 ± 4.7
+ 3 mM cyclohexane oxide	–	1222 ± 47	–	1300 ± 69

[a]*p*-Bromophenacyl bromide.

TABLE 3

Phospholipase A_2 Activity (*C. adamanteus*) against Phosphatidylcholine and Phosphatidylcholine Epoxide

	PC (nmol/mg lipase/min)	PC epoxide (nmol/mg lipase/min)
Enzyme	51.0 ± 6.9	95.0 ± 10.4
+ EDTA	18.2 ± 0.74	11.8 ± 1.42
+ 1 mM pBPB[a]	23.1 ± 3.15	35.0 ± 5.09

[a]*p*-Bromophenacyl bromide.

TABLE 4

Relative Activities of Microsomal Phospholipase A_2 and Epoxide Hydrolase with Phosphatidylcholine Epoxide

	Lung (pmol/mg protein/min)	Liver (pmol/mg protein/min)
Epoxide hydrolase	4	16
Phospholipase A_2	1222	1300
EH/PLA_2	0.006	0.012

role in metabolizing the damaged phospholipid. These data indicate that phospholipase A_2 displays 300 times the activity found for epoxide hydrolase in lung and 80 times the activity in liver using phosphatidylcholine epoxide.

DISCUSSION

The significance of lipid epoxides in biological systems is a matter that is still open to investigation. Evidence from this laboratory indicates that these epoxides are of peroxidatic origin (20) and, as such, may be grouped along with other products of peroxidation such as carbonyl compounds or hydroperoxides. The relationship of these compounds as products in the propagation scheme is, as yet, uncertain. However, they may be involved in the secondary free radical chain reactions arising from lipid hydroperoxides—but preceding cleavage reactions that form volatile hydrocarbons or malonaldehyde.

Since phosphatidylcholine epoxides were commonly encountered in animal tissues (7), we felt that it would be informative to examine their properties as substrates for phospholipases. As the results of this study indicate, one such phospholipid epoxide, 1-palmitoyl/stearoyl-2-epoxystearoyl phosphatidylcholine is at the same time poorly metabolized by microsomal epoxide hydrolase, and a preferred substrate for phospholipase A_2 when compared to the monoenoic phosphatidylcholine from which it is derived. Consideration of the data presented in Tables 1 and 2 provokes a scheme by which the metabolism of phospholipid epoxides (or other oxygenated products) may occur. In Figure 3, a possible metabolic route for phosphatidylcholine epoxide is described. In this scheme, the major route of metabolism is effected by phospholipase A_2 to give the products 1-acyl-lysophosphatidylcholine and epoxystearic acid. A minor route would be direct hydration, producing 1-acyl-2-dihydroxystearoylphosphatidylcholine. The fatty acid

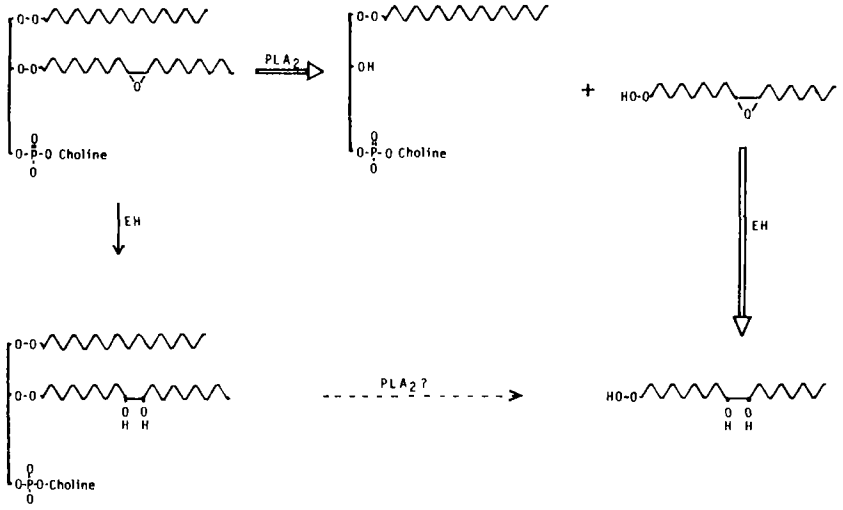

FIG. 3. A diagram describing the possible mode for metabolism of phosphatidylcholine epoxide. Alternate means for hydrolysis by phospholipase A_2 (PLA_2) or hydration by epoxide hydrolase (EH) are shown. See text for details.

epoxide released from the phospholipid is readily acted on by either cytosolic or microsomal epoxide hydrolase. Consequently, it may be converted to the fatty acid diol either in the microsomal milieu or in the cytosol, the compartment into which it is released. The physical properties of the free epoxide, but especially the diol, would favor partitioning into the cell sap from which it could eventually be eliminated through conjugate formation involving sugars or sulfur-containing compounds (17,21). Also shown in the figure is the possible hydrolysis of the phosphatidylcholine-diol product of epoxide hydrolase. This diol may be eliminated from the phospholipid with equal facility as the epoxide. When microsomes were incubated in the presence of the phospholipase A_2 inhibitor *p*-bromophenacyl bromide, a slight increase in epoxide hydrolase activity was noted (Table 1). The activities measured for epoxide hydrolase still constitute a small proportion of the activity measured for phospholipase A_2, whereas addition of the epoxide hydrolase inhibitor, cyclohexene oxide, has no effect on phospholipase A_2 activity (Table 2). This suggests that conversion to phosphatidylcholine diol has no demonstrable effect on the rate of hydrolysis; however, direct measurements using phosphatidylcholine diol are necessary to confirm this hypothesis.

Since hydrophobic interactions with phospholipid substrates have a significant effect on the observed activity of phospholipase A_2, perhaps involving the interface recognition site of the enzyme (22,23), the penetration of the catalytic site could depend on the lipid packing density of the phospholipids. In this regard, Pattus et al. (24) reported that interaction of phospholipase A_2 with lecithin monolayers decreases sharply at a packing density of 75 $Å^2$/molecule. Under an otherwise constant circumstance, the area/molecule of phospholipid increases with the degree of unsaturation, i.e., saturated<monoene<diene (25). The observations of Longmore et al. (26) support this theory, in that they found that microsomal phospholipase A_2 displays greater activity against unsaturated than saturated lecithins. Consequently, the presence of a hydrophilic center within the fatty acid matrix of a membrane would be expected to create repulsive forces resulting in greater surface areas, or "gaps" within the affected phospholipids. This could effectively facilitate penetration of membranous phospholipases. This is consistent with the theory that regulation of phospholipases can be achieved through changes in the structural arrangement of phospholipids in membranes (27). Such a hypothesis could explain the increased phospholipase action on oxidized phospholipids without the need to invoke specific phospholipases (4) or altered localization within the membrane matrix (10). It should also be noted that the measure of epoxide hydrolase activity, and perhaps phospholipase A_2, was made under conditions which probably differ from the natural interaction with phospholipids within membranes. Thus, the low activities measured for phosphatidylcholine epoxide hydrolase may be due to insufficient substrate accessibility. Such a possibility is supported by the finding that microsomal epoxide hydrolase is a deeply embedded "intrinsic" membrane protein (28).

Yet to be examined is the possibility that other phospholipases also display increased hydrolytic activity against phosphatidylcholine epoxide. Enhanced phospholipase C activity, e.g., may account for the large proportion of fatty acid epoxides found in triacylglycerol. Formation of triacylglycerols could occur through the acylation of the product diglycerides and would represent another means for eliminating damaged phospholipids from membranes.

ACKNOWLEDGMENTS

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On the Mechanisms of Fatty Acid Transformations in Membranes

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ABSTRACT

Concentrations of albumin in excess of 1% in the incubation mixture inhibited the elongation of added fatty acids and their incorporation into microsomal lipids whereas these reactions were not inhibited with endogenous microsomal membrane fatty acids. The results of these and other studies support the idea that such reactions of membrane lipid fatty acids with membrane-bound enzymes normally occur entirely within the membrane without release of free fatty acids to equilibrate with the fatty acid pool during the process.

INTRODUCTION

It has been recognized increasingly during the past few years that the usual techniques of carrying out enzymatic reactions in vitro do not accurately mimic the conditions existing in the membrane. Particularly in the case of lipid alterations, both substrates and enzymes are located in the membrane and are profoundly altered in the aqueous medium in which most in vitro enzymatic reactions are carried out. As a matter of fact, in many instances, we not only cannot accurately reproduce the in vivo conditions, but we have no clear idea of what they are. The result has been that much of our information concerning rates and mechanisms of lipid alterations in vivo may be quantitatively and, in some cases, even qualitatively inaccurate (1).

Gatt and his coworkers (1-8) and Dervichian and Barque (9,10) have studied the kinetics of reaction of enzymes with lipid substrates and Gatt (1,7,8) has proposed a scheme by which kinetic data can be more readily interpreted to reveal mechanisms in this field. While these studies have defined some of the parameters existing in the in vitro reactions, they admittedly have not defined those existing in vivo and, indeed, attempts to clarify these seem to present insurmountable difficulties involving construction of model systems truly representing the structures existing in the cell.

In this laboratory, we have been approaching this problem for several years with varying success. A basic assumption made at the start was that, in all cases in which a fatty acid is modified at the carboxyl end and in most of those in which the modification is elsewhere in the chain, the first reaction involved must be removal of the fatty acid from its position in the membrane phospholipid leaving, at least instantaneously, a lysophospholipid. Such a reaction usually involves a phospholipase and some consideration of the conditions imposed in each case will at least partially determine which phospholipase. For example, the elongation of membrane arachidonic acid (20:4) to 22:4 must be preceded by splitting of the 20:4 from carbon 2 of a phosphoglyceride and, thus, probably involves phospholipase A_2.

With these thoughts in mind, an attempt was made to inhibit the elongation of the endogenous microsomal 20:4 of brain to 22:4 by the use of a phospholipase A_2 inhibitor. Indeed, it was found that 4×10^{-4} M *p*-bromophenacyl bromide (pbpb), a phospholipase A_2 inhibitor (11), inhibited the incorporation of [1-^{14}C]-acetyl-CoA into the endogenous fatty acids of brain mitochondria completely and the incorporation of [1,3-^{14}C]malonyl CoA into those of brain microsomes by 75%, although elongation of exogenous fatty acids was not inhibited by twice this concentration (12). It thus appeared that inhibition of membrane phospholipase A_2 inhibited the elongation of membrane lipid fatty acids even though the elongation system itself was intact. However, these results were not completely satisfying in that the elongated fatty acids still labeled in the inhibited microsomal preparation included those, such as 22:4, which could be formed only by elongation of endogenous fatty acid, whereas 16:0, which could be formed by total synthesis, was drastically reduced. The reason for this finding may be that pbpb is effective in brain mitochondria, in which phospholipase A_2 is a major activity, but not in the microsomal fraction, in which it is relatively minor (13).

A second study concerned the in vitro labeling of the membrane phospholipids with specific fatty acids followed by a comparison

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of the rates of metabolism (elongation and desaturation) of these fatty acids with similar rates for the metabolism of added fatty acids (14). Although the endogenous labeled fatty acids were readily desaturated or elongated, the rates were lower than were those for the added fatty acids or acyl CoA. However, when the estimated steady-state concentrations of fatty acids released by phospholipase activity in the membrane were taken into consideration, it could be estimated that the reactions with endogenous fatty acids were more rapid than were those with the added substrates. Moreover, the product fatty acids formed from the exogenous precursors were distributed among phospholipid, free fatty acid and triacylglycerol fractions whereas those from the endogenous precursors were solely in phospholipid, thus indicating a different reaction sequence (14).

Neither of these studies, however, was completely definitive in describing a totally intramembrane pathway and a third method of approach was devised that was simple, as well as revealing. In this approach, advantage was taken of the knowledge that fatty-acid-free albumin binds 2 molecules/mol of fatty acid very tightly (15). Thus, it was considered that, in the presence of a sufficiently high concentration of albumin, any fatty acid present in the incubation medium or released from the membrane would be sequestered as the albumin complex and would be unavailable for association with the membrane-bound enzymes involved in its alteration. This paper reports the results of a study of fatty acid elongation done in the presence of albumin.

MATERIALS AND METHODS

Materials

Malonyl CoA, coenzyme A, ATP, NADPH and NADH were obtained from Sigma Chemical Co.; Pentex bovine albumin, fatty-acid-free, fraction V, is a product of Miles Laboratories, Inc. All other reagents were Baker analyzed products. Arachidonic acid was obtained from Nu-Chek-Prep Laboratory and [1-^{14}C]arachidonic acid (53 μCie/μmol) and [1,3-^{14}C]malonyl CoA (36 mCie/mmol) were purchased from New England Nuclear Corp.

Preparation of Microsomes

Whole brains from 16-day-old rats were weighed and homogenized in 9 vol of chilled 0.32 M sucrose solution with a Potter Elvehjem homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 1,000 x g for 10 min to remove cellular debris. A crude mitochondrial fraction was obtained by centrifugation of the supernatant fluid at 12,000 x g for 30 min. The resulting supernatant solution was recentrifuged at 40,000 x g for 2 hr to obtain a microsomal pellet which was washed by resuspension in 0.32 M sucrose solution followed by recentrifugation. The washed microsomal pellet was resuspended in 0.32 M sucrose solution to a protein concentration of 3-5 mg/100 μl. The procedure was, in general, similar to that described by Yatsu and Moss (16).

All procedures were done at 4 C and on the same day. Protein was determined by the method of Lowry et al. (17).

Incubation Techniques

The standard assay system contained, in a final vol of 1 ml, with the additions in the order given: 120 μmol phosphate buffer, pH 7.2; 8 μmol ATP; 0.4 μmol CoA; 8 μmol $MgCl_2$; 1 μmol NADH; 1 μmol NADPH and 10-20 nmol of fatty acid or malonyl CoA. Fresh microsomal preparation, 5-15 mg protein in 0.3 ml of 0.32 M sucrose solution, was added last.

The fatty acid substrates in micellar solution were prepared by dispersion in twice their molar equivalent of 0.1 N NH_4OH and dilution to the desired volume with 1% Triton WR 1339 (Ruger Chemical Co., Irvington on Hudson, NY).

Alongation reactions and incorporation of fatty acids into membranes were done in a nitrogen atmosphere. The incubation vials were capped with rubber stoppers and were flushed with nitrogen for at least 10 min. The vials were shaken in a Dubnoff metabolic shaker at 37 C for 20 min, unless specified otherwise.

Extraction and Separation of Lipids

The total lipids from the reaction mixtures were extracted with 20 vol of chloroform/methanol (2:1, v/v) and separated in the usual manner (18). The washed chloroform phase was evaporated and the resulting lipid was counted and separated into phospholipid, free fatty acid and triacylglycerol fractions by chromatography on precoated thin layer plates (E. Merck, Darmstadt, Silica Gel 60, 0.25 mm thick) using the solvent system pentane/diethyl ether/acetic acid (80:20:1). Radioactivity of separated fractions was monitored using a Packard Radiochromatogram Scanner. The separated lipids were subjected to methanolysis with methanolic HCl in the usual manner. Gas liquid chromatography of the methyl esters was done using a Packard gas chromatograph Model 7400 equipped with dual flame ionization detectors

and a 4 mm x 6 ft coiled glass column containing 10% Silar-10C on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, Inc.). A Packard Model 894 proportional counter was used to monitor ^{14}C activity. Identification of the radioactive peaks was accomplished by comparison with known fatty acid methyl esters.

RESULTS

When [1-^{14}C] arachidonic acid was incubated with the microsomal suspension in the presence of malonyl-CoA and other cofactors necessary for elongation, about 7% of the label appeared in the microsomal 22:4. However, in the presence of 2% albumin in the incubation mixture, no radioactive 22:4 could be detected (Table 1). It can also be seen in Table 1 that with increasing albumin concentration, there was a decrease in the radioactivity incorporated into phospholipid and triacylglycerol with a corresponding relative increase in the radioactivity of the free fatty acid fraction.

When the incubation was done with added unlabeled arachidonic acid in the presence of labeled malonyl-CoA without albumin, radioactivity was incorporated into the 22:4 (Table 2). However, in the presence of 1 and 2% of albumin, the incorporation of label into 22:4 fell to about half this value and was decreased further with 5% albumin. Labeled stearate, the only other major labeled product in this incubation, remained at the same level independent of the albumin concentration.

These effects are reflected in the incorporation of radioactivity into the membrane lipids (Table 3) in that there is a large decrease in incorporation of radioactivity into total lipids with 1% albumin and a further decrease with 5% albumin. These decreases are largely in the triacylglycerol fraction with minor decreases in the incorporation into phospholipid and free fatty acid at the 5% albumin level. The reason for the decrease at the 5% level, which appears to be entirely associated with 20:4 elongation, is unknown.

TABLE 1

Effect of Albumin on the Elongation of Added [1-^{14}C] Arachidonic Acid and on Fatty Acid Incorporation into Brain Microsomal Lipids[a]

% Albumin/ Lipid	0	1	2	3
	% Total activity			
Phospholipid	35.9	33.3	22.4	25.3
Free fatty acid	32.0	46.8	55.5	58.7
Triacylglycerol	32.1	19.9	22.1	16.0
20:4	92.7		100	
22:4	7.3		<0.1	

[a]The system contained, in a final vol of 1 ml:120 μmol phosphate buffer, pH 7.2, 8 μmol ATP, 0.4 μmol CoA, 8 μmol $MgCl_2$, 1 μmol NADH, 1 μmol NADPH, 20 nmol of malonyl CoA and 5 nmol [1-^{14}C] arachidonic acid (53 μCie/μmol. Fresh enzyme, 5-15 mg protein, in 0.3 ml of 0.32 M sucrose solution was added last. Incubation was for 20 min at 37 C.

The differences between these 2 experiments are that, with labeled 20:4 and unlabeled malonyl-CoA, the elongation of the added fatty acid was completely inhibited by albumin whereas with labeled malonyl-CoA and unlabeled added 20:4, only elongation of the added fatty acid was inhibited whereas elongation of an endogenous source of 20:4 continued.

As further confirmation that endogenous microsomal 20:4 is a substrate for elongation, the incubations were done without added fatty acid. It can be seen in Table 4 that in the absence of added 20:4, labeled 22:4 was produced in the same level as in the incubations with added 20:4 in the presence of albumin. No inhibition of elongation was produced under these circumstances by albumin until the 5% level. As in the previous experiment, the presence of albumin had no effect on the elongation of 16:0 to 18:0. Thus, it appears that

TABLE 2

Effect of Albumin on Microsomal Incorporation of [1,3-^{14}C] malonyl CoA into Exogenous 20:4 (→22:4) and Endogenous 20:4 and 16:0 (→18:0)[a]

% Albumin/ Fatty acid	0		1		2		5	
	%	Count	%	Count	%	Count	%	Count
18:0	25.5	7,800	42.8	8,100	53.3	11,600	65.2	8,300
22:4	74.5	22,900	57.2	10,800	46.7	10,100	34.8	4,400
Total incorporation				(60% of control)				(40% of control)

[a]Incubation conditions are exactly as described for Table 1 except that 20 nmol of inactive arachidonic acid instead of the labeled fatty acid and 20 nmol of [1,3-^{14}C] malonyl CoA (0.5 μCie) were included.

the elongation of membrane lipid 16:0 and 20:4 is unaffected by albumin, at least until the 5% level when an unexplained decrease in 22:4 formation occurs.

Again it can be seen (Table 5) that the incorporation of elongated endogenous fatty acids into the microsomal total lipids was not significantly affected by albumin until, at the 5% level, there was a decrease in incorporation of radioactivity into the triacylglycerol and, possibly, the free fatty acid fractions.

Finally, several experiments were done in which arachidonic acid was incubated with the microsomal preparation in order to obtain additional incorporation of 20:4 into the microsomal membrane phospholipids. Following incorporation, labeled malonyl CoA and additional cofactors were added and the incubations were continued with or without 2% albumin. It can be seen in Table 6, which is typical of several experiments, that although the presence of albumin did not decrease the elongation of 16:0 to 18:0, it markedly reduced the elongation of 20:4 to 22:4. The explanation of these results can be found in Table 3. Arachidonic acid is incorporated not only into phospholipid but into triacylglycerol and membrane-associated free fatty acid, as well. The free fatty acid is available in relatively high concentration for elongation but is re-

TABLE 3

Effect of Albumin on Incorporation of Elongated Endogenous Fatty Acids and Added Arachidonate into Microsomal Lipids[a]

% Albumin/	0		1		2		5	
Lipid	%	Count	%	Count	%	Count	%	Count
Phospholipid	40.8	12,500	57.7	10,900	58.7	12,700	56.2	7,100
Free fatty acid	21.6	6,700	22.7	4,300	18.9	4,100	25.6	3,200
Triacylglycerol	37.6	11,600	19.6	3,700	22.4	4,900	18.2	2,300
Total incorporation		30,800		18,900 (60% of control)		21,700		12,600 (40% of control)

[a]Incubation conditions are the same as those in Table 2.

TABLE 4

Effect of Albumin on Microsomal Incorporation of [1,3-^{14}C]Malonyl CoA into Endogenous Fatty Acids (18:0 and 22:4)[a]

% Albumin/	0		1		2		5	
Fatty acid	%	Count	%	Count	%	Count	%	Count
18:0	53.5	10,000	51.3	8,900	53.7	10,500	63.9	9,900
22:4	46.5	9,400	48.7	8,500	46.3	9,100	36.1	5,600
Total incorporation		20,200		17,400		19,600		15,500

[a]Incubation conditions are the same as those for Table 2 except that no unlabeled arachidonic acid was added.

TABLE 5

Effect of Albumin on Incorporation of Elongated Endogenous Fatty Acids into Microsomal Lipids[a]

% Albumin/	0		1		2		5	
Lipid	%	Count	%	Count	%	Count	%	Count
Phospholipid	45.6	9,200	51.1	8,900	49.4	9,700	69.1	10,700
Free fatty acid	17.7	3,600	22.4	3,900	26.0	5,100	16.1	2,500
Triacylglycerol	36.7	7,400	26.5	4,600	24.6	4,800	14.8	2,300
Total incorporation		20,200		17,400		19,600		15,500

[a]Incubation conditions are the same as those for Table 4.

TABLE 6

Effect of Albumin on Microsomal Incorporation of [2-^{14}C] Malonyl CoA into 20:4 Following Incorporation of Exogenous 20:4 into the Microsomal Membranes[a]

% Albumin/ Fatty acid	0 %	0 Count	2 %	2 Count
18:0	18.65	1,939	60.00	2,358
22:4	81.35	8,461	40.00	1,572
Total incorporation		10,400		3,830
	22:4 is 81% inhibited			

[a]Incubation conditions are exactly as described for Table 1 except that incubation is done in 2 steps. The first incubation was for incorporation of 20:4 (245 nmol) for 15 min in the presence of ATP, $MgCl_2$ and CoA. The second step was the chain elongation with [2-^{14}C] malonyl CoA with additional ATP, $MgCl_2$ and CoA and NADH and NADPH for a further 20 min.

moved by the albumin. This leaves only the arachidonate in membrane phospholipid to furnish a low steady state concentration of free 20:4 as a substrate for elongation and the results, including the lack of effect on 16:0 elongation, are essentially the same as those shown in Table 2. Washing the microsomal preparation with albumin solution removes the free fatty acid but inactivates the enzymes (see ref. 14).

DISCUSSION

The results of these experiments, taken together, strongly suggest that the normal pathway of microsomal fatty acid elongation does not involve release of the fatty acids from the membrane and their reincorporation during the reaction. If this occurred in the presence of 2% or more albumin, the fatty acid thus released would have been bound to the albumin with only a very low concentration available for further reaction and the overall elongation and incorporation of the 20:4 would have decreased. That this did not occur further demonstrates that the usual pathway of alteration of membrane fatty acids by membrane-bound enzymes does not involve such release from the membrane and equilibration with the medium.

On the other hand, the fact that, in the absence of albumin, added 20:4 can be elongated and incorporated into membrane phospholipids clearly indicates that the system is accessible to exogenous fatty acids and may be in relatively slow equilibrium with this pool.

These considerations raise the question of how a fatty acid can be released from its covalent bond in a phospholipid without rapid migration away from the immediate area. In the case of desaturation, it is possible that, in some cases, the fatty acid may remain attached to the glycerol backbone during the desaturation reaction. In elongation, however, this is not possible and 2 alternatives exist. First, if the enzymes involved in the elongation process are closely coupled with the phospholipase releasing the fatty acid, there may be little chance for the fatty acid to escape from the vicinity before further reaction. Second, it is possible that free fatty acid is never formed but that acyl groups are exchanged between phosphatidylcholine and, e.g., coenzyme A. These experiments would not distinguish between these possibilities.

A corollary to this proposal is that such a system may represent one means for transport of fatty acids through membranes. The incoming fatty acid could be activated at the membrane surface and incorporated through an acyl transferase into a membrane phospholipid. Action of a phospholipase, with or without further alteration of the fatty acid, could then result in its release on the same or other side of the membrane. A schematic representation of these processes is depicted in Figure 1.

While the indications from these experiments point strongly to a mechanism similar to the one proposed, there is no certainty that it is correct in all details. Obviously, many further studies employing systems not presently en-

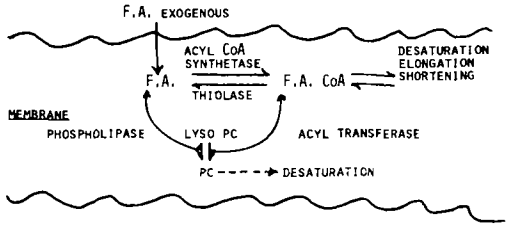

FIG. 1. Schematic representation of proposed mechanism for transfer and alteration of fatty acids in membranes.

visaged will have to take place before we can state with confidence that we know how reactions occur in the membranes.

A number of such studies are presently underway in several laboratories, in particular a consideration of phospholipid fatty acid metabolism in erythrocytes by Dise et al. (19) who have reached similar conclusions.

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Endogenous Lipolytic Activities during Autolysis of Highly Enriched Hepatic Lysosomes

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ABSTRACT

Highly enriched (50- to 70-fold) fractions of "native" lysosomes were isolated using continuous flow electrophoresis from livers of rats which had not been pretreated with Triton WR-1339. Incubation of lysosomes for 30 min at pH 5.0 in the presence of 5 mM EDTA resulted in a dramatic loss in the content of fatty acids bound to triacylglycerols (137 down to 10 μmol/mg protein) and to phospholipids and an elevation in the level of unesterified fatty acid. Phosphatidylcholine, phosphatidylethanolamine and sphingomyelin concentrations decreased whereas those of lysophosphatidylethanolamine (0.8 up to 8.5% of total lipid-P) and lysophosphatidylcholine (1.9 up to 16.7%) rose in a manner parallel to their respective, fully acylated lipids. Other phospholipids, including phosphatidylinositol, did not change in concentration during incubation. These results indicate that lysosomal phospholipase A, sphingomyelin and triacylglycerol lipase are activated by incubation at acid pH, enabling them to hydrolyze endogenous lysosomal lipids. However, lysosomal phosphatidylinositol-directed phospholipase C is apparently unable to interact with phosphatidylinositol of the lysosomal membrane.

INTRODUCTION

Hepatic lysosomes contain a variety of lipolytic enzymes which exhibit optimal activity in the acidic (pH 4 to 5) range. These include phospholipases A_1 and A_2 (1,2), sphingomyelin (3), phosphatidic acid phosphatase (4), and a triacylglycerol lipase (5,6). Recently Irvine et al. (7) showed that the soluble content of rat hepatic Triton WR-1339-filled lysosomes contained a phospholipase C which, under acidic conditions, degraded phosphatidylinositol by cleaving its inositol monophosphate moiety. The lysosomal phospholipase C showed a pH optimum of 4.8, was strongly inhibited by divalent cations, and was fairly specific for phosphatidylinositol. Matsuzawa and Hostetler (8) subsequently reported a similar phospholipase C activity in hepatic Triton WR-1339-filled lysosomes from the rat. They, however, found the enzyme to exhibit substantial activity against all phospholipids tested, including phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, although the activity against phosphatidylinositol was 3-fold greater than any other phospholipid.

Dawson and Irvine (9) have suggested that the lysosomal phospholipase C may be involved in the increased turnover of membrane-bound phosphatidylinositol in many tissues brought about by physiological stimulators. However, since the cytosolic phosphatidylinositol-specific phospholipase C has been reported to be inactive against membrane-bound substrates (10,11), a pertinent question to answer is whether the lysosomal enzyme is active against membrane-bound phosphatidylinositol, and in particular against phosphatidylinositol of the lysosomal membrane. In this regard, Richards et al. (12) have recently demonstrated that extracts of lysosomes are able to degrade the phosphatidylinositol of microsomal membranes via the action of a phospholipase C. We have, on the other hand, found that during autolysis of rat liver Triton WR-1339-filled lysosomes at pH 5.0, phosphatidylinositol is not degraded (13). This would suggest that the lysosomal phosphatidylinositol-specific phospholipase C is unable to degrade phosphatidylinositol of the lysosomal membrane. Dawson and Irvine (9) have suggested that the apparent lack of phospholipase C activity in our autolytic experiment was due to the presence of Triton WR-1339 which they claimed to be a "fairly specific" inhibitor of the lysosomal phosphatidylinositol-specific phospholipase C. In the present study, we have investigated the lipid changes associated with the lysosomal autolysis using "native" lysosomes prepared by continuous flow electrophoresis from the liver of rats which had not been pretreated with Triton WR-1339. Potential effects of Triton WR-1339 on lysosomal lipases were thus circumvented.

EXPERIMENTAL PROCEDURES

Preparation of Lysosomes

Adult male Sprague Dawley rats were maintained on Purina Laboratory chow. The rats were sacrificed by decapitation and the

livers were perfused with cold buffer and excised. Three or 4 livers generally were used for each preparation. The tissue was homogenized in 0.25 M sucrose, 0.003 M $MgCl_2$, 0.02 M Tris-HCl buffer (pH 7.2) using 10 ml/g tissue in a Sorvall omnimixer (2 x 2 sec at setting 8). Centrifugation was performed at 1,000 x g for 10 min and the resulting supernatant was spun at 20,000 x g for 20 min. The pellets were resuspended in 0.3 M KCl, 0.01 M MOPS (buffer pH 7.4) and spun again at 20,000 x g for 20 min and then similarly "washed" with 0.25 M sucrose, 0.01 M MOPS buffer (pH 7.4). The washed pellets were then resuspended in 10 to 13 ml of electrophoresis chamber buffer: 0.25 M sucrose, 8 mM Tris-acetic acid (pH 8.0), 1 mM EDTA, and spun at 1,000 x g for 10 min to remove aggregated particles. The supernatant was then applied to a continuous flow electrophoresis unit (Desaga 48 Heidelberg, West Germany) and a lysosomal isolation similar to that described by Stahn et al. (14) was done. The unit was run at 600 V and 60 mA, the sample pump was set at 5 ml/hr and the collecting pump was adjusted to a setting of 2.0. After the electrophoresis, fractions were analyzed for β-N-acetyl-glucosaminidase (15) and for protein (16) in order to determine lysosomal enrichments.

Assay of Endogenous Lipolytic Activity

Enriched (50- to 70-fold) fractions of lysosomes were pelleted and resuspended in 2.5 ml of 0.25 M sucrose, 0.04 M Tris-acetic acid (pH 5.0) and 5 mM EDTA. One-half of the available lysosomal material (after sampling for protein determinations) was incubated for 30 min in a Dubnoff shaking water bath at 37 C. The remainder of the lysosomes was kept on ice. The lipids were extracted by the method of Folch et al. (17) with minor modifications which included the re-extraction of the first upper phase with pure solvent lower phase as previously described (18).

Under these conditions, the recoveries of lysophosphatidylcholine and lysophosphatidylethanolamine were tested and found to be 96.0 ± 1.2 (SE)% and 85.0 ± 1.3 (SE)%. The lipid extracts were taken to near dryness under nitrogen and redissolved in 0.3 ml of chloroform/methanol (2:1, v/v).

Lipid extracts were analyzed for total lipid-P (19) and individual phospholipids were determined by 2-dimensional thin layer chromatography (20) followed by phosphorus analysis. Mean recovery of phosphorus content of the spots corrected for equivalent blank areas was 97.3 ± 2.0 (SE)%.

Neutral lipids were separated in a one-dimensional thin layer system (18) and the transesterification of phospholipids, triacylglycerols and free fatty acids was performed according to Morrison and Smith (21). Quantitative gas liquid chromatography was then performed on a Hewlett Packard 5830A gas chromatograph equipped with a hydrogen flame ionization detector using heneicosanoic acid as an internal standard. The columns were packed with 10% Silar 10C on 120 mesh Gas Chrom P; the temperature was programmed from 170 to 210 C.

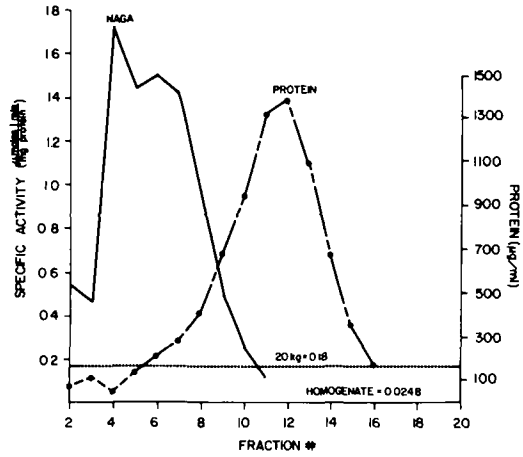

FIG. 1. The isolation of rat hepatic lysosomes by free flow electrophoresis. The distribution of activity of the lysosomal marker N-acetyl β-glucosaminidase (NAGA) and of protein in fractions separated by free flow electrophoresis. Specific activities of NAGA in the homogenate and pre-electrophoresis 5-20,000 x g (20k x g) differential centrifugation fractions are indicated for comparisons.

RESULTS

Figure 1 depicts a typical isolation of rat hepatic lysosomes after applying a 20,000 x g fraction to the continuous flow electrophoresis unit. The lysosomes (monitored using the enzyme marker β-N-acetylglucosaminidase [NAGA]) were enriched up to 70-fold in fractions nearer to the anode than in the other fractions containing the bulk of the protein (and principally containing mitochondria). The lysosomes were judged to be structurally intact as detergent was required for the full expression of NAGA activities. Highly enriched fractions of lysosomes (e.g., fractions 4-7 of Fig. 1) were pelleted and resuspended in pH 5.0 buffer for incubation as described in the methods section.

Table 1 shows alterations in the content of the major lipid classes of lysosomes following a 30-min incubation at pH 5.0 in the presence of

TABLE 1

Total Fatty Acid Content of the Major Lipid Classes of Isolated Rat Hepatic Lysosomes before and after Incubation at pH 5.0

Lipid class	Total content of fatty acid (nmol/mg protein)	
	Control (T=0)	Incubated (T=30)
Phospholipid-bound fatty acid	1111 ± 63.0[a]	844.7 ± 46.9[b]
Triacylglycerol-bound fatty acid	137 ± 16.7	10.0 ± 1.89[c]
Unesterified fatty acid	44.7 ± 8.0	384 ± 83[c]

[a]Mean ± SD of 3 samples.
[b]$p<0.01$.
[c]$p>0.001$.

5 mM EDTA. The triacylglycerols were almost totally degraded, reflecting the highly active neutral lipid lipase of the lysosomes. A substantial reduction in the content of phospholipid-bound fatty acids was also seen. The content of unesterified fatty acid rose in a parallel manner to the loss of phospholipid and triacylglycerol-bound fatty acids. There was no evidence that any particular fatty acid was being hydrolyzed preferentially as the relative fatty acid patterns of the phospholipids and triacylglycerols were not changed significantly.

The percentage composition of the lysosomal phospholipids before and after incubation at pH 5.0 is presented in Table 2. Phosphatidylcholine and phosphatidylethanolamine levels were reduced significantly whereas those of lysophosphatidylcholine and lysophosphatidylethanolamine rose in parallel. Sphingomyelin content decreased significantly upon incubation, whereas phosphatidylinositol, phosphatidylserine, cardiolipin and bis-monoacylglyceryl phosphate levels were unchanged. The total lipid phosphorus content of the lysosomes was 0.731 ± 0.132 μmol/mg protein and was not altered significantly upon incubation.

The presence of a low level of cardiolipin (2.4% of total phospholipid-P) verified that the lysosomes were being enriched by free-flow electrophoresis, but indicated that some mitochondrial inner membranes were still present in the preparation.

DISCUSSION

In this investigation, 50- to 70-fold enriched fractions of native lysosomes were incubated under conditions (pH 5.0, 5 mM EDTA) in which their lipases are known to be active against radiolabeled pure lipids. Mitochondrial

TABLE 2

Percentage Phospholipid Composition of Isolated Rat Liver Lysosomes before and after Incubation at pH 5.0 and 37 C

Phospholipid	Percent of total phospholipid-P	
	Control (0 min)	Incubated (30 min)
Phosphatidylcholine	53.1 ± 3.8[a]	39.2 ± 11.4[b]
Phosphatidylethanolamine	22.8 ± 1.8	16.7 ± 4.6[b]
Phosphatidylserine	3.2 ± 0.8	3.1 ± 0.4[c]
Phosphatidylinositol	8.70 ± 1.6	9.1 ± 2.0[c]
Sphingomyelin	4.47 ± 0.4	1.2 ± 0.4[d]
Lysophosphatidylcholine	1.9 ± 1.4	16.7 ± 8.8[d]
Lysophosphatidylethanolamine	0.8 ± 1.0	8.5 ± 2.6[d]
Cardiolipin	2.4 ± 0.8	1.8 ± 2.0[d]
Bis-monoacylglycerylphosphate	2.1 ± 0.5	1.89 ± 1.6[c]

[a]Mean ± SD for 4 samples.
[b]$p<0.01$.
[c]NS = not significantly different.
[d]$p<0.001$.

and microsomal phospholipases are active in the neutral-to-alkaline pH range and require calcium (22) and thus could not have contributed to the measured lipolytic activity. The decrease in content of triacylglycerols reflected the activity of the lysosomal neutral lipid lipase and demonstrated the accessibility of lysosomal triacylglycerol to this enzyme. Similarly, the fall in phosphatidylcholine and phosphatidylethanolamine content demonstrated the accessibility of these lipids to the lysosomal phospholipases A_1 and A_2. The parallel production of lysophospholipids verified that the phospholipases A_1 and A_2 and not phospholipase C were degrading phosphatidylcholine and phosphatidylethanolamine, and that lysophospholipase activity was not a factor. The accessibility of sphingomyelin to lysosomal sphingomyelinase was demonstrated by the degradation of 81% of the sphingomyelin during incubation. On the other hand, the lack of hydrolysis of phosphatidylinositol during incubation suggested that phosphatidylinositol was inaccessible to the lysosomal phospholipase C. Since lysosomal integrity is lost during similar incubations at pH 5.0 (10), it seems unlikely that membrane sidedness could be a complete explanation for the lack of phosphatidylinositol degradation. Conceivably, detergent-like substances such as free fatty acids or lysophospholipids might be thought to inhibit phosphatidylinositol-specific phospholipase C. In this regard, Richards et al. (12) have found that oleic acid, lysophosphatidylinositol and palmitoyl-CoA at selected levels from 1 to 10 μmol/μmol of phospholipid did not effect phosphatidylinositol hydrolysis. In any event, it appears unlikely that a lysosomal phosphatidylinositol-specific phospholipase C plays a major role in degrading the lysosomal membrane under acidic conditions. Other acid-active lipases, including phospholipases A_1 and A_2, sphingomyelinase and triacylglycerol lipase, do contribute to this lytic process in vitro and possibly under pathological conditions of acidosis.

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Identification and Quantification of Prostaglandin E_3 in Renal Medullary Tissue of Three Strains of Rats Fed Fish Oil

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ABSTRACT

Three strains of rats were fed a fish oil diet to verify their ability to incorporate and convert dietary eicosapentaenoic acid (20:5ω3) into trienoic prostaglandins. Our results show that such conversion indeed occurs in kidney medullae homogenates. Specifically, the presence of prostaglandin E_3 (PGE_3) was established by gas chromatographic-mass spectrometric (GC-MS) analysis. That compound was conclusively identified by comparison of fragment ions and their relative intensities with those obtained from authentic PGE_3. Further evidence was provided by studying the recovery of exogenously added PGE_3. The crude ethyl acetate extracts of the medullary homogenates were methylated and cleaned up by liquid-gel chromatography with Lipidex-5000 prior to conversion to PGB_3 for GC-MS analysis. The PGE_3 was quantified by selected ion monitoring (SIM) with [3,3,4,4-$^2H_4$]PGE_2 as internal standard. The levels of PGE_3 were similar, about 3 ng/mg of wet tissue, in the 3 strains of rats. Identical in vivo conversion of the 20:5ω3 fatty acid to PGE_3 could not be positively established by analysis of pooled urine specimens.

The current interest in the role of trienoic prostaglandins in hemostasis and thrombogenesis has been stimulated by results of recent studies by Dyerberg et al. (1-3). They observed that Greenland Eskimos, who have high levels of eicosapentaenoic acid (EPA, 20:5ω3) and relatively low levels of arachidonic acid (AA, 20:4ω6) in plasma lipids, also have a low incidence of myocardial infarction compared to the populations in the western world. AA is the precursor of dienoic prostaglandins (PG_2), and EPA is the precursor of trienoic prostaglandins (PG_3). Vane and coworkers (4) and Needleman and coworkers (5,6) have shown that EPA, unlike AA, inhibits platelet aggregation in human platelet-rich plasma. That fact can explain a bleeding tendency exhibited by the Eskimos, whose diet is rich in EPA, which replaces ω6 fatty acids in tissue lipids. Thus, low thrombotic tendency and low incidence of myocardial infarction appear to be associated with a high EPA/AA ratio in plasma and platelet lipids (1-3). Several mechanisms for this action have been proposed.

According to one hypothesis, EPA exerts its effect on platelets by acting as a competitive antagonist for AA (5), thus inhibiting the formation of proaggregatory (AA) metabolites such as thromboxane A_2 (TXA_2). On the other hand, the endoperoxide PGH_3 can be used in vitro by the blood vessel wall to make a substance, probably Δ^{17}-prostacyclin (PGI_3), having potent antiaggregatory activity similar to that of prostacyclin (PGI_2) (5). Conversely, the other (conceivably proaggregatory) EPA metabolite, thromboxane A_3 (TXA_3), is physiologically inactive (5,7). Thus, a diet-induced shift of the TXA/PGI balance toward a less thrombogenic state could explain the low thrombotic tendency in Greenland Eskimos.

Resolution of the question of whether dietary EPA is converted to the endoperoxides PGG_3-PGH_3 and then to trienoic PG in mammalian systems is essential if we are to explain the observed effects of dietary EPA. Previous investigations have shown that trienoic prostaglandins are formed in vitro in certain systems such as seminal vesicles (8) and platelets (9,10) via use of exogenous EPA. To our knowledge, however, the conversion of endogenous EPA to trienoic PG has not been reported. We now present evidence that EPA of dietary origin is deacylated from the renal phospholipid pool during incubation and converted to PGE_3. The experiment was conducted with 3 strains of rats, the Kyoto-Wistar (WKY), the spontaneously hypertensive (SHR), and a recently introduced (11) substrain of the latter (SHR/SP). During experiments designed to test the in vivo conversion of EPA, however, we could not positively identify PGE_3 in the urine of rats fed EPA. The measured levels of PGE_3 in the incubated tissues were similar in all 3 strains.

EXPERIMENTAL

Materials

PGE_2, PGE_3 and [3,3,4,4-$^2H_4$]PGE_2 were

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Nonstandard abbreviations: SIM = selected ion monitoring; TSI = trimethylsilylimidazole.

provided by J. Pike and U. Axen of the Upjohn Co. Piperidine was Fisher-certified (Fisher Scientific Co., Fair Lawn, NJ) and freshly distilled. Diazomethane was prepared from N-methyl-N-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Co., Milwaukee, WI) and codistilled with diethyl ether. Trimethylsilylimidazole (TSI) was purchased from Pierce Chemical Co., Rockford, IL, and Lipidex-5000 from Packard Instruments Co., Downers Grove, IL. All solvents were analytical grade and were redistilled before use.

Rat Feeding Protocol

WKY, SHR and SHR/SP weanling rats, 10 in each group, were fed a balanced diet containing 1% corn oil plus 4% menhaden oil as previously described in detail (12,13). Rats fed 5% corn oil served as control. After 22 weeks, the animals were terminated, their kidneys removed, and the medullae excised, homogenized, and incubated in 0.01 M phosphate buffer (pH 7.2) for 10 min at 37 C. Three hundred ng of [3,3,4,4-$^2H_4$]PGE_2 internal standard was added and the mixtures were immediately frozen in Dry Ice and stored at -20 C until used.

Analysis of Kidney Medullae

The homogenates were allowed to thaw, then were acidified (pH 3.5) with citric acid and extracted 3 times with a total of 12 ml AcOEt. The combined organic phases were evaporated to dryness under N_2 and the residues were methylated with diazomethane. After solvent evaporation, the residues were dissolved in heptane/chloroform (7:3) and subjected to straight phase liquid-gel chromatography with 10 x 200 mm (id) Lipidex-5000 columns with the same solvent system at 25-26 C and at a flow rate of 15 ml/hr. The first 60 ml of the eluates was discarded. The ensuing 30-ml fractions, which contained PGE_2 and PGE_3, were evaporated, and the residues were treated with 20 μl of TSI/piperidine (1:1) and analyzed by GC-MS. That reagent converts the PGE to their PGB counterparts (14). Therefore, the ions mentioned throughout the paper originated from PGB species. The detailed structure of those ions is unknown at present.

Instrumental Conditions

A Finnigan 3200 GC-MS system was used in the selected ion monitoring (SIM) mode. The glass columns, 1.50 m long x 2 mm id were treated with dimethylchlorosilane and packed with 2% SP-2330 or 2% OV-225 on 100-120 mesh Gas Chrom Q. Temperatures were: injector, 235 C; SP-2330 column, 225 C, and OV-225 column, 215 C; separator, 230 C. The carrier gas (He) was supplied at a head pressure of 1.26 kg cm^{-2}, and the flow rate was about 20 ml min^{-1}. An effluent diverter was opened manually 60 sec after each injection. The mass spectrometer was operated at 70 and 40 eV, and the electron multiplier was set at 1.7 kV. We used a PROMIM (Finnigan) modular electronic programmer for SIM, and the signals were fed to a Rikadenki 4-channel pen recorder. Peak heights were used for quantitative measurements. After elution of the PG, the column was heated rapidly to 270 C and was maintained at 270 C for 15 min before the next injection. The ions selected to monitor PGE_3 for identification purposes were: m/z 350, m/z 349, m/z 259, m/z 233, m/z 227, m/z 199 and m/z 185. For quantification of PGE_3, the ions selected were: m/z 325 and m/z 353 to monitor [3,3,4,4-$^2H_4$]PGE_2 internal standard, and m/z 349 to monitor PGE_3. The amounts of PGE_3 in the medullary homogenates were determined from standard curves.

RESULTS

Mass Spectra

Figure 1 shows the quadrupole mass spectra of derivatized PGB_2 and PGB_3 obtained from their PGE counterparts as described in Experimental. The mass spectrum of derivatized [3,3,4,4-$^2H_4$]PGB_2 has been published (15).

Identification of PGE_3 in Kidney Medullae

Under the experimental conditions just described, the chromatographic behavior of the putative PGE_3 from kidney medullae coincided with the behavior of authentic PGE_3 in the SP-2330 and the OV-225 GC columns, as well as in the Lipidex-5000 column.

Figure 2 shows a comparison of selected ion current profiles of m/z 321 (PGE_2), m/z 325 (tetradeutero-PGE_2 internal standard), and m/z 349 (PGE_3) obtained from a purified extract of renal medullae of control rats fed 5% corn oil (12) and of rats fed fish oil as the source of fat. The presence of PGE_3 in the latter sample is indicated by the additional peak at longer retention time in the m/z 349 profile. Figure 3 shows a typical ion chromatogram (12-min recording) of a derivatized kidney medullary PGE fraction from a Lipidex-5000 column. The major peak at m/z 353 is associated with the internal standard. Here, resolution of PGE_2 and PGE_3 was improved.

The PGE_3 in kidney medullae was conclu-

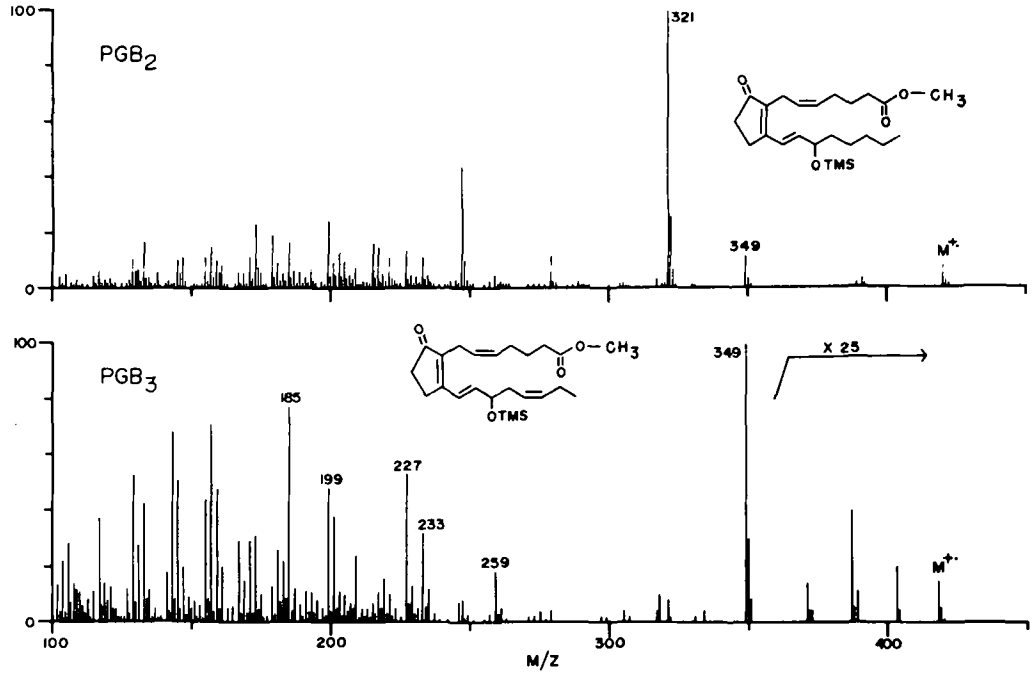

FIG. 1. Quadruple mass spectra (70 eV) of PGB_2-methyl ester-TMS ether (upper spectrum), and PGB_3-methyl ester-TMS ether (lower spectrum).

sively identified by selected ion monitoring, i.e., fragment ions and their relative intensities in a biological sample were compared to those obtained from authentic PGE_3. Tables 1 and 2 display the results of such comparisons at 70 and 40 eV, respectively.

Four medullary homogenates were pooled after thawing, and spiked with 2000 ng of [3,3,4,4-$^2H_4$]PGE_2 internal standard. The mixture was then acidified, brought up to 14 ml by adding water, and carefully divided into four 3.5-ml portions. Increasing amounts of PGE_3 were added to 3 of the 4 aliquots, and all 4 were analyzed as already described. Figure 4 is the plot of ng PGE_3 found vs ng PGE_3 added. The slope of the curve and the excellent correlation coefficient provide further confirmatory evidence of the identity of the endogenous PGE_3.

Quantification of PGE_3

Quantification was achieved by SIM with [3,3,4,4-$^2H_4$]PGE_2 as internal standard. Technical details and validation of the method will be presented elsewhere. The levels of PGE_3, expressed as ng/mg of wet tissue ($\bar{x} \pm$ SEM, n = number of observations), were: WKY, 3.15 ± 0.58, n = 6; SHR, 2.61 ± 0.16, n = 5; SHR/SP, 3.51 ± 0.18, n = 5.

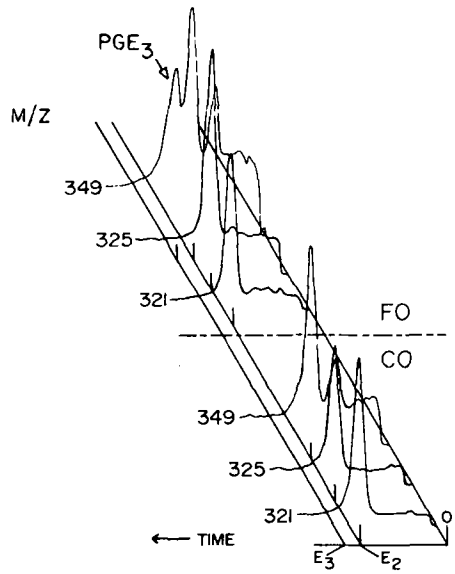

FIG. 2. Selected ion current profiles of purified and derivatized renal medullary extracts of control rats fed corn oil (CO) (lower trace) and of rats fed fish oil (FO) as the source of fat. One-tenth of the derivatized PG was injected. GC column was 2% SP-2330.

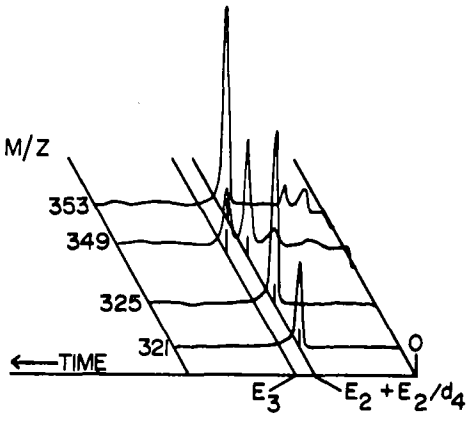

FIG. 3. Typical time-extended ion current profiles (plotted at different sensitivities) of derivatized renal medullary PGE fraction (fish oil diet) from a Lipidex-5000 column. The major peak at m/z 325 represents the internal standard. GC column was 2% SP-2330.

DISCUSSION

Recent work from our laboratory (12) confirmed the well established facts that the fatty acid composition of tissues reflects the composition of the diet and that the balance between ω6 and ω3 fatty acids in the diet profoundly influences the amounts and type of prostaglandins synthesized. Specifically, we showed that the ω3 fatty acids largely replace the ω6 fatty acids in animals fed fish oil, and that the dietary eicosapentaenoic acid reduces the maximal biosynthetic potential of dienoic prostaglandins in vitro (12). Here, we have shown for the first time that the rat is able to release EPA from kidney medullary lipids and to convert it to PGE_3 in vitro. Comparison of the present data with published data (12) on PGE_2 indicates that, in vitro, the ratio PGE_2/PGE_3 in kidney medullae is 2.7 for the WKY and SHR/SP, and 3.7 for the SHR.

We sought evidence for in vivo production of trienoic PG via SIM analysis of pooled urine samples from rats fed the fish oil diet. Urine pools were analyzed according to a modification of a published procedure (16). While the presence of PGE_2 was clearly established, the chromatographic and mass spectral evidence for the presence of PGE_3 was considered too weak for positive identification. Lands and coworkers have shown that EPA, compared to AA, is a poor substrate for the fatty acid cyclooxygenase (17) and suggested that the synthesis of PG_3 occurs only in vitro under the catalyzing effect of high peroxide concentrations normally not present in vivo (18). That suggestion, however, is contradicted by the fact that PGE_3

TABLE 1

Ion Intensity Ratios of Synthetic and Biological (Natural) PGE_3 at 70 eV

m/z	Synthetic PGE_3[a]	Natural PGE_3
350	.290	.302
349	1.000	1.000
259	.129 ± .005 (4)	.122
233	.320 ± .005 (6)	.312
227	.574 ± .011 (6)	.542
199	.580 ± .027 (4)	.535
185	1.053 ± .034 (6)	1.146

[a]Number of observations in parentheses.

TABLE 2

Ion Intensity Ratios of Synthetic and Biological (Natural) PGE_3 at 40 eV

m/z	Synthetic PGE_3[a]	Natural PGE_3
350	.292 ± .008 (5)	.269 ± .011 (6)
349	1.000	1.000
259	.358 ± .013 (5)	.316 ± .023 (6)
233	.539 ± .025 (5)	.697 ± .103 (2)
227	.958 ± .025 (5)	.995 ± .025 (5)
199	.865 ± .023 (5)	.841 ± .064 (5)

[a]Number of observations in parentheses.

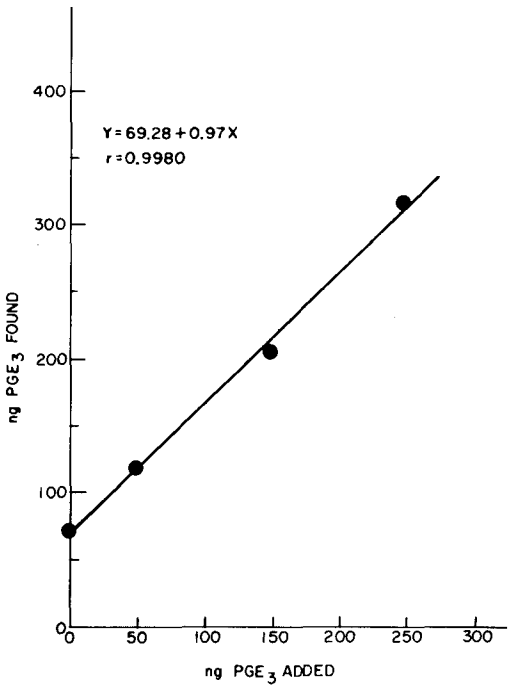

FIG. 4. Recovery of exogenously added PGE_3 to pooled kidney medullae.

is present in substantial amounts in human seminal fluid (19,20).

ACKNOWLEDGMENTS

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Phase Behavior of Triolein and Tripalmitin Detected by Differential Scanning Calorimetry

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ABSTRACT

The thermotropic behavior of triolein, tripalmitin and their mixtues was determined by differential scanning calorimetry. Polymorphic behavior was noted for the triglycerides but the triglycerides were converted to a single form after 4 to 5 successive heating scans. Melting points for each triglyceride were determined for the pure samples and mixtures, and plotted as a phase diagram. The phase diagrams indicate that a phase separation of triglycerides occurred at all concentrations of triolein and tripalmitin. However, the melting peak onset temperature of tripalmitin was shifted by triolein from 56 C at 100 mol % tripalmitin to 37 C at 15 mol % tripalmitin. Similarly, the peak onset temperature of triolein was shifted by tripalmitin from -2.5 C at 100% triolein to -4 C at 95% triolein. Enthalpies were also determined for pure samples and mixtures. These data indicated that when either triolein or tripalmitin were present as the minor component of the mixture, the enthalpy of the minor component was reduced whereas that of the major component was not greatly altered.

INTRODUCTION

Differential scanning calorimetry (DSC) is a technique which offers the advantages of measuring heats of melting and fusion as well as melting and freezing points, while requiring only mg quantities of material. The thermal behavior of VLDL has recently been determined by differential scanning calorimetry in human plasma VLDL (1) and VLDL isolated from the perfused rat liver (2). These particles are rich in triglycerides, containing 70 to 80% of their mass as triglyceride. It is believed that the structure of the VLDL triglyceride core and its chemical composition are important in determining the surface characteristics of the particle. However, the complex thermotropic transitions that were noted did not correlate well with the fluorimetrically determined transitions (2-4). The triglycerides in these investigations were highly enriched in oleic acid or palmitic acid. Although phase diagrams of other triglyceride mixtures have been reported (5-7), some of these diagrams were incomplete and contain only melting point data (5). Therefore, we have undertaken an investigation of a phase diagram of triolein, tripalmitin and several mixtures of the two. In this study, we determined the following: (1) the polymorphic behavior of triglycerides, (2) differences in melting and freezing points of pure triglycerides and their mixtures, and (3) differences in ΔH_{cal} for pure triglycerides and their mixtures. These data may help to explain the effect of different triglyceride species on each others' thermal behavior in complex mixtures as may occur in the secreted VLDL.

Abbreviations used are as follows: VLDL, very low density lipoprotein; DSC, differential scanning calorimetry.

MATERIALS AND METHODS

Differential Scanning Calorimetry

Differential scanning calorimetry was performed with a DSC-2 (Perkin-Elmer Corp., Norwalk, CT). The triglycerides (0.5-2.0 mg) were solubilized in 50 µl of chloroform and transferred to 10 µl aluminum sample pans, and warmed to about 50 C to promote solvent evaporation. The pans containing the samples were then lyophilized for 12 hr to remove any residual solvent. The pans are sealed at 24 C, cooled to -50 C at a rate of 1.25 C/min, and equilibrated for 5 min at the lower temperature limit before a scan was initiated. The samples were then reheated to 65 C at a rate of 1.25 C/min. The samples were then cooled to -50 C at 10 C/min. This cycle was repeated 4 times. The recorded traces in the figures were taken during the fourth cycle on heating unless otherwise specified. This ensured that the thermal history of the samples was the same. Sensitivities were 1.0 to 5.0 mcal/sec for large amounts of triglycerides and 0.2 for triglyceride mixtures in which the minor fraction was below 10 mol %. The areas under the peaks of the phase transition were found by weighing the paper; the weights were compared to the weight of a standard area of known enthalpy (Indium) and sample enthalpies were calculated (8,9). ΔH_{cal} is the calorimetrically measured enthalpy change (10,11). Triolein and tripalmitin were obtained from Supelco Inc., Bellefonte, PA.

RESULTS

Polymorphic Behavior of the Pure Triglycerides

Pure triolein or tripalmitin possesses multiple transitions upon first heating (Fig. 1, a and c). These are the β' and β forms typical of the polymorphism displayed by triglycerides (12, 13). α Forms were not detected since they were located at the lower temperature limit of our instrument and since samples were not specially treated to allow detection of α forms (14). As shown in Figure 1, b and d, the β' forms can be converted into β forms after multiple heating and cooling regimens (13). It should also be noted that, when multiple polymorphic forms are present, exotherms separate melting peaks of different forms. Cooling exotherms for each of the samples in Figure 1 were also determined (Fig. 2). A single transition was noted in each case at a temperature near that of the most stable β form. Multiple transitions were not noted even during the first cooling regimen (Fig. 2, a and c). Thus, the triolein and tripalmitin multiple transitions could be converted into a single, reproducible form that appeared on both heating and cooling scans. The reproducibility of the melting temperatures after the fourth run was ± 1 C.

Mixtures of Triolein and Tripalmitin

The effect of mixing tripalmitin and triolein on the onset temperatures of the transition of the respective glycerides is shown in Figure 3. The mixture did not show a single peak on either heating or cooling regimens, indicative of mixing of the 2 components. Instead, 2 transitions similar to those noted for triolein and tripalmitin, respectively, were noted on endothermic scans (fourth heating and cooling regimens). Multiple peaks due to polymorphic forms were not apparent, although they occurred during the first heating regimen. All peaks observed were reversible, as cooling peaks were observed for all samples. The onset temperature of the endothermic transition of the tripalmitin was always higher than triolein. However, increasing molar content of triolein lowered the onset temperature of the tripalmitin from 56 to 40 C on heating scans. Conversely, tripalmitin also affected the onset temperatures of the triolein transition, increasing it from -15 to -3 C on heating scans. Thus, the effect of triolein on the phase transition onset temperature of tripalmitin was greater than the

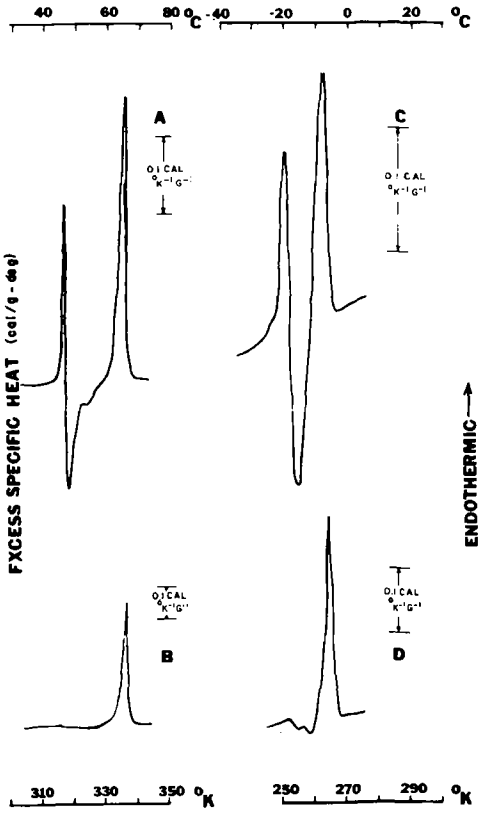

FIG. 1. Conversion of polymorphic triolein and tripalmitin forms into single phases. Triolein (0.66 mg) and tripalmitin (0.80 mg) were heated from 290 to 345 C as described in Methods. (a) Tripalmitin, first heating scan, sensitivity 2 mcal/sec; (b) tripalmitin, fourth heating scan, sensitivity 5 mcal/sec; (c) triolein, first heating scan, sensitivity 1 mcal/sec; and (d) triolein, fourth heating scan, sensitivity 2 mcal/sec.

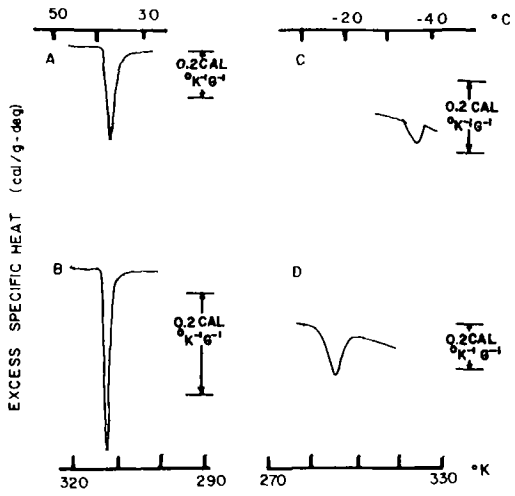

FIG. 2. Cooling scans of triolein and tripalmitin. All conditions were as described in the legend to Fig. 1.

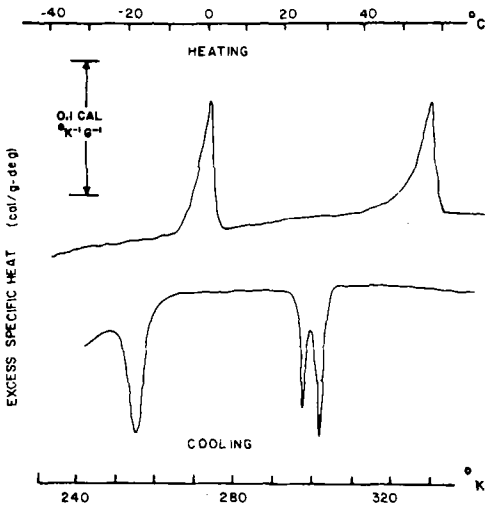

FIG. 3. DSC scans of a tripalmitin/triolein mixture. Heating and cooling scans of a 0.62 mg 60 mol % triolein/40 mol % tripalmitin mixture were determined after 4 heating and cooling cycles. The sensitivity was 1 mcal/sec on heating and cooling from 290 to 345 C.

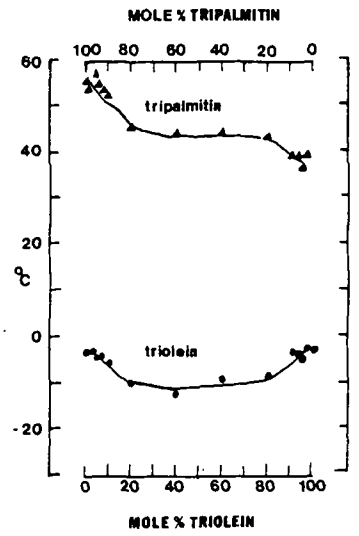

FIG. 4. Melting temperatures of triolein-tripalmitin mixtures. All methods were as described in legend to Fig. 1 except that data were taken only from the final scan (i.e., polymorphic forms were removed).

effect of tripalmitin on the onset temperature of triolein. On heating scans, the onset temperature of the phase transition of the glyceride species was relatively constant only when it constituted between 20 and 80 mol % (Fig. 4). At less than 20 mol %, the onset temperature of the glyceride was shifted toward the onset temperature of the major glyceride species. Conversely, above 80 mol %, the onset temperature of the major glyceride increased to that of the pure melting species. The cooling peak for pure triolein was at -14.0 C whereas, for equimolar mixtures with tripalmitin, it occurred at -11 C. Pure tripalmitin had a cooling peak at 37 C which occurred at a lower temperature in equimolar mixtures with triolein. It is notable that tripalmitin cooling peaks were doublets when tripalmitin was present between 20 and 80 mol % of the mixture, but pure as well as high or low mol % tripalmitin samples had a single cooling peak.

The effect of triolein on enthalpy changes of tripalmitin are shown in Figure 5a. Below 40 mol % tripalmitin, the enthalpy change of the tripalmitin peak decreased from near 35 cal/g to less than 10 cal/g. The enthalpy change of triolein (Fig. 5b) was lower than that of tripalmitin and was also decreased drastically below 50 mol % triolein (from 22 to less than 9 cal/g). Thus, when either glyceride represented about 60 mol % or greater of the mixture, its enthalpy change was not greatly altered, indicating that "impurity" of the other glyceride up to 40 mol % did not greatly alter the enthalpy change of the major species.

The thermal data for pure triolein, tripalmitin, and some mixtures thereof is presented in Table 1. At 100 mol %, the enthalpy change of 25.3 cal/g for triolein compared well with a previously reported value of 25.8 cal/g for β triolein (13). At 100 mol %, the enthalpy change of 37.9 cal/g for tripalmitin was less than the 59.0 cal/g for β tripalmitin reported elsewhere (13). However, at 91 mol %, the enthalpy change was 49.6. Thus, it seems possible that, for some of the tripalmitin

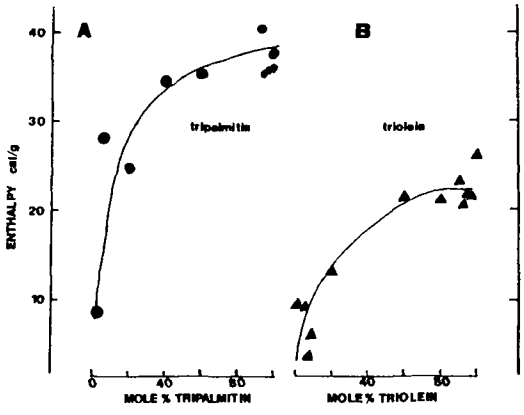

FIG. 5. Dependence of enthalpy on mol % of tripalmitin and triolein. All conditions were as described in legend to Fig. 4.

TABLE 1

Thermal Properties of Triolein-Tripalmitin Mixtures

Mol %		Temperatures (C) Peak onset		ΔH cal/g		ΔT (C)	
Triolein	Tripalmitin	Triolein	Tripalmitin	Triolein	Tripalmitin	Triolein	Tripalmitin
100	0	-2.5	–	25.3	–	8.0	–
95	4	-4	37	21.9	28.0	9.0	13.0
60	40	-8	45	21.4	33.5	11.0	15.5
5	95	-4	55	9.1	35.5	5.0	9.0
0	100	–	56	–	37.9	–	10.0

samples, not all of the sample may have been present in the β form. The effect of one triglyceride on another enthalpy change of melting is presented in Figure 5, a and b. The enthalpy change of each component increased as its mol % in the mixture increased. The width of the transition, ΔT, of triolein was decreased when this triglyceride was the minor glyceride in the mixture (5 mol %). In contrast, the width of the transition of tripalmitin was increased when it was present at 5 mol % compared to 100 mol %. The cooperativity of each of these transitions was not determined due to the complex polymorphic forms and nonideal behavior of these mixtures.

DISCUSSION

As evidenced by our data, even a single acid triglyceride will exhibit complex thermal behavior on heating. Multiple peaks, some of which disappear on successive heating and cooling regimens, do not necessarily imply that the structure of the lipid is polymorphic. In complex systems, alternate explanations such as phase separations or protein denaturation must be considered. Since no protein is present in our samples and because only a single molecular species of triglyceride is present, we interpret our results as being consistent with the presence of polymorphic forms. Such polymorphic forms have been described in detail for other single component triglycerides (13-16). Large exotherms, as noted in the present work, were also noted by others (13-16) and are also indicative of the presence of polymorphic forms (17). Their disappearance after 4 heating and cooling scans is consistent with the presence of a single triglyceride form. From Figure 1, a and c, it can be seen that more than one crystalline form of triolein and tripalmitin exist below their melting points. After a heating scan is initiated, a metastable triglyceride will convert to a stable crystalline form by a reordering of acyl chains (13-16). More unstable forms may disappear upon successive heating scans. Given the problems of multiple peaks, possible differences in sample history and reversibility, it is difficult to determine quantitative parameters on these transitions. Therefore, all samples were subjected to the same treatment regimens ensuring the same sample history. All of the polymorphic forms were converted to a single reproducible β form which was the most stable. It is known that α forms of triglyceride are not reversible and convert to β' and β forms (13-16). The β forms are the most stable and they are clearly reversible, as indicated in Figure 2. Reversibility is consistent with the interpretation that the transition noted is not metastable or due to denaturation. This procedure allowed accurate determination of enthalpies and transition temperatures.

The effect of one triglyceride component on another was determined using mixtures of triolein and tripalmitin. If a eutectic mixture is formed, then only one melting point would be expected to appear. However, the results indicated that at all mol % these triglycerides maintained at least a partial phase separation (Fig. 3). However, in mixtures of triolein and tripalmitin, the melting point of the minor triglyceride was altered in the direction of the melting point of the major triglyceride. This may be due to the partial formation of a eutectic mixture (5) or to the solubilization of the minor glyceride into the major glyceride. In contrast, the minor triglyceride had the effect of depressing the major triglyceride's melting point. Between 20 and 80 mol %, the melting points of both tripalmitin and triolein were relatively constant, but slightly below the melting points of the pure triglyceride species. This indicates that, at more equimolar ratios, there may still be small amounts of one triglyceride present in each major component. However, the data in Figure 3 clearly show phase separation between the triolein and tripalmitin transitions. Heats of melting, ΔH, were determined for each com-

ponent of the mixture by first calculating the areas of both endothermic peaks due to tripalmitin and triolein, respectively. Each area was divided by the weight of tripalmitin and triolein, respectively. This ratio was compared to that of an Indium standard of known enthalpy. Thus, the enthalpy of tripalmitin and triolein in the mixture could be estimated. Figure 5, a and b, indicated that the heat of melting of either triolein or tripalmitin was reduced by the presence of the other glyceride species when the melting glyceride represented less than 60 mol %. Thus, when these triglycerides are present as a minor component of a mixture, their enthalpies were altered. This may be caused by partial eutectic mixture formation, solubilization in the other glyceride species, or the disruption of the chain packing of the minor glyceride. Thus, although phase separation of tripalmitin and triolein clearly occurred, these phases were not pure at all molar mixtures. They appeared to approach purity when the component was present near 60-80 mol % in the mixture.

The physiological importance of these data may be relevant to the contribution of mixed triglycerides in the VLDL. It has been shown that, upon cooling freshly isolated human VLDL from 45 to 10 C, there are no thermal transitions (18). However, thermal transitions in human VLDL were noted below 0 C. Thus, the physiological significance of thermotropic transitions appears questionable. However, it should be noted that these plasma VLDL represent a heterogeneous mixture and that the triglyceride lipid composition is dependent on diet. Out laboratory recently used a perfused rat liver system to produce much more homogeneous VLDL enriched in either palmitate or oleate (2). DSC thermograms of these VLDL indicated that VLDL enriched in oleic acid had no transitions above 0 C but the transitions noted below 0 C resembled those of the human VLDL (1). In contrast, when the VLDL were enriched in palmitate, phase transitions appeared near physiological temperature at 37 C. Thus, factors that can affect the fatty acid composition of plasma VLDL (e.g., diet, drugs, endocrine status, or pathology) may also shift the location of the phase alterations.

The importance of our findings may, therefore, be extended to our previous investigations with the triglyceride-rich VLDL (2-4). The VLDL displayed multiple peaks by DSC. These transitions were due to the triglycerides and did not disappear even after 4 to 5 heating and cooling cycles. The data presented herein indicated that mixtures of triglycerides may alter the characteristic physical properties of an individual triglyceride. Some phase separation of different acid triglycerides is maintained even when a triglyceride is present at only 1 mol %. Indeed, it has been noted that saturated and unsaturated acyl chains have a tendency to partition into separate layers in mixed-acid triglycerides (12). Thus, complex thermal behavior in biological systems such as VLDL triglycerides may be caused not only by polymorphism, but also by phase separations.

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Biosynthesis of (Z,Z)-6,9-Heptacosadiene in the American Cockroach

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ABSTRACT

The biosynthesis of (Z,Z)-6,9-heptacosadiene, the major cuticular hydrocarbon component of the American cockroach, was examined by radiotracer and ^{13}C-nuclear magnetic resonance (NMR) techniques. Sodium [1-^{14}C]acetate was incorporated about equally into the saturated and diunsaturated hydrocarbons, whereas [1-^{14}C]linoleate preferentially labeled the C_{27} alkadiene and [9,10-^{3}H]oleate labeled the C_{27} alkadiene almost exclusively. ^{13}C-NMR demonstrated that [2-^{13}C]acetate labeled carbons 25 and 27 but not carbon 3 of the C_{27} alkadiene. In addition, ozonolysis of the diene labeled from [1-^{14}C]acetate followed by radio-gas liquid chromatography showed that carbons 1-6 were not labeled, whereas the fragment containing carbons 10-27 was labeled. The data presented in this paper indicate that linoleate from the diet or synthesized de novo is elongated by the addition of acetate units and is then decarboxylated.

Long chain hydrocarbons often comprise a majority of the cuticular lipids of many insect species (1,2). Functions attributed to the cuticular hydrocarbons include preventing desiccation of the insect, protecting from abrasion and environmental chemicals, and serving as pheromones, kairomones and defense chemicals (1). Because of the large amounts of hydrocarbon produced and the simplicity of the hydrocarbon pattern, the American cockroach, *Periplaneta americana*, has been the subject of a number of studies on its cuticular hydrocarbons (3-9). The major hydrocarbon components of this insect are *n*-pentacosane, 3-methylpentacosane and (Z,Z)-6,9-heptacosadiene (3-5).

n-Pentacosane arises from the decarboxylation of hexacosanoic acid (8), and 3-methylpentacosane is formed by the incorporation of a methylmalonyl-CoA in place of malonyl-CoA as the second unit during chain elongation (9). Significant amounts of methyl branched fatty acids or very long chain fatty acids are not found in the American cockroach (10), which suggests that once the branching methyl group is inserted, the growing acyl chain is not released from the enzyme system until it is decarboxylated.

The biosynthesis of (Z,Z)-6,9-heptacosadiene is of particular interest because it makes up almost 70% of the total cuticular hydrocarbons in *P. americana* (5). Also, the configuration of the double bonds in this hydrocarbon is the same as that found in linoleic acid. Previous work has implicated linoleate in the biosynthesis of (Z,Z)-6,9-heptacosadiene (1, 11). It has been suggested that linoleate could be elongated to form a C28 dienoic acid, which, in turn, could be decarboxylated to form the C27 alkadiene (11). Recently, it was demonstrated that the American cockroach can synthesize linoleic acid de novo from acetate and that oleic acid injected into the insect is readily converted to linoleate (10,12). This work examines the interrelationship among oleate, linoleate and (Z,Z)-6,9-heptacosadiene.

MATERIALS AND METHODS

Materials

Sodium [1-^{14}C]acetate (57 mCi/mmol) was obtained from Research Products International, Elk Grove, IL. Sodium [1-^{14}C]linoleate (61 mCi/mmol), and sodium [9,10-^{3}H]oleate (6 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Sodium [1-^{14}C]oleate (10 mCi/mmol) and sodium [1-^{14}C]stearate (55 mCi/mmol) were obtained from ICN, Irvine, CA. Sodium [U-^{14}C]linoleate (61 mCi/mmol) was obtained from Amersham, Arlington Heights, IL. Sodium [1-^{13}C]acetate (90 atom %) and sodium [2-^{13}C]acetate (90 atom %) were purchased from Prochem-Isotopes, Summit, NJ. All solvents were redistilled in glass prior to use.

Insects

American cockroaches were reared in 200-l galvanized containers on pulverized Purina dog chow. A 1% (w/v) agar gel was their source of water. Middle-to-late instar nymphs were used for all experiments.

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Abbreviations used: $\{^1H\}$: proton-decoupled; GLC: gas liquid chromatography; TLC: thin layer chromatography.

Radioactive Isotope Experiments

Sodium [1-^{14}C]acetate was used as an aqueous solution, and the fatty acids were used as an aqueous dispersion in a Tween 80 emulsion (0.5 mg/ml) with a radioactivity concentration of about 1 μCi/μl. Insects were injected into the abdomen, and after 6 hr, the insects were killed and the cuticular hydrocarbons extracted in hexane for 10 min. Solvent was removed under a nitrogen stream, and the hydrocarbons isolated by elution with 8 ml hexane through 6 cm x 0.5 cm columns of BioSil A (BioRad Laboratories, Richmond, CA). Saturated and unsaturated components were separated using 6 cm x 0.5 cm columns of BioSil A impregnated with 20% (w/w) silver nitrate. Saturated components were eluted with 8 ml hexane and unsaturated components with 8 ml of hexane/diethyl ether (50:50, v/v). Alternatively, alkanes and alkadienes were separated by TLC using silver nitrate (10%, w/w) impregnated TLC plates, developed in hexane. Ozonolysis of the alkadiene was performed as described by Beroza and Bierl (13).

Portions of each sample were transferred to scintillation vials and 10 ml of 0.5% diphenyloxazole in toluene was added. Samples were assayed for radioactivity by liquid scintillation counting at efficiencies of 85-90% for carbon-14 and 46-50% for tritium. Radio-GLC was performed on a Hewlett-Packard GLC interfaced with a Packard combustion flow-through proportional counter. Samples were separated on a 3% Dexsil-400 on Supelcoport column. The retention times of the ozonolysis products were compared to standards.

^{13}C-NMR Experiments

Two μl of the carbon-13-labeled acetates (0.5 g/ml) were injected daily into the abdomens of middle instar nymphs. They received no nutrition other than water in a 1% agar gel. Each experiment started with 50-60 insects. After 30 days, the insects were extracted with hexane and the C_{27} alkadiene isolated as already described. Unenriched C_{27} diene was obtained from control insects in a similar manner. Purity was confirmed by GLC. The $^{13}C\{^{1}H\}$NMR spectra were recorded in $CDCl_3$ solutions in 1-mm sample tubes at 25.00 MHz with a JEOL FX-100 Fourier transform NMR spectrometer.

RESULTS

The biosynthesis of (Z,Z)-6,9-heptacosadiene was examined by comparing the incorporation of a variety of radiolabeled precursors into the saturated and alkadiene fractions. The data (Table 1) shows that [1-^{14}C]acetate was incorporated about equally into the saturated and alkadiene fractions, whereas [1-^{14}C]- and [U-^{14}C]linoleate preferentially labeled the alkadiene, and [9,10-^{3}H]oleate was incorporated almost exclusively into the alkadiene. [1-^{14}C]Stearate preferentially labeled the saturated hydrocarbon fraction. Radio-GLC of each sample showed that the only alkadiene labeled was the C_{27} component. The majority of the radioactivity in the saturated components was distributed between *n*-pentacosane and 3-methylpentacosane. An example of the type of radio-GLC data obtained is presented in Figure 1, and shows the distribution of radioactivity into the total hydrocarbon fraction after the incorporation of [1-^{14}C]linoleate.

Since (Z,Z)-6,9-heptacosadiene comprises about 70% of the total hydrocarbon fraction (3-5) and it has been shown that linoleate is readily synthesized in this insect (12), it was expected that the distribution of radioactivity from [1-^{14}C]acetate would reflect the relative

TABLE 1

Incorporation of Radiolabeled Precursors into the Saturated and Alkadiene Components of *P. americana*

Substrate	% Incorporated into hydrocarbon	Label incorporated into hydrocarbon	
		% in alkanes	% in alkadienes
[1-^{14}C]Acetate	0.72 ± 0.20	52 ± 5	48 ± 5
[1-^{14}C]Linoleate	0.12 ± 0.02	26 ± 7	74 ± 7
[U-^{14}C]Linoleate	0.14 ± 0.05	22 ± 8	78 ± 8
[1-^{14}C]Oleate	0.51 ± 0.11	26 ± 7	66 ± 7
[9,10-^{3}H]Oleate	0.16 ± 0.07	6 ± 2	94 ± 2
[1-^{14}C]Stearate	0.03 ± 0.02	67 ± 6	33 ± 6

[a]Radiolabeled substrates were injected into the abdomens of middle instar nymphs. After 6 hr, the hydrocarbons were extracted, separated and assayed as described in Methods.

[b]Mean ± SD, n=3-6.

distribution of the hydrocarbons. As shown in Table 1, the radioactivity from [1-^{14}C] acetate incorporated into the alkadiene is less than expected. In order to examine the incorporation of acetate into the alkadiene, several experimental approaches were taken.

It has been demonstrated that ^{13}C can serve as an excellent tool for studying hydrocarbon biosynthesis in algae (14) and insects (9,15). To determine whether acetate was incorporated throughout the entire 27:2 molecule or was added only to a preformed linoleate chain, the $^{13}C\{^1H\}$NMR spectra of (Z,Z)-6,9-heptacosadiene isolated from untreated, [1-^{13}C] acetate-treated and [2-^{13}C] acetate-treated insects were obtained (Fig. 2). The natural abundance $^{13}C\{^1H\}$NMR spectrum (Fig. 2A) showed one large resonance at 29.6 ppm (carbons 12-23) and 9 smaller resonances. Carbon assignments for each resonance are given in Figure 2A, and were made based on the assignments for linoleic acid (16) and alkanes as described by Levy and Nelson (17). It was not possible to unambiguously assign the partially resolved resonances at 22.5 and 22.6 ppm. They arise from carbons 2 and 26.

When the C_{27} alkadiene was labeled by [2-^{13}C] acetate, enrichment was observed in the resonances which correspond to carbons 25 and 1 + 27. Since carbons 1 and 27 produce a common resonance, it was not possible to determine which one was labeled. However, by comparing the relative amount of enrichment in the resonances from carbons 25 and 1 + 27, and taking into account that the resonances from carbons 3, 5 + 11 and 7 + 9 were not enriched, it was concluded that the enrichment observed in the signal for carbons 1 + 27 came entirely from enrichment in carbon 27. The $^{13}C\{^1H\}$ NMR of the alkadiene labeled from [1-^{13}C]-acetate showed enrichment in the signals which correspond to carbons 4 + 24 and one of the partially resolved resonances from carbons 2 + 26. Based on the considerations already dis-

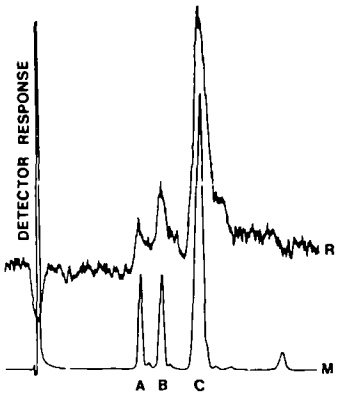

FIG. 1. Radio-GLC of the cuticular hydrocarbons of *P. americana* labeled in vivo by [1-^{14}C] linoleic acid and isolated as described in Methods. Radio-GLC was performed on a 1.8 m × 3 mm 3% Dexsil 400 on Supelcoport column temperature-programmed from 240 to 300 C at 2 C/min. Components are (A) *n*-pentacosane, (B) 3-methylpentacosane, and (C) (Z,Z)-6,9-heptacosadiene. The upper trace is radioactivity (R) and the lower trace is mass (M).

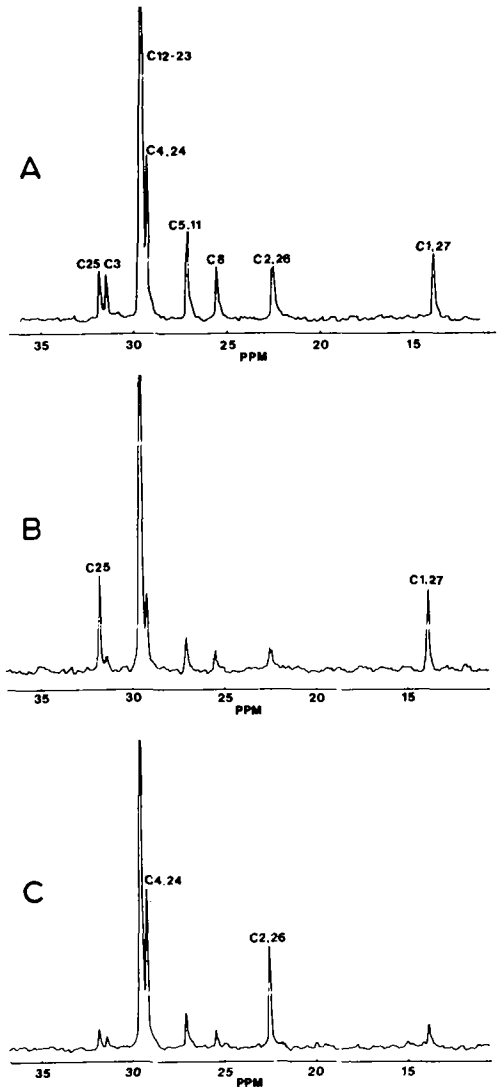

FIG. 2. $^{13}C\{^1H\}$NMR spectra of (Z,Z)-6,9-heptacosadiene: (A) natural abundance, (B) from [2-^{13}C] acetate-treated insects, and (C) from [1-^{13}C] acetate-treated insects. Labeled substrates were injected, the alkadiene isolated, and spectra were recorded as described in Methods.

cussed, it appears that the observed enrichments in this spectrum came exclusively from carbons 24 and 26.

Further data which suggest that acetate is added to a preformed linoleate were obtained by examining the ozonolysis products of the alkadiene labeled from [1-^{14}C]acetate. An examination of the radio-GLC of the ozonolysis products (Fig. 3) clearly shows that no radioactivity was incorporated into hexanal, whereas octadecanal was radiolabeled. Since the C_6 fragment contains carbons 1-6 of the alkadiene and the C_{18} fragment carbons 10-27, these data are consistent with the radiolabeled acetate being added to a preformed linoleate molecule, and not being incorporated into the portion of the molecule that came from linoleate.

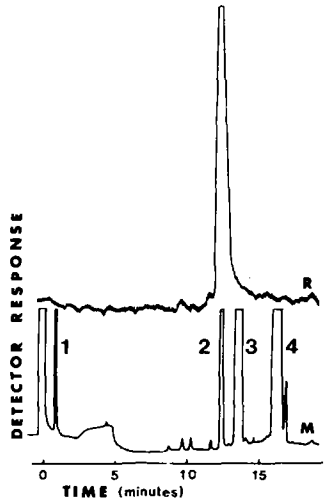

FIG. 3. Radio-GLC of the ozonolysis products of (Z,Z)-6,9-heptacosadiene labeled from [1-^{14}C]acetate. The labeled acetate was administered and the insects were extracted; the alkadiene was isolated and ozonolysis was performed as described in Methods. Radio-GLC traces were obtained on the same column described in the legend to Fig. 1, temperature-programmed from 50 to 280 C at 4 C/min. Components were identified by comparison to standards: (1) hexanal and (2) octadecanal. Peaks 3 and 4 correspond to triphenylphosphine. The upper trace is radioactivity (R) and the lower trace is mass (M).

DISCUSSION

Radioactivity from [9,10-^{3}H]oleate that was incorporated into hydrocarbon was recovered almost exclusively in the alkadiene fraction. This suggests that oleate was desaturated to linoleate which was then incorporated into the C_{27} alkadiene. Indeed, the in vivo desaturation of oleate to linoleate and the de novo synthesis of linoleate from [1-^{14}C]acetate have been demonstrated in this insect (12). The distribution of radiolabel from [1-^{14}C]oleate and [9,10-^{3}H]oleate in the cuticular hydrocarbons were compared. The results suggest that a portion of the oleate is desaturated and converted to alkadiene, and that a portion is β-oxidized to acetate with subsequent incorporation of the acetate into hydrocarbon. Since most of the tritium atoms would be lost during β-oxidation and resynthesis, such a hypothesis would explain the higher distribution of the tritiated oleate into the C_{27} alkadiene. Likewise, the radiolabel incorporated into the saturated hydrocarbons from linoleate probably arise from β-oxidation and subsequent resynthesis from acetate.

The incorporation of labeled acetate into the alkanes and alkadienes did not correspond to the relative distribution of components. Saturated hydrocarbons comprise ca. 30% of the total hydrocarbons (5), yet 52% of the radiolabel from [1-^{14}C]acetate that was incorporated was recovered in the alkanes. In contrast, the C_{27} alkadiene comprises 70% of the hydrocarbon fraction (5) and yet received only 48% of the radiolabel. The most likely explanation for this is that the radiolabeled acetate is incorporated only during the elongation of linoleate, and consequently labels only carbons 19-27. The results of the ozonolysis experiment and the data from the incorporation of [2-^{13}C]-acetate strongly support this hypothesis. These data are somewhat surprising in light of the evidence that [1-^{14}C]acetate readily labels both oleate and linoleate (10,12), and both of these fatty acids specifically label the alkadiene.

The following hypothesis is suggested to explain the radio- and stable-isotope data. Linoleic acid is synthesized in the American cockroach (12), but is not directly converted to the alkadiene in an elongation decarboxylation complex; rather, it is stored in the insect. Since *P. americana* contains a large amount of linoleate, the linoleate radiolabeled from [1-^{14}C]-acetate could be diluted in the fatty acid pool. Thus, as alkadiene synthesis proceeds, the cockroach could use linoleate from the fatty acid pool, and because of isotopic dilution, no detectable radioactivity from acetate would be observed in carbons 1-18 of the C_{27} diene. Thus, if acetate was added only to preformed linoleate, it would give rise to the observed labeling patterns. This hypothesized pathway is in contrast to the biosynthesis of 3-methylpentacosane in *P. americana*, in which once the methyl branch is added as the second unit, it is apparently not released from the enzyme complex (9) until the alkane is formed.

Indirect evidence supporting this argument is the observation that neither oleate nor linoleate contain detectable label from [1 or 2-^{13}C]-acetate, even in experiments where the hydrocarbon was readily labeled (G.J. Blomquist et al., unpublished). Dilution of the ^{13}C label by large amounts of stored lipid and rapid turnover of acyl moities could account for the lack of ^{13}C label observed in fatty acids. In contrast, hydrocarbons are accumulated as relatively stable end-products, and thus, are more amenable to ^{13}C studies.

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Inhibition of LCAT in Plasma from Man and Experimental Animals by Chlorpromazine

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ABSTRACT

Chlorpromazine (CPZ), a major tranquilizer, was found to be a potent inhibitor of lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) in the plasma of normal man, rat, rabbit and dog in vitro. The inhibitory effect of CPZ reached 35-50% at 0.5 mM depending on species; dog plasma LCAT appeared to be somewhat more sensitive than that of the other species. In rats fed CPZ or lidocaine for 14 days (0.05% in the diet), there was no statistically significant change in total plasma cholesterol levels or the size of the plasma-free (unesterified) cholesterol pool. However, 5 hr after an intracardial injection of [^{14}C]cholesterol, the percentage of plasma [^{14}C]cholesterol that was esterified was significantly lower (ca. 6%, $p<0.05$) in the CPZ-treated group, suggesting that CPZ may also inhibit LCAT to some extent in vivo. The percentage of plasma [^{14}C]cholesterol esterified in the lidocaine-treated group was similar to control values and did not reflect its ability to inhibit LCAT in vitro.

INTRODUCTION

The plasma from man and experimental animals contains the enzyme lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43). This enzyme esterifies plasma cholesterol to fatty acid derived from the 2-position of lecithin and preferentially uses lecithin in the high density (HDL, d 1.063-1.21 g/cm^3) and very high density (VHDL, d 1.21-1.25 g/cm^3) lipoprotein classes (1-3). We have recently reported that various local anesthetics inhibit plasma LCAT from man and experimental animals in vitro (4). Since cholesterol esterification by LCAT represents the integration of a lipase and a transferase step (5-7), the anesthetics could exert their inhibitory effect by a direct interaction with one or both components of the LCAT reaction (4). However, the inhibition by the anesthetics seems to parallel their order of anesthetic potency (4,8), suggesting that their effect may be nonspecific or indirect and involve alterations in the physical state of HDL-lipids, particularly lecithin, or the displacement of ApoA-1, the physiological LCAT activator. In this report, data are provided which show that chlorpromazine, a major tranquilizer which is also highly lipophilic and capable of binding to all plasma lipoproteins (9), is an inhibitor of LCAT in vitro, and to some extent in vivo. The studies presented here and those described previously (4) suggest that the local anesthetics and chlorpromazine may be useful agents in studying the factors controlling plasma LCAT activity, particularly since LCAT is important to the overall metabolism of the plasma lipoproteins (10-12).

MATERIALS AND METHODS

Animals

Rabbits were male New Zealand (3-3.5-kg) which were maintained on a diet of Purina Chow. Rats were male (Upj:TUC(SD)spf) weighing 200-225 g and were maintained on Purina Chow or Purina Chow to which was added 0.05% (w/w) chlorpromazine·HCl or lidocaine·HCl (both obtained from Sigma Chemical Co., St. Louis, MO). The dogs were 3-5-yr-old male mongrels obtained through a licensed supplier and were fed a diet of Purina Chow.

Blood

Blood was drawn from normal male rabbits, rats, and dogs, and a normal male volunteer (18-hr fasted, 60-yr-old), into heparinized syringes. The plasma was separated immediately by centrifugation at 4 C.

Injections

Rats were anesthetized with ether and injected intracardially with 1 μCi [^{14}C]cholesterol which was introduced into 0.5 ml of freshly prepared, heat-inactivated (57 C for 30 min), homologous normal rat plasma. The [^{14}C]cholesterol-labeled plasma used for the injections was prepared as previously described (13) by mixing the labeled sterol dissolved in 125 μl ethanol with 13.5 ml of plasma; the labeled sterol was added via a microsyringe.

Assay of Lecithin:Cholesterol Acyltransferase

LCAT activity was assayed in fresh plasma samples at 37 C by following the incorporation of [4-^{14}C]cholesterol (SA 54 mCi/mmol, New England Nuclear Corp., Boston, MA) into [^{14}C]cholesteryl esters. The plasma was labeled with [^{14}C]cholesterol (0.7 μCi/ml plasma) either by the direct addition of the isotope in 25 μl acetone (4) or by adding the isotope as an albumin-stabilized suspension (4). The former

method, which is simpler to perform, does not permit the calculation of initial reaction rates but does permit a calculation of net esterification and hence can be used to evaluate the potency of inhibitors (4). In the latter method, [^{14}C] cholesterol added as the albumin-stabilized suspension was allowed to equilibrate for 4 hr with endogenous lipoprotein cholesterol while LCAT was inhibited by DTNB (5,5-dithiobis-2-nitrobenzoic acid, Aldrich Chemical Co., Rochester, NY) (4) at a final concentration of 0.7 mM. After the 4-hr preincubation, LCAT inhibition was reversed with β-mercaptoethanol (11.8 mM) to initiate the assays. Equilibration of endogenous cholesterol with exogenous [^{14}C] cholesterol is ca. 90% complete by this method and permits a calculation of initial rates of esterification (14,15). After establishing that chlorpromazine (CPZ) inhibited LCAT, i.e., reduced initial rates, subsequent studies were conducted using the "direct addition" method of labeling the plasma.

Analyses

Plasma samples in which LCAT assays were performed were extracted with 20 vol $CHCl_3$/MeOH (2:1, v/v) and the cholesteryl esters were isolated from the extracts by thin layer chromatography (TLC) (16). Assay of cholesteryl ester radioactivity was done by liquid scintillation spectrometry as previously described (17). In rats previously injected with [^{14}C] cholesterol, blood was drawn into heparinized syringes 5 hr postinjection and placed in ice water for about 20 min prior to centrifugation at 4 C. Portions of the plasma samples obtained were extracted as already described and the extracts were fractionated by TLC (16). The cholesteryl ester and unesterified cholesterol fractions were scraped from the chromatoplates and assayed for radioactivity as described. Another portion of each plasma sample was taken for the measurement of total cholesterol (18) and free (unesterified) cholesterol (19).

RESULTS

Initial studies on the effect of CPZ on plasma LCAT are shown in the time course for [^{14}C] cholesterol esterification by the enzyme in freshly prepared plasma (Fig. 1) in which the lipoprotein cholesterol had been permitted to equilibrate with albumin-stabilized [^{14}C] cholesterol. Addition of CPZ to a concentration of 0.5 mM inhibited LCAT activity by ca. 50% throughout the 40-min assay. Calculation of initial rates indicated 0.40 mμmol/ml/min in control samples and 0.22 mμmol/ml/min in CPZ-treated samples. Inhibition was also independently determined by measuring net esterification in pooled rat plasma incubated for 24 hr with and without 0.5 mM CPZ. Plasma free cholesterol levels decreased from 15.6 mg/dl to 9.4 mg/dl in the control samples and to 12.6 mg/dl in the CPZ-treated samples, i.e., net esterification was 48% less in the presence of CPZ. Inhibition of LCAT by CPZ was also observed using the "direct addition" of [^{14}C] cholesterol to rat plasma (Fig. 2). At a level of 0.2 mM, CPZ inhibited LCAT ca. 20% throughout the course of a 2-hr assay.

The inhibitory effect of CPZ on LCAT was examined in greater detail in freshly isolated plasma from normal man, dog and rabbit (Fig. 3) while employing the "direct addition" method for labeling the plasma. Plasma LCAT in all species showed sensitivity to CPZ. Inhibition of the enzyme was concentration-dependent over the range 0.1-0.5 mM CPZ. The level of inhibition achieved in the dog plasma appeared to be somewhat greater than in the plasma from rabbit and man; 50% inhibition was reached at 0.5 mM CPZ in dog plasma whereas this concentration gave 35-40% inhibition in rabbit and man. In order to determine to what extent CPZ could inhibit LCAT in vivo, CPZ was administered to rats as an addition to the diet (0.05% CPZ, w/w) for 14 days. On the 14th day, the CPZ-treated rats and a

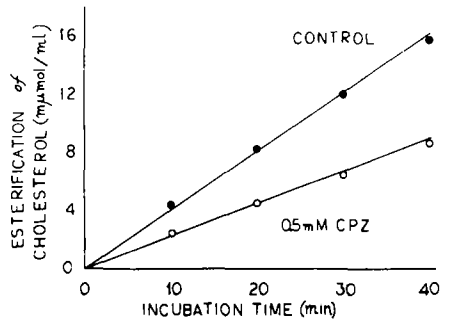

FIG. 1. Time-course of cholesterol esterification in rat plasma. Fresh plasma from 2 normal male rats was pooled. LCAT was inhibited by the addition of DTNB and lipoprotein cholesterol was permitted to equilibrate for 4 hr at 37 C with a suspension of albumin-stabilized [4-^{14}C] cholesterol as detailed in Methods. After the equilibration period (free cholesterol sp act 7530 dpm/μg), the pool of plasma was divided into 2 parts. Assays were initiated by adding β-mercaptoethanol to one portion (control, –●–●–), and β-mercaptoethanol and 0.5 mM chlorpromazine·HCl (CPZ, –o–o–) to the other. Incubations were terminated by the addition of 20 vol $CHCl_3$/MeOH (2:1, v/v). [^{14}C]-Cholesteryl esters were fractionated from the samples and assayed for radioactivity as detailed in Methods.

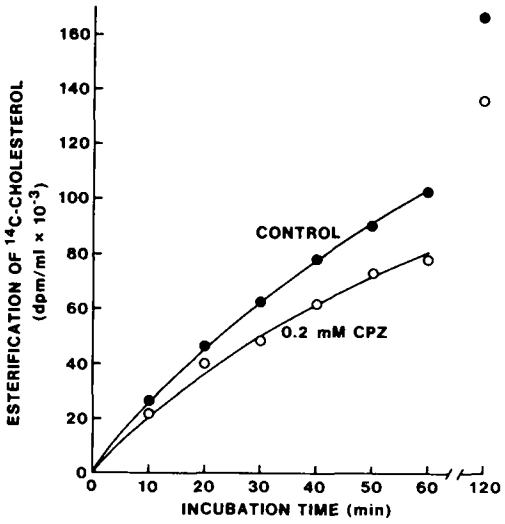

FIG. 2. Time-course of cholesterol esterification in rat plasma. Fresh plasma from 3 normal male rats was pooled. Aliquots (1.0-ml) were labeled with 0.7 μCi [4-^{14}C] cholesterol in 25 μl of acetone and assayed for LCAT in the absence (control, –●–●–) or presence of 0.2 mM chlorpromazine·HCl (CPZ, –o–o–) at 37 C. CPZ was added to the samples dissolved in 50 μl saline. Samples were analyzed as described in the legend to Fig. 1.

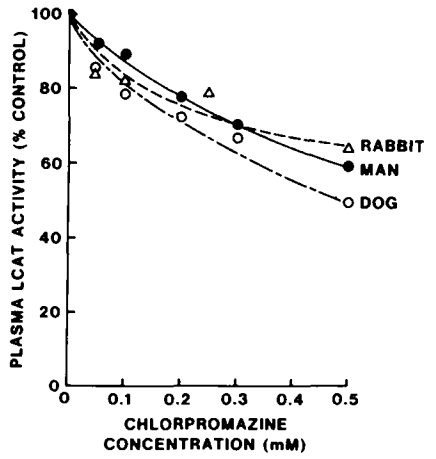

FIG. 3. Effect of chlorpromazine on LCAT activity in plasma from various species. Volumes (1.0-ml) of plasma obtained from normal male rabbit, normal male rat, normal male dog, and normal man (18-hr fasted, 60-yr-old) were labeled with 0.7 μCi [4-^{14}C]-cholesterol and assayed for LCAT in the presence of various concentrations of chlorpromazine as described in Methods and in the legend to Fig. 1. Incubations were conducted at 37 C for 2 hr.

control group were injected intracardially with [^{14}C] cholesterol as described in Methods. Another group of rats that had been fed 0.05% lidocaine in the diet was also injected since we have previously observed lidocaine to be an inhibitor of LCAT in vitro, as well (4). Analysis of the plasma 5 hr postinjection (Table 1) showed that control animals esterified 62.6% of their circulating [^{14}C] cholesterol whereas the CPZ group esterified 57.9%; this difference, though small, was statistically significant ($p<0.05$). The rats treated with lidocaine did not differ from the control group in percentage of [^{14}C] cholesterol esterified (63.7%). The total [^{14}C] cholesterol activity in the plasma of the animals showed no statistically significant differences among the groups; this indicates that drug pretreatment did not affect the clearance rate of the injected [^{14}C] cholesterol. Similarly, there was no statistically significant difference in total plasma cholesterol levels among the groups or in the plasma free cholesterol pool sizes (Table 1).

DISCUSSION

The studies presented here demonstrate that CPZ is an inhibitor of LCAT in plasma from man, rabbit, rat and dog. The inhibition can be observed in plasma in which the exogenous [^{14}C] cholesterol added has been pre-equilibrated with endogenous lipoprotein cholesterol (Fig. 1) as well as in plasma to which exogenous [^{14}C] cholesterol was added immediately prior to assay (Fig. 2). This inhibition was confirmed by decreased net esterification as determined chemically. We have previously reported that various local anesthetics are also inhibitors of LCAT (4). Although the local anesthetics (e.g., lidocaine) and CPZ (a phenothiazine tranquilizer) represent different chemical classes, they share the common property of being amphiphilic. The diversity of chemical structures represented by the local anesthetics and CPZ suggests that their ability to inhibit LCAT may not represent a direct effect of the agents on the enzyme but, rather, may be the result of an indirect effect associated with their amphiphilic nature or their ability to bind to specific phospholipids (20-22). CPZ, which binds to all the plasma lipoprotein fractions (chylomicrons; very low density lipoproteins, VLDL; low density lipoproteins, LDL; high density lipoproteins, HDL) (9), is known to alter the physical state of membrane lipids (23-26); a similar effect of CPZ on HDL lipids could alter the availability of HDL-lecithin for the LCAT reaction. Another possibility is that CPZ (and the local anesthetics) displace or compete with the physiological LCAT activator, ApoA-1,

TABLE 1

Effect of Lidocaine and Chlorpromazine on the Esterification of Circulating [^{14}C]Cholesterol in the Rat in vivo

	Plasma cholesterol (mg/dl)		Total [^{14}C]Cholesterol in plasma (dpm/ml)	[^{14}C]Cholesterol esterified (%)
	Free	Total		
Control (n=5)	15 ± 2	78 ± 3	7720 ± 1500	62.6 ± 1.7
Lidocaine (n=6)	15 ± 1	78 ± 4	5660 ± 640	63.6 ± 2.1
Chlorpromazine (n=6)	18 ± 1	84 ± 3	6725 ± 325	57.9 ± 0.7[a]

Male rats weighing 200-225 g were fed for 2 wk on a stock diet (Purina Chow) containing 0.05% lidocaine·HCl or 0.05% chlorpromazine·HCl (w/w). The animals were then injected intracardially with 0.5 ml of normal rat plasma containing 1.95 X 10^6 dpm [4-^{14}C]-cholesterol. The animals were bled 5 hr later and the plasma extracted with $CHCl_3$/MeOH (2:1, v/v). The extracts were fractionated by thin layer chromatography to separate unesterified and esterified cholesterol. Plasma free cholesterol and total cholesterol were measured as described in Methods.

[a]Significantly different from control value ($p<0.05$) by Student's independent t-test.

which is amphiphilic in nature (27). Studies with purified human LCAT show the enzyme to be Ca^{++}-independent (6,7), thus the ability of CPZ to displace Ca^{++} (8) is unlikely to account for its effect on LCAT.

Esterification of circulating [^{14}C] cholesterol injected into the circulation of rats fed CPZ for 14 days (0.05% in the diet) was statistically significantly reduced relative to the control group (62.6±1.7% vs 57.9±0.7% esterified, $p<0.05$). Since esterification of the [^{14}C]-cholesterol by hepatic ACAT (acylCoA:cholesterol acyltransferase) is likely occurring in these animals, as well (28), interpretation of such a small reduction in plasma [^{14}C] cholesteryl esters is compromised. In any event, the reduction in [^{14}C] cholesterol esterified in the CPZ-treated rats does not indicate that tolerable doses of CPZ have a strong effect on LCAT in vivo. The data do suggest, however, that an in vivo effect of CPZ on LCAT may occur to some extent. Based on food consumption, this small in vivo effect was achieved at higher levels of CPZ (ca. 30 mg/kg) than therapeutic doses which have an upper limit of ca. 10 mg/kg in man (29,30). It is, therefore, uncertain how CPZ treatment might affect LCAT in man in vivo. CPZ treatment in man has been reported to be associated with mild hypercholesterolemia (29,30) but the distribution of the plasma cholesterol pool between free and esterified sterols was not measured. Lidocaine, which inhibits LCAT in vitro (4), did not affect esterification of circulating [^{14}C] cholesterol in rats pretreated with 0.05% lidocaine in the diet. The lack of effect may be the result of a rapid clearance of lidocaine; it is absorbed from the gastrointestinal tract (31) but is almost completely removed from the plasma compartment by a single pass through the liver (32). This is in contrast to CPZ which persists in plasma for up to 24 hr (33) and can undergo an enterohepatic circulation (34).

The studies presented here show that CPZ inhibits LCAT in the plasma of various species in vitro and high-dose levels in the rat appear to marginally inhibit cholesterol esterification in vivo, as well. In comparison to our previous studies (4), CPZ appears to be about 10 times more potent (mol:mol) than lidocaine as an inhibitor of plasma LCAT in vitro.

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Effect of Water Mobility on Lateral Diffusion of Phospholipids in Liposomes

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ABSTRACT

Recently, several studies have shown that the rate of lateral diffusion of liquid-crystalline phospholipids in various phospholipid/water lamellar systems depends markedly on the hydration over the water content of 15-40 w/w%. In this paper, we calculated the change in lipid lateral diffusion as a function of water content according to the theory of Brownian motion in thin sheets developed by Saffman and Delbrück. These calculations, based on experimental data of lipid dimensions and bulk water diffusion coefficients obtained for the egg phosphatidylcholine/water system at various water contents, clearly indicate that the dependence of lipid lateral diffusion on the hydration can be attributed primarily to the change in bulk water mobility in the multilamellar phospholipid/water system.

Since the first measurement of the rate of lateral diffusion of an androstan spin label and a spin-labeled phosphatidylcholine in 2-dimensional phospholipid bilayers by, respectively, Sackman and Träuble (1) and Devaux and McConnell (2), lateral diffusion coefficient of phospholipid and its analogs in phospholipid bilayers at the liquid crystalline state has been well established by McConnell and associates to be of (1-2) x 10^{-8} cm^2/sec (3,4). Recently, the fluorescence recovery after photobleaching technique and pulsed gradient ^{1}H-NMR spin-echo technique have been elegantly applied to study lipid lateral diffusion in various phospholipid/water systems at temperatures above the gel ↔ liquid crystalline phase transition temperature (5,6). These studies indicate that lipid lateral diffusion decreases significantly with decreasing hydration over the water content of 15-40%, even though the acyl chains are in the liquid crystalline state. Since it has been suggested by LeNeveu et al. (7) that a decrease in the thickness of water layer separating individual egg phosphatidylcholine bilayers in the multilamellar liposomes will yield an increase in lateral compression of the bilayer which, in turn, results in an increase in the bilayer thickness and a simultaneous decrease in surface area of the lipid headgroup, the observed hydration effect on lateral diffusion is thus explained in terms of the conformational change of phospholipids in the bilayer (5). In this communication, we use the theory of Brownian motion in thin sheets of viscous fluid developed by Saffman and Delbrück (8) and Saffman (9) to calculate the relative lateral diffusion of egg phosphatidylcholine as a function of water content. Calculations indicate that the relative change in lipid lateral diffusion as a function of water content over the experimentally determined range of hydration can be reasonably expected to result from changes in bulk water mobility in the aqueous space between lipid bilayers in the multilamellar liposome structure.

CALCULATION AND RESULTS

According to Saffman and Delbrück (8), the lateral mobility of a cylindrical particle embedded in a lipid bilayer, b_T, depends, in a rather complex way, both on the size of the cylindrical particle and on the logarithm of the relative viscosity of the bilayer interior to that of the surrounding medium according to the following equation:

$$b_T = \frac{1}{4\pi\mu h}\left(\ln \frac{\mu h}{\mu' a} - \gamma\right)^*, \qquad [I]$$

where a and h are, respectively, the radius and height of the cylindrical particle, μ and μ' denote, respectively, the viscosity of the fluid bilayer interior and that of the surrounding medium, and γ is Euler's constant, 0.5772. (*Saffman and Delbrück use the symbol log $\frac{\mu h}{\mu' a}$ instead of ln $\frac{\mu h}{\mu' a}$ in Eq. I. Derivation of this equation indicates that the natural logarithm is a correct representation). Equation I is derived under the no-slip boundary condition that $\mu' << \mu$ in compressible fluid. Since the diffusion coefficient, D, can be expressed according to the Einstein relation as kTb_T, where k is the Boltzmann's constant and T is the absolute temperature, we can calculate the ratio of lateral diffusion of the phospholipid molecule, assumed as a cylindrical particle, in the bilayer in the presence of various degrees of hydration as follows:

$$\frac{D_d}{D} = \frac{\mu h}{\mu_d h_d} \times \left(\frac{\ln \frac{\mu_d h_d}{\mu' d a_d} - 0.5772}{\ln \frac{\mu h}{\mu' a} - 0.5772}\right), \qquad [II]$$

where D can be assigned as the lateral diffusion coefficient at 40% (w/w) water content and D_d is that at a different dehydrated state.

In order to calculate the ratio of lateral diffusion coefficient according to Equation II, one needs to know the value of h and a of phospholipids at various water contents. In addition, the ratio of $\frac{\mu}{\mu_d}$, $\frac{\mu}{\mu'}$, and $\frac{\mu_d}{\mu'_d}$ are needed. However, if $\frac{\mu'_d}{\mu'}$ is known, then $\frac{\mu_d}{\mu'_d}$ can be readily calculated from $\frac{\mu'_d}{\mu'}$, $\frac{\mu_d}{\mu}\frac{\mu'_d}{}$, and $\frac{\mu}{\mu'}$. The translational diffusion coefficient of bulk water molecules between individual lamellar bilayers has been measured as a function of water content (10); results indicate that the bulk water diffusion coefficient decreases markedly with decreasing water content. The relative bulk diffusion coefficient of water molecules can be used to calculate μ'_d/μ', since the diffusion coefficient is inversely related to the viscosity according to the Einstein-Stokes relation ($D = kT/6\pi\mu' R_s$). The values of h and a of phospholipids in bilayers at various water contents have been reported by Parsegian et al. (11). Since viscosity μ represents a measurement of the effective hydrodynamic volume of the cylindrical molecule, the ratio μ_d/μ can be estimated from the ratio of $\frac{a_d^2 h_d}{a^2 h}$ which is, in fact, extremely close to unity. This, of course, is not surprising, since the thickness and cross-sectional area of phospholipid molecules may undergo relatively large change whereas the corresponding change in volume can be rather small. This is analogous to the observed small change in volume ($\sim$ 3.5%) when phospholipids undergo gel $\leftrightarrow$ liquid crystalline phase transition (12). Finally, the ratio of μ/μ' is assumed to be 100 (8); this basic assumption is important because Eq. II is derived for the case $\mu >> \mu'$. In fact, we can increase the ratio of μ/μ' from 100 to 200 and examine the effect of this ratio on the change of the lipid lateral diffusion coefficient as a function of hydration. Results of our calculation are given in Table 1. It is clearly seen that the relative lateral diffusion coefficients of phospholipids calculated at μ/μ' = 100 and 200 decrease with decreasing hydration (columns 5 and 6, Table 1); the calculated changes over the concentration range of 15-40% (w/w) water content agree reasonably well with the data obtained experimentally for egg phosphatidylcholine and dipalmitoylphosphatidylcholine multibilayers in the liquid crystalline state (Fig. 1).

DISCUSSION

Several points deserve discussion. First, all the experimental data presented in Table 1 were obtained with hydrated egg phosphatidylcholine bilayers at room temperature; the calculated relative lateral diffusion coefficients can, therefore, be considered results for phospholipids in the liquid crystalline state. Consequently, the calculated relative diffusion coefficient can be used to compare with both the experimental data derived from the egg phosphatidylcholine/H_2O system and the dipalmitoylphosphatidylcholine/D_2O system at 57 C (Fig. 1). Second, our calculations are limited to the hydration range of 15-40% (w/w), because bulk water diffusion coefficients have been determined within this limited range only for the egg phosphatidylcholine/H_2O system. Third, the Einstein-Stokes relation ($D = KT/6\pi\mu' R_s$) is not strictly correct for the calculation of μ' from D for water molecules, since the value of 6π is, according to the reaction rate theory, incorrectly high (13). Nevertheless, the relative ratio of μ'_d/μ' is not affected by the factor of 6π, and our relative value of water viscosity presented in column 4, Table 1, can be applied with accuracy to calculate the relative lateral diffusion coefficient according to Equation II. Fourth, we believe that our estimation of μ_d/μ to be unity is quite reasonable, since the relative change in partial specific volume of phosphatidylcholines in bilayers

TABLE 1

Relative Lateral Diffusion Coefficients

H_2O (% w/w)	h(Å)	a(Å)	μ'_d/μ'	D_d/D at $\mu_d/\mu = 1$	
				$\mu/\mu' = 100$	$\mu/\mu' = 200$
40	17.5	4.89	1	1	1
35	17.8	4.84	2.85	0.80	0.82
30	18.1	4.80	3.3	0.76	0.79
25	18.6	4.74	3.3	0.75	0.80
20	19.4	4.64	5	0.65	0.70
15	20.0	4.56	9	0.54	0.58

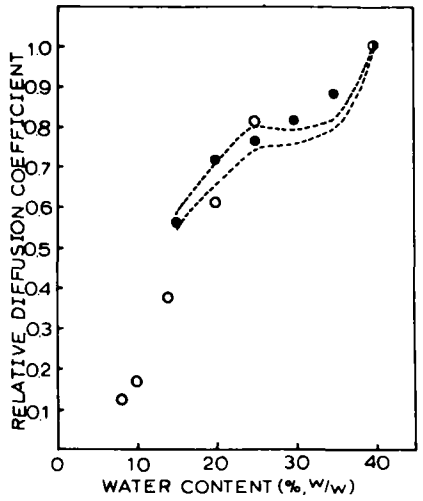

FIG. 1. Relative lateral diffusion coefficient of phospholipids in multilamellar phospholipid/water systems plotted as a function of degree of hydration. The data represented by open and filled circles are taken from McCown et al. (5) andKuo and Wade (6) for the egg phosphatidylcholine/H_2O system at room temperature and the dipalmitoyl phosphatidylcholine/D_2O system at 57 C, respectively. The upper and lower broken lines show the calculated change in relative lateral diffusion coefficient as a function of hydration according to Equation II assuming μ/μ' = 200 and 100, respectively.

at the liquid crystalline state can be assumed to be extremely small. Another term which we used for our D_d/D calculation is μ/μ'. This ratio was first estimated to be 100 by Saffman and Delbrück (8). We extend the value from 100 to 200 for our calculations as indicated in Table 1. The effect of μ/μ' on the relative lateral diffusion of phospholipids is not very large over the hydration range calculated (columns 5 and 6 in Table 1). Taking the ratio of D_d/D at 25% hydration as an example, it varies merely 6.7% when the ratio of μ/μ' is altered from 100 to 200. Finally, we want to emphasize that of all the relative values used in Equation II, μ'_d/μ' is the most dominant term in determining the relative value of the lateral diffusion coefficient; other terms have secondary, if not totally negligible, effect on the ratio of D_d/D.

In summary, we can conclude that if phospholipid lateral diffusion in bilayers can be adequately described by the theory developed by Saffman and Delbrück (8) and Saffman (9), one would expect that the bulk water will exert a drag on the motions of phospholipid molecules in the 2-dimensional plane of the bilayers, thus modulating the lipid lateral diffusion coefficient. As the water content in the phospholipid/H_2O system is lowered, the bulk water properties have been observed to change in a nonlinear fashion as indicated by the drastic decrease in the translational diffusion coefficient of H_2O molecules (10). This change in the bulk water properties, as reflected in column 4, Table 1, can be attributed as the major factor in determining the relative change of lateral diffusion coefficient of phospholipids in the liquid crystalline bilayer, according to our calculation, provided the water content in the phospholipid/water system is within the range of 15-40% (w/w). Recent fluorescent and NMR studies on the lateral diffusion of phospholipid molecules in the multilamellar bilayers in the liquid crystalline state indeed show that the lateral diffusion coefficient is hydration-dependent. Moreover, these studies show that the rate of lateral diffusion decreases by about a factor of 2 over the range of water content (15-40%) studied as indicated in Figure 1 (5,6). We suggest that the interpretation of the experimentally observed data should seriously take into account the bulk water properties. In fact, we believe, based on Figure 1, that the observed hydration dependence of lipid lateral diffusion in multilamellar liposomes in the liquid crystalline phase can be attributed primarily to the change in bulk water mobility in the aqueous space separating individual bilayers.

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Influence of Lipid Peroxidation on Lipoprotein Secretion by Isolated Hepatocytes[1]

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ABSTRACT

Isolated rat liver cells have been exposed to 3 different lipid peroxidation-inducing agents, CCl_4, $FeCl_3$ and cumene hydroperoxide, and the rates of malonaldehyde production and of lipoprotein secretion have been compared. Results indicate that it is possible to induce a high degree of lipid peroxidation without inducing strong changes in lipoprotein secretion. Only in CCl_4-poisoned hepatocytes is lipoprotein secretion strongly impaired. In this experimental condition, the effect of free radical scavengers, or inhibitors of lipid peroxidation, has been studied; the degree of covalent binding of CCl_4 metabolites to hepatocyte proteins, as well as the behavior of both lipid peroxidation and lipoprotein secretion, have been evaluated. Promethazine and propyl gallate prevented malonaldehyde production, but neither agent reduced covalent binding nor improved secretion. Menadione, on the contrary, besides inhibiting malonaldehyde production, decreased covalent binding and protected against the impairment of secretion. These data lead to the conclusion that covalent binding of CCl_4 metabolites, rather than lipid peroxidation products, accounts for the derangement of lipoprotein secretion in CCl_4-poisoned liver cells.

Carbon tetrachloride (CCl_4) still represents one of the most used model agents for investigating the mechanisms of liver injury. It is postulated that its toxicity is due to homolytical cleavage in the smooth endoplasmic reticulum with the production of chloride (Cl^-) and the trichloromethyl radical ($CCl_3^\cdot$) (1-3). Recent in vitro experiments have shown that $CCl_3^\cdot$ is rapidly converted, in the presence of O_2, into the much more reactive trichloromethylperoxy radical ($CCl_3O_2^\cdot$) (4,5). The cell damage (i.e., enzyme inactivation, inhibition of protein synthesis and of protein and lipoprotein secretion, fat accumulation within the liver cells) is the consequence either of covalent binding of such free radicals to liver macromolecules, or of lipid peroxidation, through hydrogen subtraction by free radicals from membrane polyunsaturated fatty acids (PUFA) (1,3,6).

With regard to lipid peroxidation, cell changes may be produced either directly, by membrane derangement, or indirectly, by production of several different reactive compounds such as lipid free radicals, lipoperoxides, lipohydroperoxides, aldehydes and others (7).

Some evidence supports the hypothesis that, in CCl_4 poisoning, changes in cell sites far from the endoplasmic reticulum are probably due to diffusible substances. These may include substances derived from the peroxidative breakdown of PUFA (7,8).

Whether the CCl_4-induced damage to membrane enzymes and to the lipoprotein secretory pathway is due to its prooxidant effect or to the covalent binding of its metabolites to cell structures is not yet completely clear.

The use of isolated hepatocytes represents a good model to further investigate the mechanism of cell damage in CCl_4 poisoning. In isolated liver cells, as well as in vivo, CCl_4 stimulates lipid peroxidation (9-11), inhibits protein synthesis and protein and lipoprotein secretion (11). Under these conditions, accumulation of fat in the cells was also seen (11).

One of the aims of studies reported in this paper was to determine whether the CCl_4-induced block in lipoprotein secretion was due to covalent binding or to lipid peroxidation. In order to check the relative influence of either covalent binding or lipid peroxidation on the secretory pathways, several criteria may be followed.

The use of free radical scavengers or of inhibitors of lipid peroxidation seems particularly useful. Previous experiments using CCl_4-poisoned hepatocytes have demonstrated that promethazine and propyl gallate strongly attenuate the peroxidative breakdown of PUFA up to aldehydic products (10,12). Furthermore, since we observed that menadione (vitamin K_3) is able to reduce CCl_4 covalent binding in liver cells, we also investigated the effect of this drug at a very early stage of derangement of lipoprotein secretion.

Other studies reported in this paper represent another approach to the problem. We used 2 experimental conditions other than CCl_4-poisoning, in which an increased lipid peroxidation occurs, i.e., the cell treatment with

[1]Presented in part at the ISF-AOCS World Congress, New York, April 1980.

$FeCl_3$ (13), or with cumene hydroperoxide (14).

EXPERIMENTAL PROCEDURES

Animals

Male rats of the Wistar strain (Nossan, Correzzana, Milano, Italy) of 200-250 g body wt were used. They were fed a semisynthetic diet, free of any antioxidant (Piccioni, Brescia, Italy) with free access to water. All experiments started between 10:00 a.m. and 11:00 a.m.

Reagents

All chemicals were of reagent grade and were obtained from the following sources: collagenase type I, menadione, propyl gallate, amino acids, ethyleneglycol-bis-(β-amino-ethyl-ether)-N,N'-tetraacetic acid (EGTA), and N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) from Sigma Chemical Co., St. Louis, MO; promethazine-HCl from May and Baker, Dagenham, U.K.; cumene hydroperoxide from Fluka AG, Buchs, Switzerland; [U-^{14}C] palmitic acid from The Radiochemical Centre, Amersham, U.K.; other chemicals from BDH Chemicals Ltd., Poole, U.K., and Merck, Darmstadt, West Germany.

Preparation of Intact Liver Cells

The open, nonrecirculating, in situ liver perfusion technique used was essentially that described in previous works (11,12). In order to prevent loss of cell glutathione content during hepatocyte isolation, the following modifications were made, as suggested by Viña et al. (15): in the saline buffer used in the first perfusion step, 0.2 mM EGTA was included; in the cell incubation medium, the amino acid mixture was replaced by 1 mM methionine during the first 10-min incubation step.

Triglyceride Secretion from Prelabeled Hepatocytes

Hepatocytes were suspended (10^7 cells/ml) in Ham's F-12 medium containing 10% horse serum (11,12). Cell triglycerides were prelabeled by incubating 10 ml of cell suspension with 5 ml of 3 mM [^{14}C] sodium palmitate (sp act 0.33 mCi/mMol) complexed with albumin as previously reported (11,12). After 60 min incubation at 37 C, the cell suspension was diluted with 100 ml of incubation medium and then centrifuged at 400 × g for 4 min. Labeled hepatocytes were resuspended in incubation medium to 5 × 10^6 cells/ml. Aliquots of 2 ml of the suspension were poured into the main compartment of 50-ml flasks fitted with center wells and closed with screw caps.

Promethazine, propyl gallate or menadione, when indicated, were added directly to the cell suspension.

To initiate lipid peroxide formation, CCl_4 (5 μl, 86 μM final concentration) was added to the center well and allowed to diffuse in the closed system. On the other hand, when used, $FeCl_3$ or cumene hydroperoxide were added (at the different concentrations hereafter reported) directly to the cell suspension. Flasks were incubated at 37 C for 10 or 30 min. Aliquots of 2 ml of prelabeled hepatocytes were centrifuged without incubation to determine the time-zero secretion. At the end of incubation, cell suspensions were centrifuged. Triglycerides in supernatants were purified and processed for radioactivity measurements as described elsewhere (16).

Determination of Thiobarbituric Acid Reacting Materials

Malonaldehyde production was estimated by measuring the thiobarbituric acid (TBA)-reacting compounds (17). After the incubations, portions of the cell suspensions were added to 10% (w/v) trichloroacetic acid and water to give a final concentration of 5% trichloroacetic acid. After centrifugation, 1.5-ml portions of the supernatant solutions were treated with the same volume of 0.67% TBA, incubated in boiling water for 10 min and made alkaline with KOH (final concentration 0.29 M). Absorbance at 543 nm was determined with a Beckman Acta III spectrophotometer.

Determination of Covalent Binding of CCl_4 Metabolites to Membrane Proteins

Hepatocyte incubation at 37 C for 10 min was carried out with [^{14}C] CCl_4 (sp act 22 mCi/mMol) in the presence or in the absence of promethazine, propyl gallate or menadione. At the end of the incubation, the radioactivity bound to cell proteins was determined according to Rao and Recknagel (18).

RESULTS AND DISCUSSION

Previous results in our laboratory showed that promethazine, a very strong antioxidant, is able to completely inhibit the production of TBA-reacting compounds (mainly malonaldehyde) induced in liver cells by CCl_4 poisoning. On the other hand, the drug, up to the concentration of 10 μM, does not protect against blockage of protein and lipoprotein secretion in hepatocytes incubated for 40 min at 37 C in the presence of 129 μM CCl_4 (11, 12).

This result suggests that the CCl_4-induced impairment of the hepatic protein and lipid secretion is mainly due to a mechanism that is different from lipid peroxidation.

In the present investigation, in order to confirm the inability of promethazine in preventing this cell damage, a lower (86 μM) concentration of CCl_4 has been used. In fact, to avoid direct effects of the scavenger on the secretion pathway, it was not possible to increase its concentration over 10 μM. Experiments to check the effect of promethazine on CCl_4-induced lipid peroxidation and on covalent binding of CCl_4 metabolites to cell proteins have been done simultaneously. In similar ways, we tested the effects of 2 other antioxidant drugs, propyl gallate and menadione, which inhibit lipid peroxidation, differing from promethazine for the site of action and the mechanism, respectively. Propyl gallate acts as antioxidant by electron donation, like promethazine, but mainly reacting at the NADPH flavoprotein level (1). Menadione, a lipophilic drug, inhibits lipid peroxidation by reducing the NADPH available to sustain it, and, under the form of semiquinone, by electron donation. (19,20).

Table 1 shows that 86 μM CCl_4 inhibits the hepatocyte release of lipoprotein triglycerides by 60% as early as 10 min after poisoning. No significant protection was detected when CCl_4 treatment was done in the presence of 10 μM promethazine. On the other hand, this scavenger completely inhibits the CCl_4-induced increase of malonaldehyde production, but it does not significantly affect CCl_4 covalent binding to cell proteins.

The evidence that the pretreatment with promethazine does not prevent the CCl_4-induced block of lipoprotein secretion is consistent with earlier studies showing that the antioxidant protects against CCl_4-induced necrosis, but has little effect on the accumulation of fat (21). All these results support the hypothesis that different mechanisms are primarily responsible for the 2 main hepatotoxic effects of CCl_4, necrosis and fatty degeneration. The 2 different CCl_4-reactive metabolites, i.e., $CCl_3O_2^{\cdot}$ and $CCl_3^{\cdot}$, respectively, might be implicated in the already mentioned types of liver injury. Indirect support to this speculation comes from recent pulse radiolysis studies on CCl_4 and promethazine interaction. This substance, in fact, reacts very quickly with the trichloromethylperoxy radical and very slowly with $CCl_3^{\cdot}$ (4,5). In other words, the first radical would initiate lipid peroxidation, whereas the $CCl_3^{\cdot}$ might be implicated in tissue changes not protected by the antioxidant, i.e., dependent on CCl_4 covalent binding to cell structures.

The following studies using propyl gallate instead of promethazine (Table 2) give similar conclusions, even if the first scavenger, at the most suitable concentration (50 μM), shows lower antioxidant activity than the second one. Another reason that neither promethazine nor propyl gallate inhibit [^{14}C]CCl_4 binding may be related to their hydrophilic nature. These 2 substances do not easily diffuse through the lipid membranes, so they could not be very effective in scavenging lipid peroxidation initi-

TABLE 1

Effect of Promethazine on Lipoprotein Secretion, Malonaldehyde Production and CCl_4-Protein Covalent Binding in CCl_4-Poisoned Hepatocytes[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]	[^{14}C]CCl_4-protein covalent binding[d]
Control (not treated)	2,913 ± 288	0.090 ± 0.010	–
+ Promethazine (10 μM)	2,675 ± 462[e]	0.082 ± 0.006[e]	–
CCl_4 (86 μM)	1,134 ± 473[f] (61%)	0.243 ± 0.025[f]	689 ± 38
+ Promethazine (10 μM)	1.085 ± 234[g] (59%)	0.095 ± 0.011[h]	665 ± 46[g]

[a]Cell aliquots (10^7 hepatocytes) were incubated 10 min at 37 C in the presence or in the absence of CCl_4 and/or promethazine. All data represent means of 2 experiments in triplicate ± SD.

[b]Values are radioactivities (cpm) of lipoprotein triglycerides released from 10^7 cells prelabeled with [^{14}C]-palmitic acid. Values in parentheses are percent inhibition with respect to the corresponding control.

[c]Values are optical densities at 543 nm of thiobarbituric acid (TBA)-reacting compounds produced by 10^7 hepatocytes.

[d]Values are radioactivities (cpm) of [^{14}C]CCl^4 metabolites covalently bound to proteins of 10^7 cells.

[e]Not significant as to control group ($p > 0.05$).

[f]Significant as to control group ($p < 0.001$).

[g]Not significant as to CCl_4 group ($p > 0.05$).

[h]Significant as to CCl_4 group ($p < 0.001$).

TABLE 2

Effect of Propyl Gallate on Lipoprotein Secretion, Malonaldehyde Production and CCl_4-Protein Covalent Binding in CCl_4-Poisoned Hepatocytes[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]	[^{14}C]CCl_4 protein covalent binding[d]
Control (not treated)	2,030 ± 168	0.102 ± 0.015	–
+ Propyl gallate (50 μM)	1,850 ± 195[e]	0.092 ± 0.010[e]	–
CCl_4 (86 μM)	858 ± 288[f] (58%)	0.215 ± 0.024[f]	720 ± 48
+ Propyl gallate (50 μM)	923 ± 169[g] (51%)	0.153 ± 0.018[h]	696 ± 27[g]

[a]Cell aliquots (10^7 hepatocytes) were incubated 10 min at 37 C in the presence or in the absence of CCl_4 and/or propyl gallate. All data represent means of 2 experiments in triplicate ± SD.

[b]See Table 1.

[c]See Table 1.

[d]See Table 1.

[e]Not significant as to control group ($p > 0.05$).

[f]Significant as to control group ($p < 0.001$).

[g]Not significant as to CCl_4 group ($p > 0.05$).

[h]Significant as to CCl_4 group ($p < 0.003$).

ation products.

The lack of protection by the 2 scavengers against CCl_4 covalent binding is in agreement with the results obtained with liver microsomes by Cheeseman and Slater (22). In fact, in their experimental system, 100 μM promethazine or 50 μM propyl gallate inhibited by 70-80% the CCl_4-induced lipid peroxidation, producing at the same time only a very small decrease in [^{14}C] CCl_4 radioactivity bound to microsomal proteins (10-20%). These results strongly favor the assumption that covalent binding of CCl_4 metabolic products is likely to be the most important mechanism for lipoprotein secretion derangement in CCl_4-poisoned hepatocytes.

This hypothesis is strengthened by identical experiments done on CCl_4-poisoned liver cells using menadione as free radical scavenger. Table 3 shows the effects of menadione addition to liver cells just before CCl_4 poisoning. The impairment of lipoprotein secretion due to CCl_4, as well as the covalent binding of CCl_4 metabolites to hepatocyte proteins, are partly prevented by 100 μM menadione. The effectiveness of menadione in scavenging CCl_4 metabolites, probably CCl_3^*, may be related not only to the mechanism of action, but also to the lipophilic nature of the drug. It is notable that these 2 protective effects show a similar degree of intensity, whereas the stimulation of malonaldehyde production is almost completely inhibited.

We also studied lipoprotein secretion in hepatocytes treated with 2 other lipid peroxi-

TABLE 3

Effect of Menadione on Lipoprotein Secretion, Malonaldehyde Production and CCl_4-Protein Covalent Binding in CCl_4-Poisoned Hepatocytes[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]	[^{14}C]CCl_4 protein covalent binding[d]
Control (not treated)	1,878 ± 50	0.077 ± 0.010	–
+ Menadione (100 μM)	1,740 ± 29[e]	0.053 ± 0.008[f]	–
CCl_4 (86 μM)	882 ± 44[f] (53%)	0.183 ± 0.013[f]	811 ± 42
+ Menadione (100 μM)	1,270 ± 25[g] (27%)	0.083 ± 0.007[g]	566 ± 58[g]

[a]Cell aliquots (10^7 hepatocytes) were incubated 10 min at 37 C in the presence or in the absence of CCl^4 and/or menadione. All data represent means of 2 experiments in triplicate ± SD.

[b]See Table 1.

[c]See Table 1.

[d]See Table 1.

[e]Not significant as to control group ($p > 0.05$).

[f]Significant as to control group ($p < 0.003$).

[g]Significant as to CCl_4 group ($p < 0.001$).

dation stimulators, Fe^{3+} and cumene hydroperoxide. Our data indicate that, in isolated hepatocytes, it is possible to induce a high degree of lipid peroxidation without inducing strong changes in lipoprotein secretion.

Table 4 shows that 25 μM $FeCl_3$ stimulates malonaldehyde production to the same degree as 86 μM CCl_4; higher $FeCl_3$ concentrations (50-100 μM) stimulate malonaldehyde production much more than does CCl_4. Only with 100 μM $FeCl_3$ is lipoprotein secretion reduced; this reduction, however, is very low.

Experiments with cumene hydroperoxide (Table 5) further strengthen the dichotomy between degree of lipid peroxidation and degree of inhibition of lipoprotein secretion. Cumene hydroperoxide, at concentrations (100-200 μM) that are much more active than CCl_4 in stimulating malonaldehyde formation, is much less active than CCl_4 in reducing lipoprotein secretion.

These results may be interpreted either in terms of poor influence of lipid peroxidation on lipoprotein secretion (this interpretation would fit with the results obtained with free radical scavengers) or in terms of different peroxidation pathways. In other words, differences between CCl_4, $FeCl_3$ and cumene hydroperoxide stimulated lipid peroxidation may be: (a) "topographical," i.e., different cell sites are involved; (b) "chemical," i.e., different sequences, different intermediates or end-products are operative.

Regarding iron-induced lipid peroxidation, peroxidative breakdown of membrane lipids in the presence of NADPH, ADP and Fe^{3+} has

TABLE 4

Lipoprotein Secretion and Malonaldehyde Production from [^{14}C]Palmitic Acid Prelabeled Hepatocytes Treated with CCl_4 or $FeCl_3$[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]
Control	5,947 ± 125	0.160 ± 0.032
CCl_4 (86 μM)	1,198 ± 145[e] (80%)	0.450 ± 0.060[e]
$FeCl_3$ (25 μM)	5,806 ± 55[f]	0.481 ± 0.029[e]
$FeCl_3$ (50 μM)	5,765 ± 238[f]	0.730 ± 0.018[e]
$FeCl_3$ (100 μM)	4,654 ± 254[d] (22%)	0.770 ± 0.046[e]

[a]Cell aliquots (10^7 hepatocytes) were incubated 30 min at 37 C in the presence or in the absence of CCl_4 or $FeCl_3$. All data represent mean of 3 experiments in triplicate ± SD.

[b]See Table 1.

[c]See Table 1.

[d]Significant as to control group ($p < 0.05$).

[e]Significant as to control group ($p < 0.001$).

[f]Not significant as to control group ($p > 0.05$).

TABLE 5

Lipoprotein Secretion and Malonaldehyde Production from [^{14}C]Palmitic Acid Prelabeled Hepatocytes Treated with CCl_4 or Cumene Hydroperoxide[a]

Experimental groups	Lipoprotein secretion[b]	Malonaldehyde production[c]
Control	5,853 ± 261	0.135 ± 0.020
CCl_4 (86 μM)	463 ± 18[e] (92%)	0.360 ± 0.020[e]
Cumene hydroperoxide (50 μM)	4,953 ± 178[d] (15%)	0.212 ± 0.017[d]
(100 μM)	3,872 ± 249[e] (34%)	0.480 ± 0.029[e]
(200 μM)	1,814 ± 220[e] (69%)	1.480 ± 0.063[e]

[a]Cell aliquots (10^7 hepatocytes) were incubated 30 min at 37 C in the presence or in the absence of CCl_4 or cumene hydroperoxide. All data represent means of 2 experiments in triplicate ± SD.

[b]See Table 1.

[c]See Table 1.

[d]Significant as to control group ($p < 0.05$).

[e]Significant as to control group ($p < 0.001$).

been observed not only in liver microsomes (23), but also in liver mitochondria (24) and lysosomes (25). This may explain why, in whole hepatocyte systems, only the cell treatment with very high Fe^{3+} concentrations induces peroxidative inactivation of the microsomal enzyme glucose-6-phosphatase (26,27), whereas, to give a similar impairment, one-half or one-third the CCl_4 concentration is sufficient (27).

Furthermore, CCl_4 and iron lipoperoxidative effects show chemical differences in terms of initiating reactions, free radical intermediates (1,28) and probably in terms of aldehydic end-products. In fact, recent analyses of the aldehydic patterns produced by CCl_4- or iron-mediated peroxidation of microsomal lipids have shown remarkable qualitative differences between the 2 treatments (Esterbauer, Cheeseman, Dianzani, Poli and Slater, manuscript in preparation).

As for lipid peroxidation stimulated by cumene hydroperoxide, several hemoproteins, including cytochrome P-450, cytochrome b_5 and cytochrome c, have been demohstrated to act as catalysts (14,29); cytochrome P-450 is 10 times more effective than the 2 other cytochromes (14). Several arguments exist against the involvement of a free radical chain reaction in the initiation step of this lipoperoxidative model system, suggesting a mechanism more like that of lipoxygenases (14). Cumene hydroperoxide seems to exert its effects mainly through the microsomal cytochrome P-450-dependent enzyme system; so, it should impair lipoprotein secretion mainly at the step of combination of phospholipid, triglyceride and lipid-carrier protein to form lipoprotein, whereas CCl_4 has been also demonstrated to affect the triglyceride transport from the liver to the plasma (11). Experiments to determine types and biological activity of aldehydes produced by cumene hydroperoxide catalyzed lipid peroxidation are now in progress.

The other possibility, that lipid peroxidation per se has little influence on liver lipoprotein secretion, and that this cell mechanism is mainly deranged by covalent binding of CCl_4 cleavage products, deserves maximal attention.

A major role of covalent binding has been proposed for some CCl_4-induced in vivo damages, such as cytochrome P-450 inactivation (30) and polyribosome dissociation (31), as well as for the reduction of aminopyrene demethylase activity and cytochrome P-450 content of isolated hepatocytes poisoned with CCl_4 (10). In addition to these data, it was observed (32) that protein synthesis in liver cell-free systems was also inhibited by $CBrCl_3$, a halogen derivative of methane that sparks lipid peroxidation in a way very similar to CCl_4 (1). The effect of $CBrCl_3$ on protein synthesis, however, seemed to be dependent on a mechanism involving free radical attack, unrelated to lipid peroxidation (32).

In conclusion, there is increasing evidence for a prominent role of covalent binding of reactive metabolites of CCl_4 in early changes induced by this haloalkane in liver cells. In other words, CCl_4-induced liver injury probably results from both covalent binding of CCl_4 metabolites and lipid peroxidation.

ADDENDUM

While this paper was in press, our attention was drawn to the work reported by Griffin, B.W. (in "Microsomes, Drug Oxidations, and Chemical Carcinogenesis," Vol. 1, pp. 319-322, Academic Press, New York, 1980). This author gives evidence for the involvement of free radical species in the reaction of cumene hydroperoxide with hemoproteins. This must be considered when examining our discussion on the mechanism of action of the compound.

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Glycerolipid Biosynthesis in Rat Adipose Tissue: VIII. Effect of Obesity and Cell Size on [^{14}C]Acetate Incorporation into Lipids

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ABSTRACT

[^{14}C] Acetate incorporation into different lipid fractions was measured as a function of adipocyte size by using the larger and smaller adipocytes derived from Sprague-Dawley rats. In both the larger and smaller adipocytes, [^{14}C] acetate was incorporated into phospholipid, diacylglycerol, free fatty acid and triacylglycerol fractions. Although the rates of lipid formation were significantly higher in the larger adipocytes compared to the smaller ones, the proportions of the various lipids formed from [^{14}C] acetate did not change significantly as a function of cell size. In some experiments, isolated adipocytes derived from obese Zucker rats were fractionated further to isolate an adipocyte preparation which was similar in size to those obtained from lean animals. The matching adipocytes derived from lean and obese animals did not differ significantly with respect to lipid formation from [^{14}C]-acetate. These studies suggest that the larger adipocytes are more active in lipogenesis from [^{14}C]-acetate than the smaller ones and that the increased capacity of lipogenesis in obese adipose tissue noted previously (Biochem. J., 170, 153-160, 1978) is not an intrinsic property of all the obese adipocytes, but is limited mainly to the larger adipocytes.

Genetically transmitted obesity has been described in several strains of rodents (1). In obese Zucker 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 hyperphasic and show increased body fat, as a result of an increase in adipocyte size and number (1). Obesity in this animal model is accompanied by enhanced fatty acid synthesis, increased triacylglycerol formation from [^{14}C] acetate, [^{14}C] glucose and [^{14}C]-glycerol-3-phosphate, and increased lipoprotein lipase activity in adipose tissue (1-4).

In this investigation, a new approach was used to study adipocyte metabolism in lean and obese animals. Adipocytes isolated from obese animals were fractionated further to obtain an adipocyte preparation which was similar in size to those present in lean animals. The resultant adipocyte preparations from lean and obese animals were then used to study [^{14}C] acetate incorporation into lipids. Since obesity is associated with the enlargement of adipocytes and the fact that several metabolic processes have been shown to increase with the adipocyte size (5-11), it is thought that such an approach would identify the specific metabolic changes associated with obesity and separate them from those related to the cellular enlargement. Recently, we have used this approach successfully to dissociate the age-related changes in glycerolipid metabolism from those related to the cellular enlargement (12). We have examined here the incorporation of [^{14}C] acetate into lipid, since this parameter is known to be altered in the adipose tissues of obese rats (2). In addition, various problems encountered in processing the obese adipocytes have been described.

MATERIALS AND METHODS

[1,2-^{14}C] Acetate (sp radioactivity 96.8 mCi/mmol) was purchased from New England Nuclear Corporation, Boston, MA. Collagenase (158 units/mg protein, lot 4196 CLS 49 S 240) was obtained from Worthington Corp., Freehold, NJ. Most of the other chemicals were of AR-grade quality and were purchased from the sources reported previously (2,13). Male obese Zucker rats (fa/fa) and their lean controls (FA/FA) were obtained from Dr. P.R. Johnson, Vassar College, Poughkeepsie, NY. Rats were maintained on a laboratory chow diet (Purina Laboratory, St. Louis, MO) and sacrificed by decapitation when they were 8-10 weeks old.

Initial studies were conducted with the adipose tissues from albino rats to determine optimal incubation conditions. Sprague-Dawley rats of the same age as the obese rats were from our animal colony. The incubation conditions developed with adipose tissues from these animals were found to be satisfactory to measure [^{14}C] acetate incorporation into lipids in the adipocytes derived from both lean and obese rats.

Isolation of Adipocytes and Radiochemical Assays

Epididymal fat pads from individual animals were used to isolate adipocytes by the method of Rodbell (14) with slight modification (8).

During isolation and washing, a considerable loss of obese adipocytes was apparent. This was evident from the presence of oil droplets and also from the measurements of the cell diameters between the isolated adipocyte preparations and the adipocytes present in the tissue fragments. To avoid contamination of adipocytes from oil droplets, these droplets were removed as much as possible, using a syringe, without disturbing the adipocyte layer.

In obese rats, the cells from the tissue fragments were 174 ± 24 μ in diameter (mean ± SD from 5 different experiments using 5 different rats) whereas the isolated adipocytes were 92 ± 4.5 μ in diameter ($p<.001$). In lean Zucker rats, these values were 72 ± 7 and 60 ± 8 μ in diameter, respectively (5 experiments with 5 different rats). In Sprague-Dawley rats, these values were 70 ± 3 and 66 ± 4 μ, respectively (3 experiments with 3 different rats). Thus, some loss of larger adipocytes was apparent from lean Zucker and Sprague-Dawley rats. However, it was less than the loss encountered with the obese animals. As far as the cell size was concerned, adipocytes derived from Sprague-Dawley rats did not differ significantly from those obtained from lean Zucker rats. For cell size determination, isolated adipocytes were fixed in O_sO_4 for 24-48 hr at 37 C (15). After processing (8,15), the cell diameter of the osmium-fixed cells was measured using a micrometer eyepiece (8).

In order to obtain adipocytes of similar sizes, the adipocytes derived from lean and obese rats were filtered through a 52-μ nylon screen (Tetco Inc., Elmsford, NY) as described previously (8).

To determine the conversion of [^{14}C] acetate into lipid, the smaller and larger adipocytes (1-2 × 10^5 cells/vial) were incubated in the presence of 2 ml Krebs-Ringer bicarbonate buffer containing 4% albumin, 0.5 mM acetate, 1 μCi [^{14}C] acetate, and 20 mM glucose at 37 C for 60-90 min under O_2CO_2(19:1) (2,16). The reactions were terminated by the addition of 5 ml of chloroform/methanol (2:1, v/v). Tissue lipids, along with the medium, were extracted with chloroform/methanol (2:1, v/v) and purified as described by Folch et al. (17). One portion (100 μl) of the lipid sample was counted for radioactivity to measure incorporation of label into total lipids. The other portion (50 μl) was subjected to thin layer chromatography (Silica Gel G, E. Merck, Darmstadt, W. Germany) by using the solvent system hexane/diethylether/acetic acid (70:25:2, by vol) (2). The radioactivie lipids formed were processed as described earlier (2).

The rates of lipid formation were expressed in relation to adipocyte number which was determined using 0.2 mm hemocytometer (8).

RESULTS AND DISCUSSION

Figure 1 shows the conversion of [^{14}C]-acetate into lipids as a function of time, cell size and number. At 3 × 10^5 to 8 × 10^5 adipocytes, [^{14}C] acetate incorporation into lipids was linear with time up to 90 min in the case of both large and small adipocytes. In the standard assays, incubation was routinely continued for 60 min and the concentration of adipocytes was adjusted between 1 × 10^5 to 2 × 10^5 adipocytes/sample.

The rates of [^{14}C] acetate incorporation into lipids were significantly higher in the larger adipocytes compared to the smaller ones (Fig. 1, Tables 1 and 2). This was also true for the adipocytes derived from lean or obese Zucker rats. A similar effect of cell size on [^{14}C]-acetate incorporation into lipid was reported recently by Hood and Thornton (11) for a sheep adipose tissue by using a different technique for the isolation of adipocytes of various sizes.

The smaller adipocytes derived from obese and lean Zucker rats were similar in size (B and C adipocyte samples from Table 2) and did not differ significantly with respect to [^{14}C] acetate incorporation into lipids. In all the adipocyte preparations, the [^{14}C] acetate was incorporated into phospholipid, diacylglycerol, free fatty acid and triacylglycerol fractions. Although the rates of lipid formation in the larger adipocytes were significantly higher compared with the smaller ones, the distribution of [^{14}C] acetate into different lipid fractions did not differ significantly as a function of cell size or obesity.

Previously, we had noted that obese adipose tissue is several times more active in lipid synthesis from [^{14}C] acetate than lean adipose tissue (2). If this is an intrinsic property of the obese adipose tissue, it should be present in all the adipocytes regardless of their sizes. However, when the adipocytes of equal sizes from lean and obese animals were compared for their capacity for lipid synthesis from [^{14}C] acetate, no substantial differences were observed. The larger adipocytes from obese rats were significantly more active in lipid synthesis than the smaller adipocytes derived from lean and obese animals. Thus, it seems that the increased capacity of lipogenesis from [^{14}C] acetate in the obese adipose tissue noted previously (2) is not an intrinsic property of the obese adipocytes, but probably resulted from the presence of

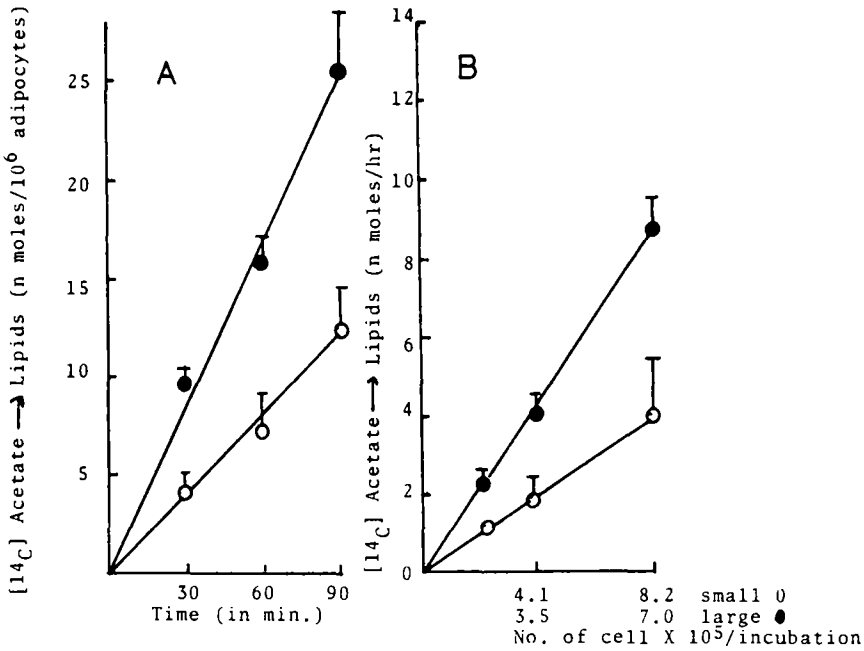

FIG. 1. Effect of cell size on [^{14}C]acetate incorporation into lipid. In these experiments, the larger (•) and the smaller (○) adipocytes derived from Sprague-Dawley rats were incubated in triplicate with [^{14}C]acetate for various times (A) and at various adipocyte concentrations (B). For A, the concentrations of the larger and smaller adipocytes were, respectively, 0.35 X 10^6 cells/incubation and 0.41 X 10^6 cells/incubation. For B, the incubation time was 1 hr.

larger adipocytes in the obese adipose tissue. The other possibility is that this subpopulation of obese adipocytes may be atypical in nature in the sense that the increased capacity of lipogenesis associated with obesity is not fully developed in these adipocytes. Since, in this study, mainly basal capacity of lipogenesis is measured, one also cannot rule out the possibility that the adipocytes of equal sizes derived from lean and obese animals, having a similar capacity of lipid synthesis in the basal state, may respond differently to lipogenic hormones, such as insulin. Possibly, these differences in hormonal response could be responsible for the various abnormalities in the obese adipocytes, including their larger size and increased lipid synthetic capacity. Nevertheless, it is clear from these studies that the larger adipocytes are more active in lipogenesis from [^{14}C]acetate than the smaller adipocytes and that these differences are present in the adipose tissues of both lean and obese rats.

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TABLE 1

Effect of Cell Size on [^{14}C] Incorporation into Lipids[a]

Body wt (g)	Adipose wt (g)	Adipocyte sample	Mean cell size (μ)	[^{14}C]acetate→lipid (nmol/hr/10^6 adipocytes)	Distribution of (%), ^{14}C into: PL	DG	FA	TG
294 ± 31	2.49 ± 0.95	Small	41 ± 7	6.25 ± 1.71	16 ± 5	26 ± 7	17 ± 5	41 ± 8
		Large	66 ± 9[b]	12.42 ± 4.12[b]	19 ± 10	30 ± 7	20 ± 4	31 ± 6

[a]Assays were conducted in triplicate as described in Methods by using large and small adipocytes derived from epididymal fat-pads from Sprague-Dawley rats. Each value is the mean ± SD from 5 or 6 experiments. PL = phospholipid; DG = diacylglycerol; FA = fatty acids; TG = triacylglycerol. The smaller adipocytes were separated from the larger ones by washing through 52-μ nylon filter with the use of a 1=ml Biotip pipette and several small volumes of buffer as described previously (8). In each experiment, epididymal fat-depots from 1 animal were used.

[b]Significantly different from small adipocytes, $p<.01$.

TABLE 2

[^{14}C]Acetate Incorporation into Lipids as a Function of Adipocyte Size in Lean and Obese Rats[a]

Group	Body wt (g)	Adipose wt (g)	Adipocyte sample	Mean cell size (μ)	[^{14}C]Acetate→lipid (nmol/hr/10^6 adipocytes)	Distribution (%) of ^{14}C into: PL	DG	FA	TG
Obese	267 ± 27	4.26 ± 0.59	A	85 ± 12	16.38 ± 4.19	34 ± 11	33 ± 9	14 ± 8	19 ± 6
			B	39 ± 4	4.18 ± 1.60	36 ± 14	22 ± 9	23 ± 13	18 ± 7
Lean	205 ± 21[b]	0.84 ± 0.15[c]	C	37 ± 5	5.57 ± 1.28	24 ± 2	21 ± 11	24 ± 5	30 ± 9
		p value between	A & B	<.01	<.01	ns	ns	ns	ns
			B & C	ns	ns	ns	ns	ns	ns
			A & C	<.01	<.01	ns	ns	ns	ns

[a][^{14}C]Acetate incorporation into lipids was studied as described in Methods. Assays were conducted in duplicate. Obese adipocytes were subjected to filtration to obtain an adipocyte preparation which was similar in size to those present in lean animals. Each value is mean ± SD from 5 different experiments. A = large adipocytes from obese rats; B = small adipocytes from obese rats; C = small adipocytes from lean rats; no substantial amounts of large adipocytes from lean rats were available to conduct various measurements. In each experiment, epididymal fat depots from one animal were used.

[b]$p<.01$; [c]$p<.001$.

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Absence of Cholesterogenesis Regulation in the Liver and Prostate of the BIO 87.20 Hamster

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ABSTRACT

Normal, adult golden Syrian hamsters and the inbred stain BIO 87.20 Syrian hamsters were maintained on either control, cholesterol, candicidin or clofibrate diets for time periods of up to 4 months. The ventral prostate gland in both species was found to synthesize cholesterol at a greater rate than the liver. Also, our results show that, while hepatic cholesterol synthesis in the normal Syrian hamster is under feedback control with dietary cholesterol, hepatic cholesterol synthesis in the BIO 87.20 hamster, and prostatic cholesterol synthesis in either species, is under no such control. This apparent regulatory defect in the BIO 87.20 hamster, which results in a dramatic accumulation of cholesterol in the liver and serum, renders this animal a potentially valuable in vivo model for the study of cholesterol-related disorders.

INTRODUCTION

The inbred Syrian hamster (*Mesocricetus auratus*), designated as BIO 87.20, was first described by Homburger and Nixon (1) in 1970, to exhibit in the male, genetic and age-related cystic prostatic hypertrophy. After the hypocholesterolemic drug, candicidin, had been reported for its effect on benign prostatic enlargements in dogs (2) and in man (3-5), further studies in our laboratory (6) revealed that candicidin and the structurally unrelated hypocholesterolemic ion exchange resin, colestipol, both reduced the enlargement of the prostate gland in the BIO 87.20 male hamster and also reduced the overall cholesterol content of the gland. It was suggested that this effect on the prostate gland was related in some way to the known inhibitory action of candicidin and colestipol on absorption-resorption of cholesterol and bile acids, respectively, in the enterohepatic circulation. Both drugs thus effectively prevented the micellar solubilization of cholesterol in the intestinal tract.

Since the reduction of the enlarged prostate with hypocholesterolemic drugs might be related to decreased body cholesterol levels or possibly to changes in cholesterogenesis in the gland, studies were designed to compare the effects of dietary cholesterol, candicidin, and clofibrate on the content and rate of synthesis of cholesterol in the prostate gland and liver of the BIO 87.20 and normal male Syrian hamsters. In this study, the polyene macrolide antibiotic candicidin, as an inhibitor of cholesterol absorption-resorption (7) and the synthetic drug clofibrate or [ethyl-2-(4-chlorophenoxy)-2-methyl propionate], as a known inhibitor of cholesterol synthesis (8) were selected for study.

MATERIALS AND METHODS

Male BIO-RB golden Syrian hamsters as controls and male BIO 87.20 Syrian hamsters, 6 months of age, were purchased from Telaco, Bar Harbor, ME. Cholesterol was purchased from Fisher Scientific Co., Pittsburgh, PA; the polyene antifungal antibiotic candicidin (lot 671NPF-7) was obtained from S.B. Penick and Co., Lyndhurst, NJ. The animals, in groups of 6 or more, were placed on control diet (Purina Lab Chow) or on control diets supplemented with either cholesterol (1%), candicidin (75 mg/kg body wt/day), or clofibrate (Atromid S, 0.3%) for a period of up to 6 months. All animals were maintained on daily rations comparable to the average for the candicidin-treated group. All animals were also maintained with water ad libitum and under an alternating 12-hr light and 12-hr dark schedule.

At 2-month intervals, the animals were sacrificed. After anesthetization with intraperitoneal injections of sodium barbital, the animals were exsanguinated and the blood used for the preparation of sera. The 2 lobes of the ventral prostate, free of the fat covering, and the liver were excised for the determination of cholesterol content and rate of cholesterogenesis.

The total amount of cholesterol in sera and in the liver was determined colorimetrically by the procedure of Parekh and Jung (9). Serum and liver cholesterol concentrations were expressed as mg cholesterol/100 ml serum (mg %) and mg cholesterol/g tissue, respectively.

For the determination of the in vitro incorporation of radiolabeled acetate into cholesterol, the excised ventral prostate and the liver were minced and weighed in tared Teflon test tubes. Approximately 30-mg and 200-mg samples of minced prostate and liver tissues on a

wet wt basis, respectively, were used to study the incorporation of acetate into cholesterol. The minced tissues were kept in ice until further use. Subsequently, the tissues were incubated with 2 ml Hank's Balanced Salt solution supplemented with 0.2% glucose and 1 μCi/ml of 2-[^{14}C]acetate, sp act 50 mCi/mmol, New England Nuclear, Boston, MA, (pregassed with 95% O_2 and 5% CO_2) at 37 C for 2 hr on a constant speed shaker. At the end of the incubation period, the reaction was terminated by instant freezing of the tubes in a Dry Ice/acetone bath.

For the analysis of radioactivity in cholesterol, the tissues were saponified by the addition of alcoholic KOH to a final concentration of 10% KOH and 50% ethanol (95%) at 75 C for 75 min. Nonsaponified lipids were pooled by repeated extractions with *n*-hexane. The hexane extracts were evaporated under nitrogen and digitonin precipitation was done according to the procedure of Sperry (10). The cholesterol-digitonin complex was dissolved in 1 ml of methanol and 0.1-ml aliquots were counted in duplicate for [^{14}C]activity in a Packard Scintillation Counter. The rates of cholesterol synthesis were expressed as counts/min/g tissue $\times 10^5$.

RESULTS

In a preliminary study with the BIO 87.20 male Syrian hamster, the effects of dietary cholesterol and candicidin on serum cholesterol concentration was determined over a period of 6 months. Compared to animals on a control diet, it was immediately apparent that 1% cholesterol feeding produced a dramatic and marked hypercholesterolemia in this animal model. With an initial 0-time serum cholesterol level of 93.6 ± 7.7 mg % whereas the control animals exhibited levels of 112.0 ± 5.2, 105.2 ± 7.0 and 95.7 ± 3.5 mg %, respectively, after 2, 4 and 6 months, the cholesterol-treated animals exhibited levels of 177.6 ± 30.9, 286.3 ± 32.5, and 476.6 ± 35.0, respectively. Animals on a candicidin diet (75 mg/kg body weight) exhibited, in contrast, serum cholesterol levels of 85.9 ± 9.5, 67.3 ± 5.0, and 45.9 ± 4.3, respectively, after 2, 4 and 6 months of treatment. Examination of the liver of the cholesterol-treated animals also revealed a dramatic accumulation of cholesterol compared to the livers of control and candicidin-treated animals. In fact, 1% cholesterol feeding to these animals over a period of 6 months resulted in a ca. 50% mortality rate. For this reason, all further studies were terminated at 4 months.

This initial study was followed by a second study in which the effects of dietary cholesterol, candicidin and clofibrate on the serum and liver cholesterol content of BIO 87.20 and normal male Syrian hamsters were compared. In this study, male BIO 87.20 Syrian hamsters and golden Syrian hamster controls were treated, respectively, with control, cholesterol, candicidin and clofibrate diets over a period of 4 months. In all groups, no significant differences in food intake were noted. The serum cholesterol levels for these animals after 2 and 4 months of treatment are given in Table 1. In these results, there is no significant difference in serum cholesterol levels between the BIO 87.20 and control Syrian

TABLE 1

Cholesterol Content of the Serum of Normal Syrian and BIO 87.20 Male Hamsters

Regimen	Normal Syrian hamster–serum cholesterol (mg %)		
	0 Time	2 Months	4 Months
Control diet	81.42 ± 6.88*(6)**	87.55 ± 7.56(6)	78.48 ± 6.42(6)
Cholesterol diet		101.42 ± 9.33(6)	90.17 ± 8.48(6)
Candicidin diet		51.38 ± 5.18(6)[b]	46.72 ± 7.13(6)[b]
Clofibrate diet		71.88 ± 6.42(6)	67.32 ± 6.13(6)
Regimen	**BIO 87.20 hamster–serum cholesterol (mg %)**		
	0 Time	2 Months	4 Months
Control diet	86.15 ± 5.20*(6)**	94.20 ± 7.35(6)	88.50 ± 7.10(6)
Cholesterol diet		108.40 ± 22.60(6)	206.55 ± 20.70(6)[b]
Candicidin diet		71.45 ± 6.25(6)[a]	53.50 ± 5.85(6)[b]
Clofibrate diet		78.63 ± 7.10(6)	72.51 ± 6.27(6)

*Mean ± SE.
**Number of animals/group.
Significance: p value [a]<0.05; [b]<0.01.

TABLE 2

Cholesterol Content of the Liver of Normal Syrian and BIO 87.20 Male Hamsters

Regimen	Normal Syrian hamster—liver cholesterol (mg/g)		
	0 Time	2 Months	4 Months
Control diet	1.72 ± .24*(6)**	2.01 ± .31(6)	1.84 ± .29(6)
Cholesterol diet		2.94 ± .22(6)[a]	3.07 ± .38(6)[a]
Candicidin diet		2.48 ± .36(6)	2.71 ± .34(6)
Clofibrate diet		1.26 ± .10(6)[a]	1.43 ± .13(6)
	BIO 87.20 hamster—liver cholesterol (mg/g)		
Regimen	0 Time	2 Months	4 Months
Control diet	2.63 ± .31*(6)**	2.91 ± .38(6)	3.17 ± .28(6)
Cholesterol diet		19.42 ± 3.12(6)[b]	48.11 ± 5.63(6)[b]
Candicidin diet		2.17 ± .44(6)	2.83 ± .32(6)
Clofibrate diet		1.12 ± .10(6)[b]	1.27 ± .16(6)[b]

*Mean ± SE.
**Number of animals/group.
Significance: p value [a]<0.05; [b]<0.01.

hamsters after 2 months on the cholesterol diet. On the other hand, serum cholesterol levels in the BIO 87.20 hamsters increased by 130% after 4 months. The whole blood upon removal also appeared quite milky. In contrast, both the candicidin and clofibrate treatments decreased serum cholesterol levels in the normal Syrian hamsters and the BIO 87.20 hamsters after 2 and 4 months of treatment.

The cholesterol content of the livers of both groups of hamsters fed control diet and diets containing cholesterol, candicidin and clofibrate was also measured and is given in Table 2. On a cholesterol diet, the liver cholesterol content after 2 and 4 months increased by 46 and 67%, respectively, in the control hamsters compared to ca. 500 and 1500% in the BIO 87.20 hamsters. This dramatic increase of liver cholesterol content in the BIO 87.20 hamsters resulted in a wholly abnormal greyish-white liver. With the clofibrate diet, the liver cholesterol content of the normal hamsters decreased by 37 and 22%, respectively, compared to animals on control diet after 2 and 4 months. In the BIO 87.20 hamster, the decrease of liver cholesterol content was ca. 60% after both intervals of time. There was no significant change in liver cholesterol levels in all animals maintained on a candicidin-containing diet.

In order to determine the effects of the different dietary treatments on rates of cholesterol synthesis in both groups of animals after 2 and 4 months, the liver and prostate gland were specifically selected for study here. The incorporation of carbon-14 labeled acetate into cholesterol was specifically measured in minced tissues of excised liver and prostate gland. The results obtained with the livers of both groups of Syrian hamsters are presented in Table 3.

In the normal control hamster maintained on a cholesterol diet for 2 and 4 months, the rate of cholesterol synthesis in the liver decreased by 88 and 82%, respectively, compared to that of the animals on a control diet. In the BIO 87.20 hamsters on a cholesterol diet, there was no apparent decrease in the rate of cholesterol synthesis compared to those animals on a control diet. It is also notable that the rate of liver cholesterol synthesis in the BIO 87.20 hamster was significantly lower than in the normal control hamster. Also quite apparent is that the synthesis of cholesterol in the liver of the BIO 87.20 Syrian hamster is not under feedback regulation as it is in the Syrian hamster control. For all animals on the candicidin diet, the rate of cholesterol synthesis approximately doubled after 2 and 4 months of treatment. In contrast, on a clofibrate diet, the rates of cholesterol synthesis in the liver of the control hamsters decreased 78 and 69%, respectively, after 2 and 4 months whereas in the BIO 87.20 hamster, the decrease in synthesis was 30 and 50%, respectively, compared to that of the animals on a control diet.

The rates of cholesterol synthesis in the prostate gland of all animals were also determined and are given in Table 4. It is immediately apparent that the rate of cholesterol synthesis in the prostate gland of all animals was 2- to 3-fold higher than in the liver. It is also apparent that the rate of prostate cholesterol

TABLE 3

In Vitro Incorporation of [^{14}C] Acetate into Cholesterol in the Liver of Normal Syrian and BIO 87.20 Male Hamsters

Regimen	Normal Syrian hamster—rate of synthesis (cpm/g tissue × 10^5)		
	0 Time	2 Months	4 Months
Control diet	.523 ± .021*(6)**	.658 ± .016(6)	.627 ± .020(6)
Cholesterol diet		.076 ± .011(6)[b]	.113 ± .023(6)[b]
Candicidin diet		1.527 ± .173(6)[b]	1.463 ± .168(6)[b]
Clofibrate diet		.147 ± .033(6)[b]	.190 ± .051(6)[b]
Regimen	BIO 87.20 hamster—rate of synthesis (cpm/g tissue × 10^5)		
	0 Time	2 Months	4 Months
Control diet	.462 ± .097*(6)**	.380 ± .088(6)	.403 ± .093(6)
Cholesterol diet		.351 ± .072(6)	.375 ± .091(6)
Candicidin diet		.756 ± .127(6)[b]	.800 ± .113(6)[b]
Clofibrate diet		.266 ± .043(6)	.200 ± .036(6)[a]

*Mean ± SE.
**Number of animals/group.
Significance: p value [a]<0.05; [b]<0.01.

synthesis was not affected by cholesterol feeding in either the normal hamster control or the BIO 87.20 hamster, reflecting the lack of negative feedback regulation. On the candicidin diet, the rate of prostate cholesterol synthesis increased by ca. 60-70% in both Syrian hamster groups whereas on the clofibrate diet, the inhibitory effect on cholesterogenesis was more pronounced in the BIO 87.20 hamsters than it was in the normal hamster controls. After 2 and 4 months of clofibrate treatment, the rates of cholesterol synthesis decreased by 45 and 49%, respectively, in the normal hamster whereas in the BIO 87.20 hamster, the decline was 82 and 86%, respectively.

DISCUSSION

In the current studies with the male BIO 87.20 Syrian hamster, it was our original intention to determine the effect of dietary cholesterol on cholesterol metabolism of the prostate gland as possibly related to the age-dependent and spontaneous cystic prostatic hypertrophy exhibited by this animal model (1). Since our earlier investigations (6) had revealed that the chemically unrelated hypocholesterolemic drugs, candicidin and colestipol, both prevented this enlargement and reduced the cholesterol content of the prostate gland, the exact role, if any, of cholesterol in this animal disease process remained obscure.

It was immediately apparent from the results of the first study presented here that cholesterol feeding to the BIO 87.20 hamster produced an accumulation of cholesterol in the liver and hypercholesterolemia over a period of 6 months of treatment.

Although significant increases of cholesterol in serum and liver of hamsters on a cholesterol diet have been previously observed by Ho (11), it is well known that cholesterogenesis in the liver of common laboratory rodents is usually quite responsive to dietary cholesterol (12). The presence of moderate amounts of cholesterol in the diet leads to a rapid and, depending on the species, near total cessation of liver cholesterol synthesis. This is achieved by an efficient negative feedback control system with the rate-limiting enzyme β-hydroxy-β-methyl glutaryl CoA reductase (13,14).

Considering the marked increase of cholesterol in the serum and liver of the BIO 87.20 hamsters on a cholesterol diet, it became important to determine the presence of feedback control of cholesterogenesis in the liver of this animal disease model compared to normal Syrian hamsters. While the BIO 87.20 hamsters on a cholesterol diet after 4 months revealed significant increases in serum cholesterol levels and most surprising increases in liver content, the control Syrian hamsters exhibited no significant increases in serum cholesterol and only slight increases in liver content.

The difference in both hamster groups could be readily explained from the present results obtained in the stereogenesis studies. The determination of the rates of cholesterol synthesis in the liver and prostate of the BIO 87.20 hamster clearly revealed the lack of

TABLE 4

In Vitro Incorporation of [^{14}C] Acetate into Cholesterol in the Prostate of Normal Syrian and BIO 87.20 Male Hamsters

Regimen	Normal Syrian hamster—rate of synthesis (cpm/g tissue X 10^5)		
	0 Time	2 Months	4 Months
Control diet	1.897 ± .233*(6)**	2.130 ± .184(6)	2.031 ± .212(6)
Cholesterol diet		2.214 ± .167(6)	2.163 ± .185(6)
Candicidin diet		3.369 ± .187(6)[b]	3.421 ± .203(6)[b]
Clofibrate diet		1.168 ± .134(6)[b]	1.030 ± .117(6)[b]
Regimen	BIO 87.20 hamster—rate of synthesis (cpm/g tissue X 10^5)		
	0 Time	2 Months	4 Months
Control diet	.939 ± .122*(6)**	.919 ± .143(6)	.972 ± .157(6)
Cholesterol diet		1.065 ± .166(6)	1.122 ± .138(6)
Candicidin diet		1.479 ± .238(6)	1.630 ± .217(6)[a]
Clofibrate diet		.165 ± .033(6)[b]	.127 ± .021(6)[b]

*Mean ± SE.
**Number of animals/group.
Significance: p value [a]<0.05; [b]<0.01.

negative feedback control of endogenous cholesterol synthesis in either organ. While the normal Syrian hamster on a cholesterol diet clearly exhibited feedback control of liver cholesterol synthesis, it also lacked such control in the prostate gland.

The relationship of the absence of negative feedback control of cholesterogenesis in the liver and prostate gland of the male BIO 87.20 hamster to the other pathological conditions in this inbred hamster line, viz., development of cystic prostatic hypertrophy and susceptibility to cholesterol feeding, may be more than coincidental. Compared to normal Syrian hamsters, it was quite evident that the BIO 87.20 hamster did not fare well on the 1% cholesterol diet exhibiting marked hypercholesterolemia and greyish-white mottled livers after a relatively short period of feeding.

Other pathological conditions of the liver such as hepatomas are also associated with the loss of negative feedback regulation of cholesterol synthesis seen in normal liver (15). Neoplasmas, in general, are also characteristically associated with the absence of this control of cholesterogenesis (16). Derangements in cholesterol metabolism have also been observed in some leukemias (17). In this particular case, aside from this loss of regulation in cholesterogenesis, the gross appearance of the livers of the BIO 87.20 hamsters on a normal diet appear to be, otherwise, quite normal and similar to those of the normal control Syrian hamsters.

With both the BIO 87.20 and normal Syrian hamsters, the diets supplemented with the hypocholesterolemic drugs candicidin and clofibrate produced significant reductions in serum cholesterol levels. While clofibrate reduced the liver cholesterol content in both groups of animals, candicidin produced no such significant changes.

Clofibrate and candicidin clearly exhibited opposing effects on the rates of cholesterol synthesis in the liver and prostate gland of both hamster lines. Treatment with clofibrate, which illicits its hypocholesterolemic effect by inhibiting cholesterol synthesis, clearly displayed this inhibition in both organs of both hamster lines. Candicidin is believed to lower serum cholesterol levels by preventing the absorption-resorption of cholesterol from the intestinal tract, thus interfering with the enterohepatic circulation of cholesterol. In both the normal Syrian and BIO 87.20 hamsters, dietary candicidin clearly stimulated cholesterol synthesis in the liver to a significant extent, but less so in the prostate gland. In the normal Syrian hamster, for which hepatic cholesterol synthesis is under feedback control by exogenous cholesterol, the stimulation of cholesterol synthesis by candicidin treatment is understandable. In the BIO 87.20 hamster, for which hepatic cholesterol synthesis is not under the usual negative feedback regulation by exogeneous cholesterol, candicidin treatment, in sharp contrast to the effects of dietary cholesterol, illicits a marked increase in the hepatic synthesis of cholesterol. This suggests the presence of some sort of positive feedback regulation of cholesterogenesis in the liver of both hamster

lines. When the cholesterol pool of the enterohepatic circulation is lowered by inhibitory action of candicidin on cholesterol absorption-resorption in the small intestine, the synthesis of endogeneous cholesterol in the liver and prostate gland is stimulated.

Another strain of golden hamster maintained on a standard hamster chow was reported (18) to exhibit a marked increase in liver cholesterol ester content. Here, in comparison to normal control animals, the disease model animals exhibited a marked increase in the esterification of newly synthesized cholesterol rather than any alteration in the synthesis of cholesterol from mevalonate. The animals on a normal diet also exhibited hypercholesterolemia as well as hyperlipidemia involving elevated serum triglyceride levels.

The marked accumulation of cholesterol in the serum and livers of the BIO 87.20 male hamster maintained on a 1% cholesterol diet, resulting in death, is now understandable in light of the finding that cholesterogenesis in the liver of this animal disease model is defective in its lack of negative feedback regulation. These animals on a normal hamster chow diet were otherwise quite normal in respect to their serum and liver cholesterol contents and their rates of cholesterogenesis in the liver and prostate gland were reduced compared to normal Syrian hamster controls. It is now evident that the BIO 87.20 male hamster is an important animal disease model for studies involving both the prostate gland and cholesterol metabolism of the liver.

ACKNOWLEDGMENTS

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Age-Related Changes in Biological Parameters in Zucker Rats

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ABSTRACT

Changes in a number of morphological and biochemical parameters were observed in genetically obese Zucker rats and in lean controls between 3 and 58 weeks of age. By 3 weeks, the genetically obese rats had higher proportional (wt/100 g body wt) and absolute amounts of adipose tissue, hyperlipemia affecting all the lipid fractions, and hyperproteinemia compared to lean controls. Obesity, hepatomegaly, high concentrations of hepatic lipids and hyperinsulinemia did not appear until the fifth week. In obese animals, liver lipid concentration reached a maximum at 17 weeks of age and then declined. During this time, the triacylglycerol concentrations in the serum remained stable, whereas the cholesterol and phospholipid concentrations continued to increase. The glycogen concentration in obese animals increased, both absolutely and compared to lean controls, between the 12th and 43rd week of age. From weaning, the Zucker rats, compared to lean controls, exhibited characteristics of obesity (accumulation of adipose tissue, hyperlipemia and hyperproteinemia), which persisted to the age of 58 weeks.

The genetically obese Zucker rat is often used as an experimental model in studies of obesity (1). This type of rat inherits obesity as an autosomal Mendelian recessive trait. The phenotypic expression of the obesity appears a week or 2 after weaning and is characterized not only by hyperphagia, greater weight and a large accumulation of lipids in the storage tissues, but also by hyperlipemia and hyperinsulinemia (2).

Studies done in this laboratory on the Zucker rat have shown changes in certain morphological and biochemical parameters during its lifetime. We report here the results obtained for 3- to 58-week-old rats.

MATERIALS AND METHODS

Rats

Male Zucker rats (CSEAL, 45045 Orléans Cedex, France), either genetically obese (fa/fa) or lean (Fa/fa or Fa/Fa, represented here by Fa/-), were used. The 3-week-old rats remained with their mothers up to the moment of sacrifice. All other animals were weaned at 21 days of age.

The animals were housed in a constant-temperature room (23 C) with a controlled light-dark cycle (0700-1900 hr). All rats were allowed tap-water and laboratory chow (A04; 1 g = 12.12 kJ from UAR, 91 Villemoisson-sur-Orge, France) ad libitum.

Weight Gain and Food Intake

During the week before the animals were killed, their weight gains and food intakes were measured daily (except for those of the 3-week-old batch).

Sampling

Serum. Nonfasting rats were decapitated between 0900 and 1000 hr. After the blood had been collected through a funnel into a centrifuge tube and centrifuged, the serum was decanted.

Liver. Immediately after a rat had been decapitated, while it was still being bled, the abdomen was opened and ca. 2-g piece of liver was taken by freeze-stop clamping. Several more pieces were then sampled, weighed, quickly frozen in liquid nitrogen, and stored frozen in polyethylene bags until use. Later, total lipids were extracted from some of these samples with chloroform/methanol (2:1, v/v); water content and total protein were measured in others. The rest of the liver was excised and weighed.

The first piece of liver was used in assays for various metabolites. After it had been ground under liquid nitrogen, the powder was transferred into a tared centrifuge tube and weighed. Ice-cold 6% $HCLO_4$ was added to bring the ratio of the weight of the wet liver to the vol of $HCLO_4$ solution to 1/3. The liver was ground in an Ultra-Turrax homogenizer, the homogenate centrifuged, the supernatant decanted, and an aliquot of the liver extract neutralized with 7 mol/1 KOH.

Other organs and tissues. The heart, lungs and kidneys and the pairs of subcutaneous epididymal, perirenal and inguinal fat pads were removed and weighed. The tibia of one hind

TABLE 1

Daily Energy Intake and Wt Gain

Age (wk)	Daily energy intake (kJ/rat)	Daily energy intake (kJ/100-g rat)	Daily wt gain (g/rat)
4 Fa/-	178 ± 6	167 ± 2	6.4 ± 0.3
fa/fa	275 ± 4*	193 ± 6*	8.4 ± 0.4*
6 Fa/-	206 ± 10	163 ± 3	6.2 ± 0.5
fa/fa	294 ± 5*	174 ± 6	7.5 ± 0.4
11 Fa/-	258 ± 2	90 ± 2	2.9 ± 0.5
fa/fa	328 ± 10*	79 ± 3*	5.0 ± 0.8
16 Fa/-	234 ± 7	66 ± 2	2.1 ± 0.6
fa/fa	278 ± 6*	58 ± 1	3.2 ± 0.6
23 Fa/-	218 ± 4	59 ± 1	1.9 ± 0.7
fa/fa	235 ± 8	45 ± 1*	3.2 ± 1.3
32 Fa/-	207 ± 5	55 ± 1	1.1 ± 0.5
fa/fa	221 ± 6	38 ± 1*	0.8 ± 0.3
42 Fa/-	230 ± 12	44 ± 3	0 ± 1.5
fa/fa	298 ± 7*	42 ± 1	0.7 ± 0.6
57 Fa/-	220 ± 7	47 ± 1	0 ± 0.5
fa/fa	258 ± 19	34 ± 2*	0 ± 1.0

Results are expressed as mean ± SEM (n = 8 except for 57-week-old rats, where n = 7). Each value indicates the mean of the values obtained each day during the whole week preceding the week the animals were killed. One g food: 12.12 kJ. Differences were tested by Student's t-test (*$p < 0.05$).

leg was excised, freed of surrounding muscle tissue, weighed, and its length measured.

Analytical Methods

The ketone bodies (3) and glycogen (4) in the liver were measured enzymatically. The amounts of triacylglycerols (5), phospholipids (6) and cholesterol (7) in both the plasma and the liver also were assayed. The concentration of free cholesterol in the plasma was calculated by subtracting the amount of esterified cholesterol from the amount of total cholesterol. For the 3-week-old rats, the 3 fractions of lipids in the blood were quantitated by enzymatic micromethods (Triglycerides Reagent, Dow Diagnostics, Dow Chemical Company, Indianapolis, IN; Phospholipids B-Test, Wako Biochemicals, Wako Pure Chemical Industries, Ltd., Osaka, Japan; Cholesterin enzymatisch, Merckotest, E. Merck, Darmstadt, West Germany). Triacylglycerol and phospholipid levels were calculated using average molecular weights of 885 and 801, respectively. The concentration of nonesterified fatty acids (NEFA) was measured by Soloni and Sardina's technique (8) and was expressed as palmitic acid equivalents. Free glycerol was assayed in the serum (triglycerides, Merckotest, E. Merck, Darmstadt, West Germany). The turbidity of the serum was measured at 630 nm in a spectrofluorimeter (JY3D, Jobin-Yvon, Longjumeau, France) used as a nephelometer (excitation at 630 nm, direct emission). A suspension of formazaline (9) was used to calibrate the apparatus. The total proteins in the plasma (10) and the liver (11) were determined, as was the serum albumin (12) level. The serum concentrations of urea (13), glucose (14), Na and K (Corning 460 flame photometer), Ca (15), and Mg (16) also were determined. Plasma immunoreactive insulin (IRI) was assayed by radioimmunoassay (charcoal-dextran insulin assay kit, CEA-IRE-SORIN), with rat insulin (NOVO Industrie Pharmaceutique, Paris, France) used as a standard. The activities at 30 C of the following enzymes were measured in the plasma: lactate dehydrogenase (LDH) (17), glutamic oxaloacetic transaminase (GOT) (18), and glutamic pyruvic transaminase (GPT) (19).

Statistical Analysis

Results are expressed as mean ± SEM. Differences were tested by Student's t-test, using the 5% level for statistical significance (20).

RESULTS AND DISCUSSION

Daily Energy Intake and Weight Gain: Whole-Body and Organ Weight

The obese Zucker rats ate more than the lean controls (21). As Table 1 shows, this hyperphagia was present at the 4th week of age and was maintained throughout the first year of life (22). However, if the amount of energy ingested was expressed per 100 g body wt, the hyperphagia occurred only during the 4th week of age; Dilettuso and Wangsness (23) observed this phenomenon from the 3rd to the 10th weeks. From the 11th week of age, food intake, expressed as energy ingested per 100 g body wt, was less in obese rats. Despite this observation, the daily weight gain was greater in the obese rats than the lean controls, perhaps because these obese rats retain energy much more efficiently than lean rats (24,25). As Table 1 shows, this was the case at least until the 23rd week of age; but curves of wt vs time for the 2 types of animal (Fig. 1) suggest that this difference in the daily weight gain lasts until the 58th week. This difference accounts for the obese rats' weighing 35 to 45% more than the lean ones starting from about the 5th week and lasting throughout the first year of life (Fig. 1).

When expressed per 100 g body wt (Fig. 2), the weights of the heart (26), the kidneys (24,27), the lungs and the tibia (which is lighter and shorter in the obese than in the lean rats at 12-33 weeks [28,29] [Fig. 1]) were signifi-

cantly less in the obese rats than in the lean ones. In both types of animals, the weights of all these tissues as a proportion of total body wt decreased markedly from the 3rd to the 5th week and more slowly until the 12th week, and then remained stable until the 58th week (Fig. 2).

The absolute and proportional (wt/100 G rat) weights of the liver (Fig. 1) and the adipose tissues (Fig. 3), organs that play a key role in obesity, were greater in the obese animals. This characteristic was significant for the liver from the 5th week (28) and for the adipose tissues (from whatever site) from the 3rd week. Boulangé et al. (30) observed this characteristic from the first week of age in the inguinal subcutaneous adipose tissue. Adipose tissue developed most rapidly (31) and reached a considerable weight in the older obese rats—105.6 ± 3.8 g at 58 weeks—because fat continued to accumulate progressively throughout the rat's life (32). Jamdar (33) showed that more glycerolipid was formed in the adipose tissue of obese rats than of lean ones, and that the difference increased with age. This explains the continuous accumulation of adipose tissue in obese rats.

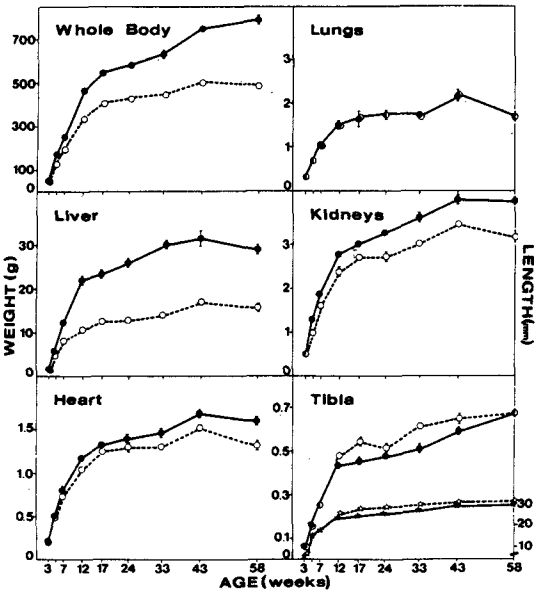

FIG. 1. Whole body and organ weights of genetically obese Zucker rats (fa/fa) and lean controls (Fa/-). Results are expressed as means ± SEM (n = 8, except for 58-week-old rats, where n = 7). Where the SEM is not shown, it was too small to be represented here. Closed symbols, fa/fa; open symbols, Fa/-. Circles = body and organ weight; stars = tibia length. *p < .05.

Serum

Lipids. Zucker and Zucker (1) showed that obese rats are hyperlipemic and that this characteristic affects all lipid fractions (34). As Figure 4 shows, from as early as the 3rd week and through the 58th week, the serum concentrations of insoluble particles (35), triacylglycerols, total and esterified cholesterol, phospholipids, nonesterified fatty acids (for which the difference was not always signifi-

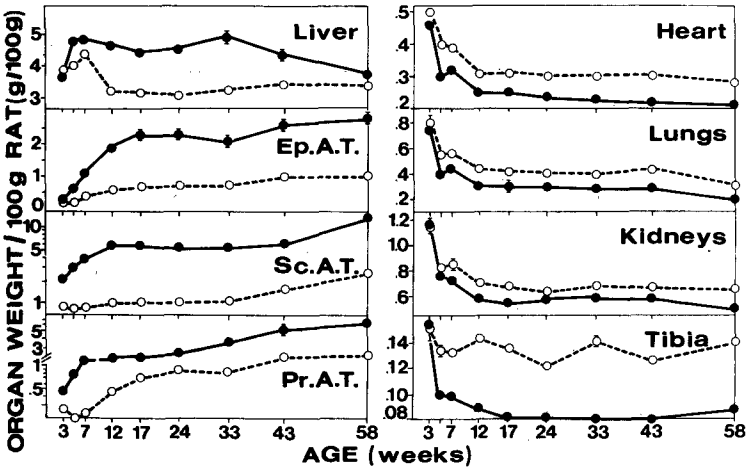

FIG. 2. Proportional organ weights (per 100 g body wt). Results are expressed as means ± SEM (n = 8, except for 58-week-old rats, where n = 7). For the adipose tissues, the values indicated represent both fat pads of a pair. Closed circles = fa/fa; open circles = Fa/-; Ep.A.T. = epididymal adipose tissue; Sc.A.T. = subcutaneous adipose tissue; Pr.A.T. = perirenal adipose tissue. *p < .05.

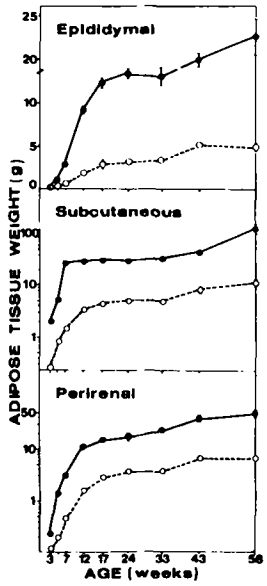

FIG. 3. Adipose tissue weights. Results are expressed as means ± SEM (n = 8, except for 58-week-old rats, where n = 7). The values indicated represent the weights of pairs of fat pads. Closed circles = fa/fa; open circles = Fa/-. *p < .05.

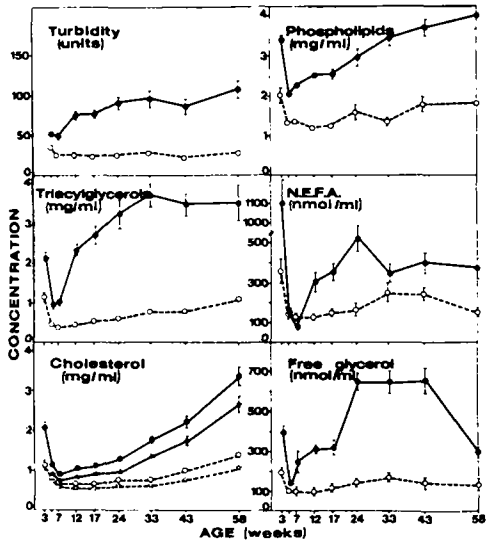

FIG. 4. Serum lipid levels. Results are expressed as means ± SEM (n = 8, except for 58-week-old rat, where n = 7). In the 3-week-olds, there was too little serum for determination of its turbidity or of the concentration of esterified cholesterol. Closed symbols = fa/fa; open symbols = Fa/-; circles = serum lipid levels; TC = total cholesterol; EC = esterified cholesterol (stars). *p < .05.

cant), and free glycerol were perceptibly higher in the fa/fa than in the Fa/- rats.

Like Jamdar (33), we observed that, in all the animals, the concentrations of triacylglycerols decreased between 3 and 5 weeks of age, as did other lipids, probably because of the change-over from food rich in milk lipids to a lipid-poor food (3% on ave. in A04). In the obese animals, unlike the lean controls, serum turbidity and serum concentration of triacylglycerols reached their highest levels about the 24th week. This was in contrast to the serum concentrations of free and esterified cholesterol and phospholipids, which continued to increase to the end of the study. The proportion of esterified cholesterol to total cholesterol (Fig. 4) was independent of both the rat's genotype and age; 77 ± 1 to 87 ± 1% of total cholesterol was esterified.

In Figure 5, the relative amounts of total cholesterol, phospholipids and triacylglycerols in each group has been expressed as a percentage of total mol of these 3 components. To compare the plasma lipid composition of the different series, we plotted the result for each group as a single point, using triangular

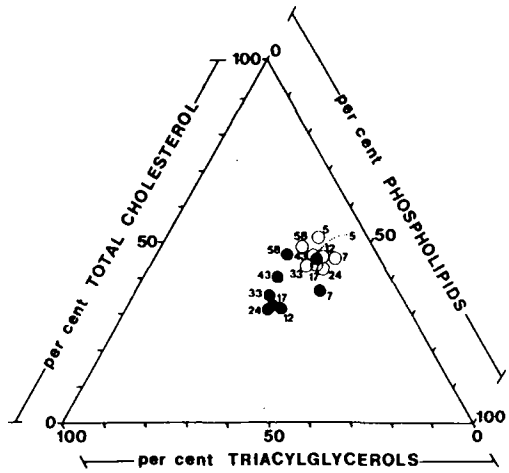

FIG. 5. Three-dimensional representation of total cholesterol, phospholipid and triacylglycerol levels in serum. The 3 substances are treated as a ternary mixture represented by an equilateral triangle whose apices correspond to the pure constituents. The levels are expressed as the percentages of molecules of the various constituents. The mean serum concentration for each batch of animals is represented by a circle, beside which is shown the age of the rats concerned (e.g., closed circle numbered 7:37% cholesterol, 45% phospholipids, 18% triacylglycerols, sum 100%). The results for the 3-week-old rats are not represented here because of their mixed diet and the special quantitation techniques (see Materials and Methods). Closed circles = fa/fa; open circles = Fa/-.

coordinates. As the obese rats get older, this point first moves downward, then toward the left, and finally upward. This shows that, with age, the relative importance of each lipid fraction varies. Passing from the 5th to the 7th week of life, the relative rate of phospholipids increases; then from the 7th to the 24-33rd week, the importance of triacylglycerols grows and then the rate of cholesterol increases. In the case of lean rats, such variations according to age do not seem to apply.

It is possible to show by a series of linear, bilateral relations the relationships between the concentration of triacylglycerols, cholesterol and phospholipids, and the quantity of subcutaneous fat/100 g body wt (Fig. 6). The test for departure from linearity (20) shows that this simplification may be used.

Glucose and insulin. The genetically obese Zucker rats had very high basal levels of immunoreactive insulin from the 5th to the 58 week of age (Fig. 7) (36,37). The level of immunoreactive insulin increased until the 17th week of age, then decreased until the 58th week. From the 17th week, the greatly elevated immunoreactive insulin concentration was

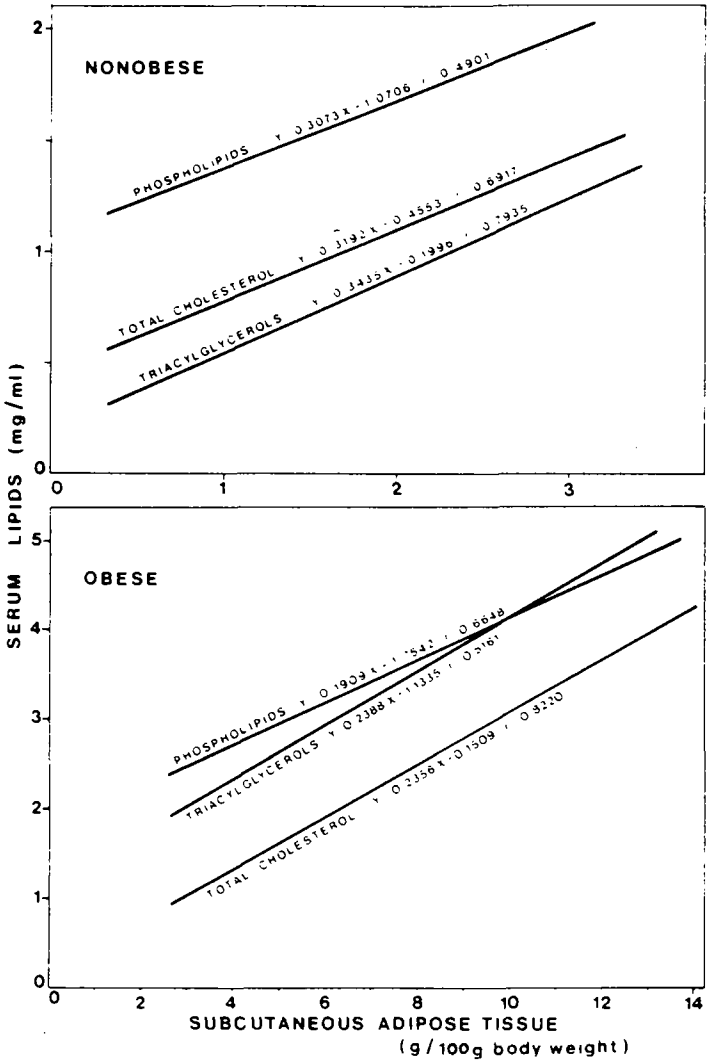

FIG. 6. Relationship between the proportional amounts (per 100 g body wt) of the subcutaneous adipose tissue and the serum lipid levels. The results for the 3-week-old rats were not used in the calculation of the regression lines (see legend to Fig. 5). For each line, $n = 63$, $p < 0.001$.

accompanied by a small but significant increase in blood glucose (32), which seems to originate in an increase in the hepatic and renal production of glucose in the obese rats (38).

Proteins. As we have already indicated (34), and contrary to the results of Fillios and Saito (39), the serum concentrations of albumin (28) and of total proteins, and hence of globulins (total proteins minus albumin), were higher in the obese animals (Fig. 7); the albumin/globulin ratio lay between 1.1 ± 0.1 and 1.8 ± 0.1 and was not significantly different in the 2 types of animals. Our results agree with Zucker's in 1965 (28), demonstrating that the albumin level is high in young, obese rats and decreases with age: the 17% decrease observed between the 12th and 58th week of life is highly significant. During the same period, the serum albumin level remains constant in non-obese rats.

Elements. There were no important differences between the 2 types of rats in the serum concentrations of Na, K, Ca and Mg.

Enzyme activities. There was no significant difference in the activities of glutamic pyruvic transaminase or lactate dehydrogenase between the obese and lean animals.

Liver (Fig. 8)

Water. Between the 12th and 33rd weeks, the hepatic water content was significantly lower in the obese rats. It must be remembered that the blood vol/100 g body wt (34) and the percentage of total body water (40) are less in obese rats.

Proteins. The obese rats had slightly less protein/g of liver than the lean rats (39), although the difference was usually not significant. Parallel decreases have been observed in the protein content of the entire bodies of obese animals (40), even though, as Zucker (28) and Fillios and Saito (39) showed, the synthesis of protein is considerably higher in their livers.

Glycogen. The hepatic levels of glycogen were higher in the obese rats from the 12th week of age (41)—contrary to Wade's (42) claim—since its synthesis is increased in the obese animals (38).

Lipids. From the 12th week, the liver of an obese rat contained more lipids than that of a lean one, gram for gram. Thus, it can be seen that the obese animals usually had higher hepatic levels of triacylglycerols and cholesterol, but lower levels of phospholipids.

Despite an enhanced lipid synthesis in the obese rat (33), the accumulation of lipids in the liver did not appear until the 12th week. Liver lipid content of obese rats (Fig. 8) reached a maximum at 17th to 24th weeks, and then decreased. This decrease is significant for triacylglycerols (24 vs 58 weeks), total cholesterol (17 vs 43) and phospholipids (7 vs 58).

Jamdar (33) showed that the hepatic formation of glycerolipids is significantly higher in obese than in lean rats at all ages, reaching a maximum at about 42 to 44 days and decreasing thereafter. It is thus possible that in the young obese rat, lipids, being synthesized rapidly in the liver, accumulate there and are then unloaded little by little once their synthesis has declined.

The triaclyglycerols enter the bloodstream, where their concentrations remain constant after the 24th week (Fig. 4), and arrive in the adipose tissue, which gradually accumulates them, whereas cholesterol and phospholipids accumulate in the blood.

In the obese rats, despite a metabolism strongly oriented toward anabolism, hepatic ketogenesis is no lower than in the livers of the lean animals (Fig. 8).

In view of the hepatomegaly of the obese rats, it is not surprising that all these results were different when based on the total liver weight. The livers of the obese rats contain, from the 5th week of age (i.e., when the hepatomegaly became clear), less water, more protein, and more triacylglycerols, cholesterol and phospholipids than did the livers of the lean animals, and from the 12th week of age, more glycogen.

The earliest anomaly that Boulangé (30,31)

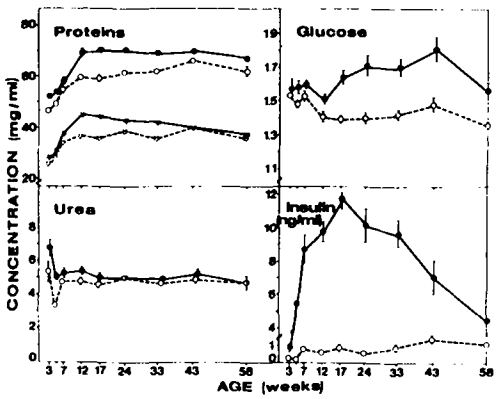

FIG. 7. Serum concentrations of proteins, urea, glucose and insulin. Results are expressed as means ± SEM (n = 8 except for 58-week-old rats, where n = 7). The insulin determination was performed in duplicate, except at 3 weeks, when it was performed only once. Closed symbols = fa/fa; open symbols = Fa/-. For protein levels: circles = total proteins; stars = albumin. *$p < .05$.

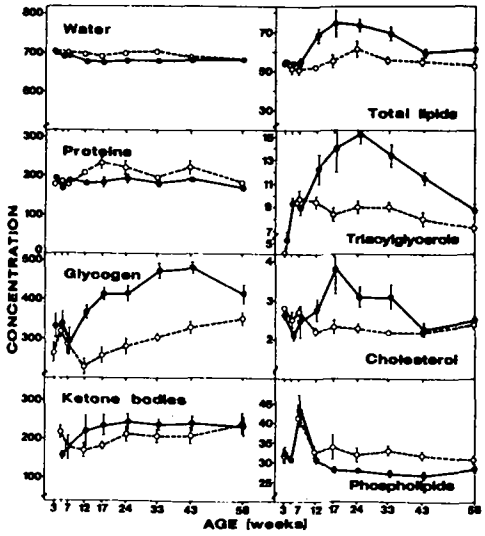

FIG. 8. Liver composition. Results (mg/g wet liver, except for the ketone bodies (nmol/g wet liver) and glycogen [nmol glucosyls/g wet liver]) are expressed as means ± SEM (n = 8, except for 58-week-old rats, where n = 7). The ketone bodies (the sum of β-hydroxy-butyrate and acetoacetate) were not quantitated at 3 weeks. The concentration of total lipids was obtained after extracting the hepatic lipids with a solvent and weighing the dry extract. The concentrations of triacylglycerols, cholesterol and phospholipids were obtained by colorimetric determination on a sample of the dry extract. Closed symbols = fa/fa; open symbols = Fa/-. *$p < .05$.

finds in obese rats, when they are only 1 week old, is the overdevelopment of white fat. Next, there is a drop in the activity of lipoprotein lipase in the muscles and a shift in the division between storage and consumption of energy substrates. This explains why the main characteristics of genetic obesity–the accumulation of adipose tissue, and hyperlipemia–are already detectable at the time of weaning. With the change in diet at weaning, hyperphagia appears. From the 7th week of life, the liver starts to accumulate triacylglycerols and cholesterol, as well as glycogen; during that same week, the excess of lipids in the blood and storage tissues, observed from the age of 3 weeks, increases considerably. Between 17 and 24 weeks of age, the hepatic lipids begin to decrease whereas the serum cholesterol and phospholipid concentrations continue to increase, and the triacylglycerols reach their peak, probably because the adipose tissues continue to accumulate them. Around the end of the first year of life, the various hepatic parameters–except the hepatomegaly–gradually subside toward normality, whereas the hyperlipemia and the accumulation of lipids in the adipose tissue persist or increase.

ACKNOWLEDGMENTS

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Essential Fatty Acid Supplemented Diet Increases Renal Excretion of Prostaglandin E_2 and Water in Essential Fatty Acid Deficient Rats[1]

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ABSTRACT

Weanling male rats were fed an essential fatty acid (EFA)-deficient diet for 25 weeks and then switched to an EFA-supplemented diet for 3 weeks. Control rats received the EFA-supplemented diet for 25 weeks and then the EFA-deficient diet for 3 weeks. Throughout the last 19 weeks, the rats were housed in metabolic cages once a week for a 24-hr period. Urinary excretion of prostaglandin E_2 (PGE_2) was estimated by radioimmunoassay. Throughout a period of 12 weeks (weeks 13-24) water consumption increased ca. 60%, and urine output and PGE_2 excretion decreased ca. 45% and 70%, respectively, in the EFA-deficient rats. Feeding EFA-supplemented diet to the EFA-deficient rats for 3 weeks decreased the water consumption and raised the urine output to that observed in the controls. However, the urine output was corrected within 1 day whereas the water consumption was not corrected until the second measurement 8 days after the dietary change. The PGE_2 excretion increased more than 9-fold (from 18 ± 8 ng/24 hr to 165 ± 51 ng/24 hr) 1 day after EFA-supplementation, followed by a decrease to 86 ± 29 ng/24 hr over the following 2 weeks. On the basis of the present data, it is suggested that EFA deficiency in rats causes diminished PGE_2 excretion, which can be normalized by EFA supplementation. The normalization of the urine flow may, in part, be caused by the concomitant considerable increase in endogenous PGE_2 synthesis.

EFA are precursors for the prostaglandins in mammalian tissues (1). Young rats fed an EFA-deficient diet for several weeks develop symptoms characteristic of EFA deficiency, e.g., disturbed water balance, observed as increased water consumption and decreased urine output (2). Prostaglandins, especially PGE_2, which are synthesized in the kidney and excreted in the urine (3), seem to be involved in the control of renal excretion of water (4). From in vitro studies with rat kidneys, it has been showed that EFA deficiency results in reduced levels of the prostaglandin precursor, arachidonic acid, in the tissues, and in reduced PGE_2 formation (5,6). However, these studies also showed that the activity of prostaglandin synthetase was increased during EFA deficiency. Furthermore, in vitro prostaglandin production may not reflect the in vivo situation, since prostaglandin synthesis is stimulated by tissue homogenization (7) as well as being highly dependent on cofactors and on substrate concentration (8). We had found earlier that the activity of the prostaglandin catabolic enzyme, 15-hydroxy-prostaglandin dehydrogenase, which could contribute to a regulation of endogenous prostaglandin levels, is unchanged in EFA-deficient rat kidney (9), indicating that changes in urinary PGE_2 excretion probably reflect changes in renal PGE_2 biosynthesis. Renal PGE_2 production in vivo in relation to EFA deficiency has not been studied previously. The aim of this study was to investigate the effect of EFA deficiency and EFA-feeding on urinary PGE_2 excretion, and on urine output and water consumption in conscious male rats.

MATERIALS AND METHODS

Animals and Collections

Ten 21-day-old male Wistar rats (K. Møllegaard-Hansens Avlslaboratorium A/S, Ll. Skensved, Denmark) were divided into 2 groups of 5 animals each. Group 1 was reared on a semisynthetic fat-free diet. Group 2 was reared on a diet with 28% arachis oil, i.e., the fat-free diet with 28% by wt of the sucrose replaced by 28% by wt of arachis oil. Thus, linoleic acid contributed 14 cal % of the diet (10). After 25 weeks of feeding, the diets were switched between the 2 groups and fed for 3 weeks. Diets and water were supplied ad libitum throughout the experiment. The animals were housed in wire mesh cages, 2 animals/cage. Once a week, the rats were housed individually in metabolic cages for 24 hr with free access to water and food. The 24-hr urine was collected in a polyethylene tube packed in Dry Ice which ensured immediate freezing of the urine after

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Abbreviations: EFA = essential fatty acid; PGE_1, PGE_2 = prostaglandin E_1, prostaglandin E_2 ... ; TXB_2 = thromboxane B_2; RIA = radioimmunoassay.

voiding. The collected urine samples were kept at -20 C for no more than 3 months before PGE_2 determinations were done. The change of diet between the 2 groups was made 1 day before the weekly urine collection. The experimental feeding period started in June and ended in December. The humidity decreased from ca. 60% to ca. 40% within this period. Throughout the experiment the temperature was 23 ± 1 C. The rats were kept in a 12-hr/12-hr light-dark period.

At weeks 16 and 28 of the experiment, both groups of rats received indomethacin (Dumex, Copenhagen, Denmark) per os. Indomethacin was dissolved in 0.05 M $NaHCO_3$. During each test period, 3 doses of the indomethacin were given, i.e., 12 hr before, at the beginning, and 12 hr within the 24-hr urine sampling period. Thus, at week 16, the rats received 3 x 0.75 mg/kg, and at week 28, 3 x 10 mg/kg of indomethacin. The dose of indomethacin at week 16 was low in order to avoid gastrointestinal side effects during the rest of the feeding period (11), whereas the dose given at week 28 was high to secure efficient inhibition of prostaglandin synthesis (12).

PGE_2-Radioimmunoassay

[^{3}H]PGE_2, 160 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, England, and unlabeled prostaglandins were gifts from Dr. J. Pike, The Upjohn Co., Kalamazoo, MI. The PGE_2 antiserum was kindly provided by Dr. P. Christensen, Institute of Experimental Medicine, Copenhagen, Denmark.

The present RIA is a modification of that described by Christensen and Leyssac (13) which involves an initial purification of the prostaglandin by acidic (pH = 3) extraction followed by Sephadex LH-20 chromatography. The modification involves ethyl acetate extraction from urine diluted with phosphate buffer (pH 7.0), thereby replacing the acidic extraction and the Sephadex LH-20 chromatography. A series of analysis usually included 20 biological samples, which consisted of 10 µl and 20 µl urine in duplicate from 5 rats. The urine samples were diluted to 500 µl with 0.1 M sodium phosphate (pH = 7.0). After addition of [^{3}H]PGE_2 (600 cpm), which served as internal standard for extraction recovery, all samples were extracted twice with 1.5 ml ethyl acetate. The extract was evaporated under a stream of nitrogen and the residue was dissolved in 150 µl assay buffer (13). For the determination of recovery, 40 µl was counted, whereas 2 x 50 µl were used for the RIA as described (13). The RIA data were calculated on a Hewlett-Packard 9810 A calculator using the method of progressive fit of a quadratic equation (14). Cross reactivities for the PGE_2 antibody with PGE_1, PGE_3, $PGF_{2\alpha}$, PGA_2, PGD_2 and TXB_2 were 20.8, 4.2, 1.1, 0.6, <0.02 and <0.02%, respectively, at the 50% displacement level.

Extraction at pH 7.0 resulted in a selective loss of the more polar prostaglandins, i.e., recovery for PGE_2 was ca. 80%, whereas that for $PGE_{2\alpha}$ was around 54%. A major part of the prostaglandins in the urine was di- or tetranor-13,14-dihydro-15-keto-metabolites having 1 or 2 carboxylic groups (15,16), making them more polar than the corresponding primary prostaglandins. Measurements of urine samples with the original (x) and the modified (y) RIA produced the same results, giving a regression line ($y = -5.0 + 0.994x$; $n = 11$; $r = 0.924$: $p < 0.001$) where the slope and the intercept were insignificantly different from unity ($p > 0.5$) and zero ($p > 0.1$), respectively. The column purification resulted in a selective isolation of PGE_2 (13), whereas the pH 7.0 extraction selected only the most lipophilic prostaglandins. High amounts of PGE_1 in the urine relative to PGE_2 could lead to falsely high PGE_2-values with the modified RIA procedure, since PGE_2 and PGE_1 are not separated by a pH 7.0 extraction. Thus, it can be concluded that there is only a minor, if any, excretion of PGE_1 in the urine, in agreement with the results of others (17,18), since the 2 purification procedures resulted in the same PGE_2 values. Sequential urine dilution (y) showed a linear relationship with the measured amount of PGE_2 having the following regression line ($y = 19.6 + 5.97x$; $n = 8$; $r = 0.991$; $p < 0.001$), where the intercept is insignificantly different from zero ($p > 0.1$).

Chemical Determinations and Statistics

Urine sodium and potassium concentrations were estimated using an FLM 3 Flame Photometer (Radiometer, Denmark). Creatinine was determined spectrophotometrically after formation of the picrate in alkali (19). Urine osmolality was determined on a freezing-point osmometer (Knauer Halbmikro-Osmometer, Eppelheim bei Heidelberg, West Germany).

Statistical analysis was done using the paired and unpaired Student's t-test for intra-individual and inter-individual comparison, respectively.

RESULTS

The urinary excretion of PGE_2 was found to be ca. 70 ng/24 hr for the control group (group 2), and ca. 25 ng/24 hr for the EFA-deficient rats (group 1) throughout a 12-week sampling

period (weeks 13-24) (Fig. 1).

When the EFA-deficient rats were transferred to the control diet, it resulted in a 9-fold increase (from 18 ± 8 ng/24 hr to 165 ± 51 ng/24 hr, $p < 0.001$) in the PGE_2 excretion, which was more than twice as high as the ca. 70 ng/24 hr found for the control group. The increase in the PGE_2 excretion was seen 1 day after th dietary change. The PGE_2 excretion in group 1 subsequently fell within 2 weeks to 86 ± 29 ng/24 hr, a value not significantly different from the level seen in group 2 during the control period (weeks 13-24). When the control rats were fed the EFA-deficient diet, there was no immediate change in the PGE_2 excretion. Indomethacin treatment at week 16 resulted in 86 ± 7% inhibition of PGE_2 excretion in the EFA-deficient rats, compared to the values obtained the week before. This low dose of indomethacin gave no inhibition of PGE_2 excretion in the control rats. At week 28, the inhibition by a very high dose of indomethacin was 80 ± 13% and 97 ± 4% in groups 1 and 2, respectively.

The EFA-deficient rats showed a significantly lower urine output than the control rats (Fig. 2). One day after the switch over of diet between the 2 groups, the urine output in group 1 was normalized (from 7.5 ± 2.1 ml/24 hr to 11.3 ± 3.6 ml/24 hr, $p < 0.05$), whereas there was no change in group 2. The low urine output in the EFA-deficient rats resulted in a high urine osmolality (Fig. 3), high urine sodium concentrations (ca. 150-175 meq/l compared to values of ca. 75 meq/l for the controls, detailed data not shown), and high urine potassium concentrations (ca. 175 meq/l compared to values of ca. 75 meq/l for the controls, detailed data not shown). The latter 2 parameters were normalized just as was the urine osmolality ($p < 0.01$) 1 day after the dietary change (Fig. 3). There were no apparent differences between the 2 groups in the rates of excretion of these solutes calculated as mosmol/24 hr, meq sodium/24 hr, and meq potassium/24 hr, respectively (data not shown).

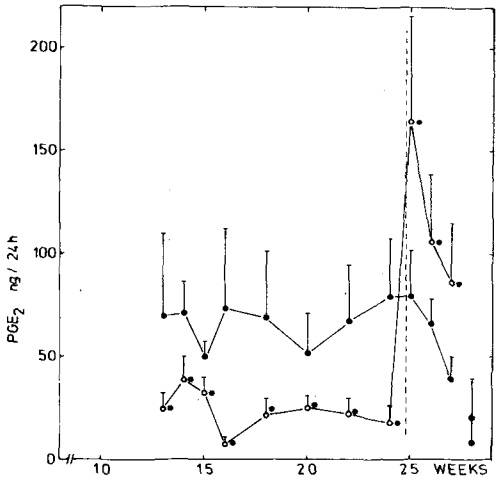

FIG. 1. PGE_2 urinary excretion of rats fed diets with or without linoleate. Twenty-one-day-old male rats were fed either a fat-free diet (group 1) o–o, or the same diet in which 28% by wt of sucrose was replaced by 28% by wt of arachis oil (group 2) •–•. The dashed line indicates that one day before the measurement at week 25, the diets were switched over between the 2 groups for the next 3 weeks. Once a week the rats were housed individually in metabolic cages, and the 24-hr urine was collected in a tube packed in Dry Ice. Urinary PGE_2 was estimated by radioimmunoassay. At weeks 16 and 28, the rats in both groups were treated with indomethacin per os, 3 times 0.75 mg/kg/12 hr and 3 times 10 mg/kg/12 hr, respectively. The bars indicate one SD for n = 5. *Statistically significant difference ($p < 0.05$) compared to group 2.

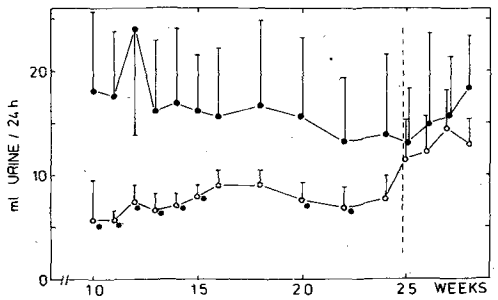

FIG. 2. Urine output from rats fed diets with or without linoleate. For details, see Fig. 1 legend. *Statistically significant difference ($p < 0.05$) when compared to group 2.

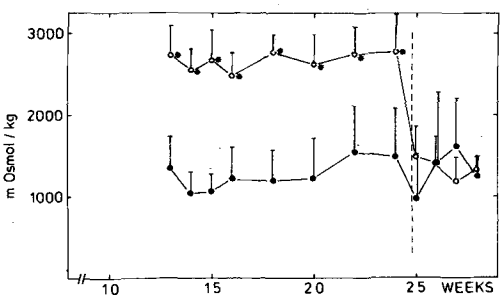

FIG. 3. Osmolality of urine from rats fed diets with or without linoleate. For details, see Fig. 1 legend. *Statistically significant difference ($p < 0.01$) compared to group 2.

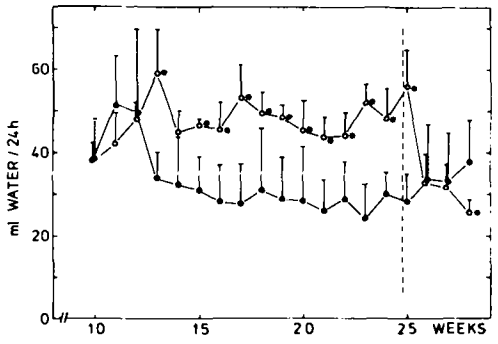

FIG. 4. Water consumption of rats fed diets with or without linoleate. For details, see Fig. 1 legend. *Statistically significant difference ($p < 0.05$) compared to group 2.

Throughout the last half of the period in which the rats in group 1 were fed the EFA-deficient diet, they had significantly higher water consumption compared to the control rats (Fig. 4). The difference between the water consumption and the urine output, the "evaporation," was also significantly higher in the EFA-deficient rats (Fig. 5). Both water consumption ($p < 0.01$) and "evaporation" ($p < 0.01$) were corrected when the EFA-supplemented diet was given, but not until the second measurement 8 days after the dietary change (Figs. 4 and 5).

In the period between weeks 13 and 24, the EFA-deficient rats (group 1) excreted from 7.5 mg creatinine/24 hr increasing to ca. 8.5 mg/24 hr, whereas the control rats (group 2) excreted from ca. 12 mg creatinine/24 hr increasing to ca. 14 mg/24 hr during the same period. The dietary change resulted in a slow increase in creatinine excretion to 11.2 ± 1.4 mg/24 hr at week 28 for the rats in group 1. There was no change in the creatinine excretion in the rats in group 2 (13.7 ± 3.1 mg/24 hr at week 28) when they were fed the EFA-deficient diet (detailed data not shown).

The EFA-deficient rats showed the characteristic cessation of growth (Fig. 6), which could be reversed by feeding the EFA-supplemented diet.

Although a marked inhibition of PGE_2 excretion by indomethacin was observed in group 1 at week 16 and in both groups at week 28, no changes were seen in any of the measured parameters except for a slight depression of water intake in group 1 (Fig. 4).

DISCUSSION

Our data (Figs. 1-5) were all calculated per rat and not per g body wt or per mg creatinine excretion. If the urine flow and the PGE_2 excretion data were calculated per g body wt or per mg creatinine excretion, no significant differences would be observed between the figures obtained for the EFA-deficient rats and those obtained for the controls over the same period (weeks 13-24), respectively. The differences in water consumption and in the "evaporation" between the 2 groups, respectively, would be more pronounced if calculated per mg creatinine excretion. Due to the following 3 reasons, the data (Figs. 1-5) were calculated per rat in spite of the existing marked differences in body wt between the 2 groups. (a) The PGE_2 excretion and urine flow were rather constant, although there was an increase in body wt (and creatinine excretion) during weeks 13-24; (b) at week 25, 1 day after the start of feeding the EFA-supplemented diet to the EFA-deficient rats, their urine output was already equal to that found in the control rats whereas the body

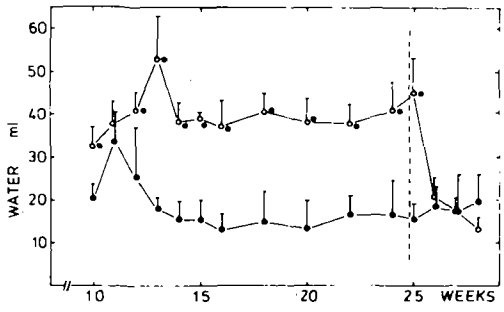

FIG. 5. "Evaporation" through the skin of rats fed diets with or without linoleate. The "evaporation" is calculated as the difference between the consumption of drinking water and the urine output. For further details, see Fig. 1 legend. *Statistically significant difference ($p < 0.05$) compared to group 2.

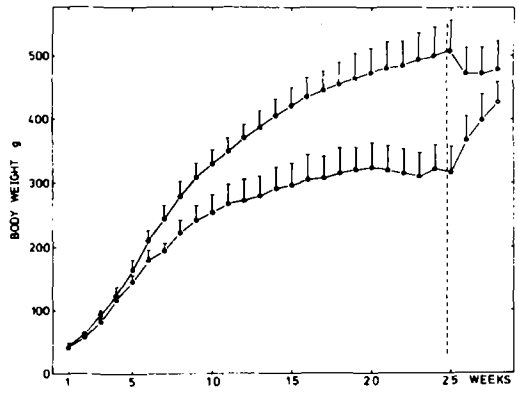

FIG. 6. Growth rate of rats fed diets with or without linoleate. For details, see Fig. 1 legend.

weight remained unchanged; (c) the urine of the EFA-deficient rats (weeks 13-24) was highly concentrated (high osmolality, and high sodium and potassium concentrations) indicating that the low urine output was not mainly due to the corresponding low body weight, but rather to an efficient water-conserving mechansim. The absolute osmotic load, mosmol/24 hr, was about the same in the 2 groups. Thus, it appeared that the relatively high osmolar excretion rate seen in the EFA-deficient rats had to occur in parallel with a low renal water excretion, which probably was provoked by extrarenal water loss.

Induction of EFA-deficiency in experimental animals results in a decrease in the total amount in the tissues of arachidonic acid, the precursor of the dienoic prostaglandins. Several in vitro studies have shown a decrease in the amount of dienoic prostaglandins produced in tissues or tissue homogenates from EFA-deficient animals (5,6,20-27). However, such in vitro studies may not necessarily reflect the situation in vivo, since the prostaglandin-precursor pool in vivo may be different from the one seen in vitro. Our data showed a lower urinary excretion rate of PGE_2 over a period of 12 weeks (weeks 13-24) in the EFA-deficient rats compared to the controls over the same period. A decreased urinary excretion of the major urinary metabolite of E-prostaglandins, 7α-hydroxy,5,11-diketotetranorprostan-1,16-dioic acid, has also been seen in EFA-deficient infants (28). Nugteren et al. (16) have also reported decreased urinary excretion of tetranorprostaglandin metabolites in rats which received a diet with a low content of linoleic acid (0.6 Joule %). When the EFA-supplemented diet was given to the EFA-deficient rats after 25 weeks, the urinary excretion of PGE_2 rose drastically 1 day after the change of diet. This may suggest that the availability of substrate had been reduced and that the enzyme activity was increased during EFA-deficiency. This explanation is in agreement with the in vitro data (5,6). Thus, although the enzyme activity may be increased, the substrate availability appears to be the controlling factor in the PGE_2 synthesis in the EFA-deficient rats. However, after the change in diet, the prostaglandin synthetase could be the controlling factor in the PGE_2 synthesis, since with the same diet there was a decline in PGE_2 excretion over the following 2 weeks. A decrease in PGE_2-synthetase activity has also been seen for in vitro PGE_2 synthesis in kidney medulla from EFA-deficient rats which had been fed an EFA-rich diet for 6 weeks (6).

In our control rats (group 2), the urinary PGE_2 excretion was ca. 70 ng/24 hr (weeks 13-24) with a considerable individual variation. However, for each rat in the group, the urinary PGE_2 excretion rate was rather constant (data not shown). When the control rats were switched to the EFA-deficient diet after 25 weeks of experiment, no immediate changes were observed in their urinary PGE_2 excretion. The low dose of indomethacin at week 16 caused an 80% inhibition of the PGE_2 excretion in the EFA-deficient rats whereas it had no effect on the controls. This could probably be explained by a different tissue distribution of indomethacin in the control rats having sizable fat depots compared to the EFA-deficient, lean rats. The high dose of indomethacin at week 28 resulted in a high degree of inhibition of the PGE_2 excretion in both experimental groups.

The EFA-deficient rats (weeks 13-24) showed a rather constant low urine output resulting in a highly concentrated urine (high osmolality, and high sodium and potassium concentrations). The EFA-deficient rats also had a high water intake and a high "evaporation," which is in agreement with previous observations (2). When the EFA-deficient rats were fed EFA-supplemented diet, their water consumption decreased and their urine output increased to the levels observed in the controls, respectively, but with a different response time. At the week-25 measurement, 1 day after the change of diet, the rats in group 1 still had high water consumption and high "evaporation," but the urine output was normalized. A possible explanation for this relatively rapid change in urine output might be that it was caused by hormonal changes, either intrarenally or in the release of vasopressin from the pituitary. There is evidence that endogenous reno-medullary PGE_2 has a diuretic effect in the rat (29), and may be involved in a negative feedback regulation of vasopressin-induced water reabsorption (4,30-32). Preliminary results indicate that the EFA-deficient rats had a very high vasopressin production, which only was corrected at the second measurement 8 days after the EFA-supplementation (unpublished results). Thus, 1 day after the EFA-supplementation, the rats in group 1 might have had a high vasopressin excretion, very high PGE_2 excretion and normal urine output. The lack of antidiuretic effect of indomethacin in this study does not exclude the possibility that the increased urine flow present 1 day after feeding the EFA-supplemented diet to the EFA-deficient rats may have been caused by the simultaneous, transient, marked increase in renal PGE_2 production. Indomethacin treatment at week

16 resulted in a decreased PGE_2 excretion in the EFA-deficient rats only. In these rats, the urine was maximally concentrated; thus, the renal water excretion could not be further decreased as there apparently was no endogenous inhibition of vasopressin-stimulated water reabsorption. The lack of an indomethacin effect on urine flow rate at week 28 may be related to the possibility that, at this time, PGE_2 excretion had already decreased to such a low level (ca. 70 ng/24 hr) that its effect on the urine flow was negligible.

It is remarkable that the changes in water consumption and "evaporation" in relation to feeding the EFA-supplemented diet to the EFA-deficient rats were rather slow compared to the change in urine flow. Feeding EFA to EFA-deficient rats resulted in a similarly slow correction of transepidermal water loss (33). A possible explanation might be that a defect in the cutaneous permeability barrier causing the high "evaporation" is corrected by a relatively slow incorporation of EFA into the lipids of the skin (34).

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METHODS

Separation of Molecular Species of Ceramides as Benzoyl and *p*-Nitrobenzoyl Derivatives by High Performance Liquid Chromatography[1]

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ABSTRACT

Ceramides were subjected to direct separation and structural analysis by high performance liquid chromatography after derivatization with benzoyl and *p*-nitrobenzoyl chloride. The structures of benzoyl and *p*-nitrobenzoyl derivatives of a representative ceramide, N-heptadecanoyl D-sphingosine, were fully characterized by mass spectrometry. Excellent separation of molecular species of ceramides was achieved with a 10-μ LiChrosorb RP18 column by isocratic elution. Both the acyl group and the long-chain base moiety were separated on this reverse-phase column. The method has been used to determine quantitatively molecular species of nonhydroxy ceramides derived from bovine brain and egg yolk. Comparison of gas liquid chromatographic and high performance liquid chromatographic assays of intact ceramides was made.

INTRODUCTION

Ceramides are the biosynthetic precursors of complex sphingolipids which include cerebrosides, sulfatides, gangliosides and sphingomyelins. Ceramides also play important roles in the catabolism of these sphingolipids. Although free ceramides are minor components of tissue lipids, their level in tissues and urine of patients with Farber's disease has been reported to rise (1). The structural constituents of ceramides, i.e., long-chain bases and fatty acids, have been analyzed extensively by gas liquid chromatography (GLC). However, the structures of individual molecular species of ceramides in tissues cannot be obtained accurately from analysis of long-chain bases and fatty acids. Two laboratories (2,3) reported that molecular species of synthetic and natural ceramides could be separated as trimethylsilyl (TMS) ether derivatives by GLC and GLC-mass spectrometry. However, on these chromatograms, the ceramides were separated mainly according to the number of carbon atoms, resulting in poor resolution of ceramides containing unsaturated fatty acids from those containing saturated fatty acids of the same carbon number. Furthermore, the TMS derivatives of ceramides containing longer chain fatty acids showed lower detector responses than those containing shorter chain fatty acids. This required the determination of the molar response factor of each peak to an internal standard if one wished to analyze the accurate composition of molecular species of ceramides by GLC. Recently, separation of ceramides containing nonhydroxy and 2-hydroxy fatty acids by high performance liquid chromatography (HPLC) on normal and reverse-phase columns has been reported in the literature (4-6). This report describes procedures for the separation and quantitation of molecular species of nonhydroxy ceramides as their benzoyl and *p*-nitrobenzoyl derivatives by reverse-phase HPLC. Application of the methods to the estimation of composition of nonhydroxy ceramides derived from bovine brain and egg yolk is also described.

MATERIALS AND METHODS

Materials

Authentic fatty acids, their methyl esters, bovine brain nonhydroxy cerebrosides, hexamethyldisilazane/chlorotrimethylsilane/pyridine

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(3:1:9, v/v), Silica Gel G and GF Prekote plates, HI-Flosil (60/200 mesh) silica gel were obtained from Applied Science Division, Milton Roy Company Laboratory Group, State College, PA. Triphenylphosphine, dipyridyldisulfide, benzoyl chloride and *p*-nitrobenzoyl chloride were obtained from Aldrich Chemical Co. Inc., Milwaukee, WI. The preparation of egg yolk nonhydroxy ceramides and D-sphingosine from egg yolk sphingomyelins has been described elsewhere (7). Bovine brain nonhydroxy ceramides were prepared from nonhydroxy cerebrosides by the procedures of Carter et al. (8). All solvents used for HPLC were spectral grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI) and were degassed by ultrasonication.

Preparation of Reference Ceramides

The reference ceramides were prepared according to the procedure of Kishimoto (9). To a mixture of a fatty acid (0.1-0.15 mmol each of palmitic, heptadecanoic, stearic, arachidic, 11-eicosenoic, behenic, erucic, tricosanoic, lignoceric, nervonic or cerotic acid) and 2 molar equivalents of triphenylphosphine and of dipyridyldisulfide was added 1 molar equivalent of D-sphingosine in 20 ml of methylene chloride. The reaction mixture was stirred at room temperature overnight. The resulting ceramides were purified by preparative thin layer chromatography (TLC) (development with chloroform/methanol/acetic acid, 90:2:8, v/v) and dried over phosphorus pentoxide in a vacuum desiccator for 3 hr at 30 C.

Preparation of Benzoyl and *p*-Nitrobenzoyl Derivatives of Ceramides

Various ceramides (10-100 μmol) were derivatized with a 10-fold molar excess of either 10% (w/v) benzoyl or *p*-nitrobenzoyl chloride in pyridine at 60 C for 3 hr or at room temperature overnight. The reaction mixture was dried under a stream of nitrogen at 45 C.

The residue derived from benzoyl chloride was extracted with 60 ml of ether. The ether extracts were washed successively with 15 ml of water, twice with 15 ml of 3% sodium bicarbonate solution and twice with 15 ml of water and then evaporated to dryness. For characterization of the benzoyl derivatives, the crude benzoyl derivatives were purified by a HI-Flosil column (20 cm × 2 cm id). The column was eluted successively with 200 ml of hexane/chloroform (8:2) and 200 ml of hexane/chloroform (6:4). The hexane/chloroform eluted the benzoyl derivatives.

The residue derived from *p*-nitrobenzoyl chloride was extracted with 60 ml of chloroform/methanol (2:1). The extracts were partitioned by adding 0.2 vol of water, washed with the theoretical upper phase of Folch et al. (10) and evaporated to dryness. For characterization by mass spectrometry (MS), the resulting *p*-nitrobenzoyl derivatives were purified by preparative TLC by use of chloroform as the developing solvent (Fig. 1).

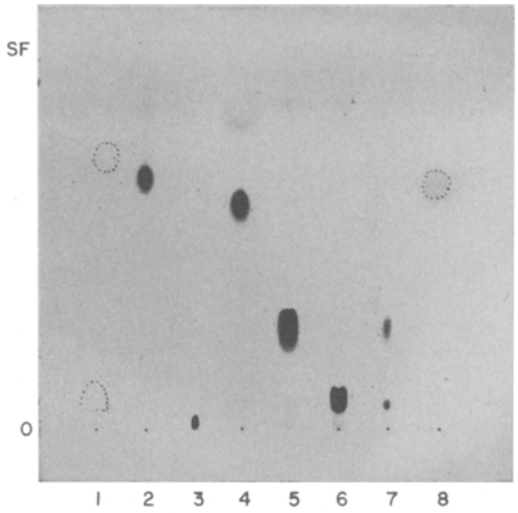

FIG. 1. Separation of ceramide derivatives by thin layer chromatography. Lane 1: methyl benzoate (upper spot) and benzoic acid (lower spot); lane 2: tribenzoyl, nonhydroxy ceramides; lane 3: nonhydroxy ceramides; lane 4: tri-*p*-nitrobenzoyl, nonhydroxy ceramides; lane 5: di-*p*-nitrobenzoyl, nonhydroxy ceramides; lane 6: mono-*p*-nitrobenzoyl, nonhydroxy ceramides; lane 7: reaction products of nonhydroxy ceramides with *p*-nitrobenzoyl chloride at 15 C; and lane 8: methyl-*p*-nitrobenzoate. The solvent was chloroform and the spots were visualized by short-wavelength UV light (dotted circles) and 50% aq H_2SO_4.

High Performance Liquid Chromatography

HPLC analyses were done on a Laboratory Data Control (Riviera Beach, FL) Constametric IIG high performance liquid chromatograph equipped with a Laboratory Data Control Spectromonitor II variable wavelength UV monitor. Three Excalibar reverse-phase columns (4.6 mm id × 25 cm, stainless steel tubing), LiChrosorb RP18 (5 μ and 10 μ), LiChrosorb RP8 (10 μ) and Spherisorb S5 ODS (5 μ and 10 μ), were tested for the separation of ceramide derivatives. The 10-μ LiChrosorb RP18 was the most suitable for the separation of both benzoyl and *p*-nitrobenzoyl derivatives of nonhydroxy ceramides. Isocratic

elution of ceramide derivatives was attempted with acetonitrile/methanol/water mixture, hexane/acetonitrile mixture, chloroform/acetonitrile mixture and 2-10% chloroform in methanol. Satisfactory resolution of benzoyl and *p*-nitrobenzoyl derivatives of ceramides was achieved by 5-10% chloroform in methanol and 2-5% chloroform in methanol, respectively. Therefore, all data presented in this report were obtained by use of the chloroform/methanol system. The optimal wavelength for the detection of *p*-nitrobenzoyl derivatives was 260 nm. Although the benzoyl derivatives of ceramides had the maximal absorption at 230 nm, 240 nm was chosen for the optimal wavelength since there was a strong UV absorption from the eluting solvent, 5-10% chloroform in methanol. The flow rate was maintained at 1.5-2.0 ml/min and the attenuation was usually set at a full scale of 0.01 or 0.02 absorbance unit. For the quantitative determination, the peak areas were measured by a Laboratory Data Control Model 308 computing integrator or by triangulation.

Other Analytical Methods

TLC of ceramides and their derivatives was done on Silica Gel G plates (0.25 mm thick) with chloroform as the developing solvent. The spots were visualized under short-wave length UV light and then by heating the plate at 120 C for 5 min after spraying with 50% aqueous sulfuric acid. TMS ether derivatives of nonhydroxy ceramides and fatty acid methyl esters derived from ceramides were analyzed by GLC on a Hewlett-Packard 7610 or 5730A gas chromatography, both equipped with flame ionization detectors. TMS derivatives of ceramides were analyzed on a Series 3000-3 HI-EFF coiled glass column (2 mm id x 0.9 m) packed with 3% OV-101. Fatty acid methyl esters were analyzed on a U-shaped glass column (4 mm id x 1.8 m) packed with 3% Silar 10C. Mass spectra were determined by use of direct probe with A.E.I. 902 mass spectrometer at the Pennsylvania State University. The ion source was 260 C and the ionizing electron energy was 70 eV.

RESULTS

Characterization of Ceramide Derivatives

Separation of ceramide derivatives by TLC is presented in Figure 1. Examination of the *p*-nitrobenzoyl derivative of ceramides formed at room temperature shows the presence of 2 derivatives (Fig. 1, lane 7) whose R_f values were lower than that (Fig. 1, lane 4) formed at 60 C for 3 hr. Therefore, the reaction products (Fig. 1, lanes 2 and 4) of a representative ceramide, N-heptadecanoyl D-sphingosine, formed at 60 C for 3 hr with both benzoyl and *p*-nitrobenzoyl chloride, were characterized by MS. Figure 2a shows the mass spectrum of benzoyl derivative of N-heptadecanoyl D-sphingosine. To facilitate the understanding of the spectrum, the structural formula and the possible fragmentation patterns are shown together. The mass spectrum shows a pronounced molecular ion at m/e 863 corresponding to N-benzoyl-N-heptadecanoyl-1,3-di-*O*-benzoyl-D-sphingosine. Ions due to elimination of a, (b+1), d, e, 2 (e+1), (d+e) and (e+C_6H_5CO) groups from the molecular ion are clearly revealed in the spectrum. The fragments at m/e 253 and 294 provide information on the fatty acid moiety and the rearranged fragments at m/e 354 and 367 indicate the structure of D-sphingosine. The rearranged fragments at m/e 502 and 515 give information on both the fatty acid moiety and D-sphingosine. In addition, there are peaks due to the benzoyl group and benzoic acid at 105 and 122, respectively. The spectrum exhibits fragmentation patterns which are very similar to that of tribenzoyl N-oleoyl sphingosine recently reported in the literature (5). Figure 2b shows the mass spectrum of *p*-nitrobenzoyl derivative of N-heptadecanoyl D-sphingosine. Although the molecular ion is not observed, the correct molecular weight is indicated by ions at m/e 831 [M-(e+1)], m/e 682 [M-(e+$CO.C_6H_4.NO_2$)], m/e 664 [M-2(e+1)] and m/e 651 [M-2(d+e)]. The ions at m/e 253, 294, 502 and 515 observed in the spectrum of tribenzoyl derivative of N-heptadecanoyl D-sphingosine (Fig. 2a) are also prominent in this spectrum. The rearranged fragments at m/e 399 and 412 indicate the structure of D-sphingosine. The base peak at m/e 150 is due to *p*-nitrobenzoyl group and the peak at m/e 167 is due to *p*-nitrobenzoic acid. From these fragmentation patterns, the structure is identified as N-*p*-nitrobenzoyl-N-heptadecanoyl-di-*O*-*p*-nitrobenzoyl D-sphingosine.

HPLC of Ceramide Derivatives

When nonhydroxy ceramides were derivatized at 60 C for 3 hr, the yield of both tribenzoyl and tri-*p*-nitrobenzoyl derivatives based on ceramides was consistently over 98%. These derivatives were routinely subjected to the HPLC analysis without prior chromatographic purification. However, for the accurate determination of molecular species of nonhydroxy ceramides derived from bovine brain and egg yolk, these derivatives were purified by column chromatography or TLC as described in "Methods" to remove various by-products derived

from the derivatizing reagents. For the quantitation of ceramides, the standard curves of tri-*p*-nitrobenzoyl and tribenzoyl derivative of N-heptadecanoyl D-sphingosine were obtained by injecting increasing amounts of the derivatives dissolved in 5-10 μl of carbon tetrachloride. Inspection of Figure 3 reveals that the ratio of peak area to amount of tri-*p*-nitrobenzoyl derivative injected is linear, indicating that the *p*-nitrobenzoyl derivative is satisfactory for the quantitative determination of ceramides. However, the ratio of peak area to amount of tribenzoyl derivative injected showed a biphasic linearity (not shown) mainly due to strong UV absorption from the eluting solvent, 10% chloroform in methanol. Since the *p*-nitrobenzoyl derivative gave linear UV response and 2-3 times higher sensitivity than the tribenzoyl derivative, all the quantitative data were obtained by use of tri-*p*-nitrobenzoyl derivatives. The lower limit for the detection of tri-*p*-nitrobenzoyl ceramides at 260 nm was ca. 50 pmol. Figure 4 shows the HPLC separation of molecular species of reference ceramides derived from saturated and unsaturated fatty acids as tri-*p*-nitrobenzoyl derivatives. As expected on a reverse-phase column, ceramides derived from shorter chain fatty acids were eluted earlier than those derived from longer chain fatty acids. Ceramides derived from

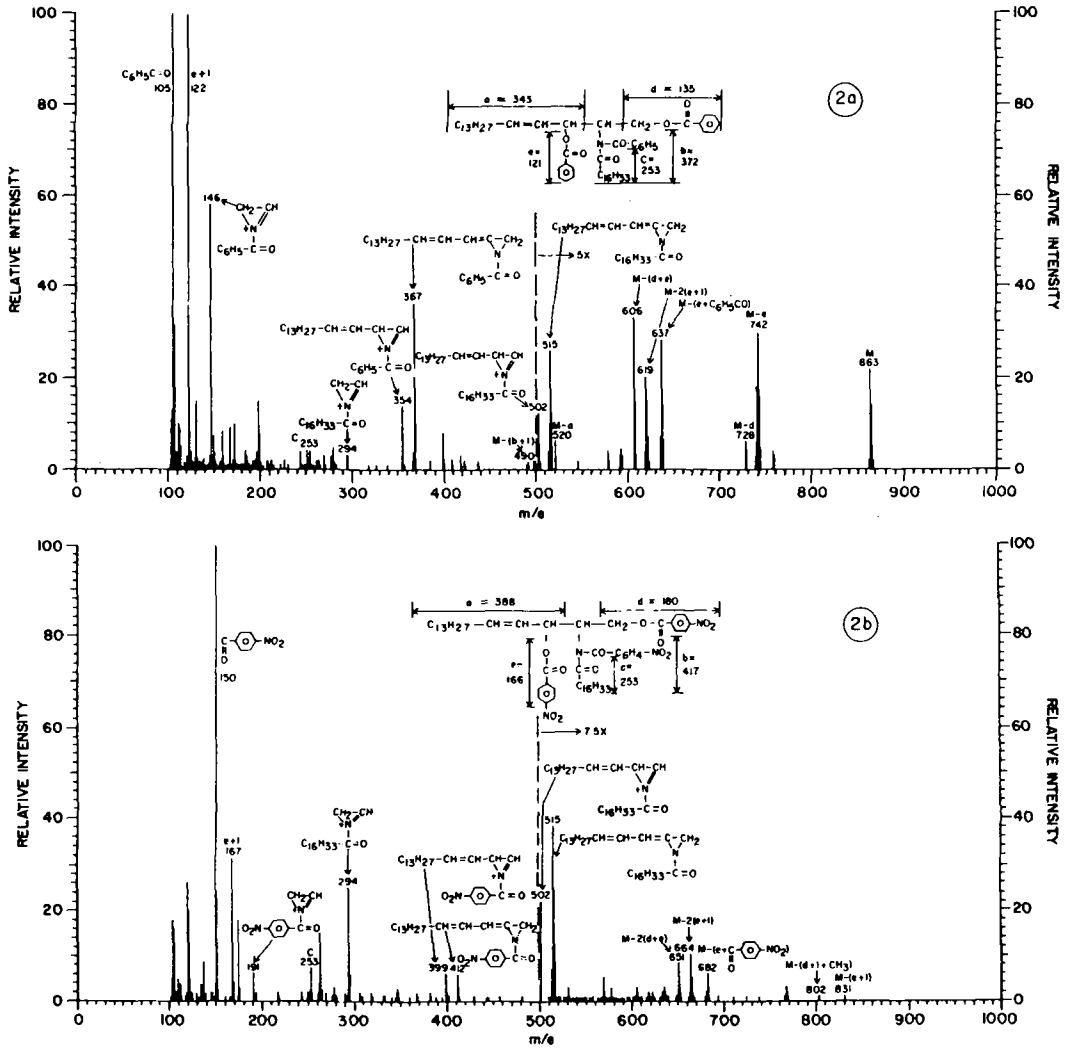

FIG. 2. Partial mass spectra of ceramide derivatives. (a) Tribenzoyl N-heptadecanoyl D-sphingosine; and (b) Tri-*p*-nitrobenzoyl N-heptadecanoyl D-sphingosine.

monoenoic fatty acids were eluted earlier than those derived from saturated fatty acids of the same carbon atoms and eluted together with ceramides derived from 3 carbonless saturated fatty acids on the 10-μ LiChrosorb RP18 column. For example, 11-eicosenoic acid/heptadecanoic acid overlap on the chromatogram. When ceramides derived from 6 molar equivalents of D-sphingosine and a mixture of 1 molar equivalent of palmitic, stearic, arachidic, behenic, tricosanoic and lignoceric acid were derivatized with a 10-fold excess of *p*-nitrobenzoyl chloride at 60 C for 3 hr, the relative peak ratio of one peak to another was 1, which agreed well with the theoretical molar ratio. This feature was very useful since it eliminated the need for determining the individual molar response factor of each peak to an internal standard.

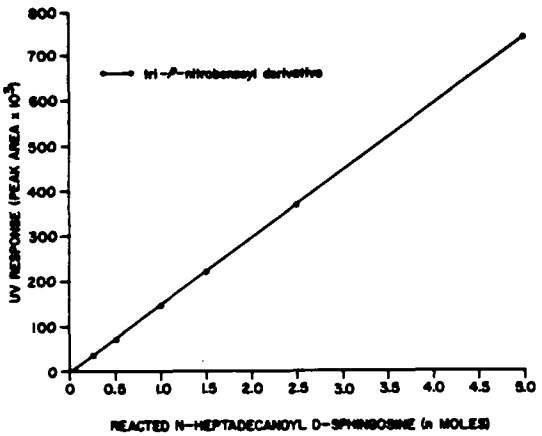

FIG. 3. The standard curve for tri-*p*-nitrobenzoyl N-heptadecanoyl D-sphingosine.

Analysis of Molecular Species of Nonhydroxy Ceramides Derived from Egg Yolk Sphingomyelins and Bovine Nonhydroxy Cerebrosides

A typical HPLC of intact egg yolk nonhydroxy ceramides derived from sphingomyelins by the action of phospholipase C is shown in Figure 5a. The predominant species is N-palmitoyl D-sphingosine. Moderate amounts of ceramides containing stearic and nervonic acid and small amounts of other ceramides containing saturated and monoenoic acids are also present. The typical GLC of the same intact egg ceramides is presented in Figure 5b. The GLC separation of ceramides is achieved mainly according to the number of carbon atoms. However, the peak corresponding to N-palmitoyl D-sphingosine is resolved from that corresponding to N-palmitoyl D-dihydrosphingosine in a manner similar to the previous HPLC analysis. Ceramides containing monoenoic acids are eluted together with those containing saturated fatty acids of the same carbon atoms. Unfortunately, the peak of N-myristoyl D-sphingosine overlaps with that of an impurity which has not been removed even after vacuum desiccation for 4 hr. Table 1 shows the quantitative composition of fatty acids derived from bovine brain nonhydroxy cerebrosides and egg yolk nonhydroxy sphingomyelins. It also compares the composition of fatty acid moiety of intact egg yolk ceramides determined by HPLC with that determined by GLC.

DISCUSSION

The structures of *p*-nitrobenzoyl derivatives formed at room temperature were not characterized by MS. However, since the 2 derivatives showed a gradual decrease in their R_f values on the TLC (Fig. 1) and in their retention volumes on the reverse-phase HPLC (not shown) relative to the R_f value and retention volume of the tri-*p*-nitrobenzoyl derivative, the upper and lower spot in Figure 1, lane 7, were assumed to be di- and mono-*p*-nitrobenzoyl derivative of ceramides, respectively.

The HPLC of *p*-nitrobenzoyl derivatives of

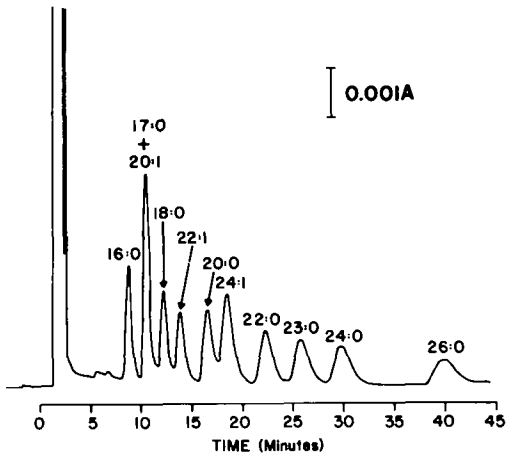

FIG. 4. The high performance liquid chromatogram of tri-*p*-nitrobenzoyl derivatives of reference nonhydroxy ceramides derived from saturated and unsaturated fatty acids. The number before and after the colon indicates the number of carbon atoms and the number of double bonds in fatty acid moiety, respectively. The long chain base of the ceramides is D-sphingosine. Conditions for chromatography were: 10 μm LiChrosorb RP18 column (4.6 mm id X 25 cm); eluting solvent, 3% chloroform in methanol; flow rate, 2 ml/min.

ceramides had the following advantages over that of their benzoyl derivatives: (a) higher sensitivity, (b) more stable baseline due to weaker UV absorption from the eluting solvent and (c) wider range of linear detector response. From the comparison of Figures 5a and 5b, the HPLC analysis of intact ceramides appears to be superior to their GLC analysis because of its greater sensitivity for ceramides containing longer chain fatty acids, its better resolution of ceramides containing unsaturated fatty acids, its nondestructive assay conditions at room temperature and the absence of an overlapping peak due to an impurity.

The fatty acid composition of bovine brain nonhydroxy cerebrosides and egg yolk nonhydroxy sphingomyelins in Table 1 clearly reveals the dramatic compositional difference. Some overlapping peaks (15:0 + 18:1, 17:0 + 20:1, 19:0 + 22:1, 21:0 + 24:1 and 22:0 + 25:1) on the HPLC of natural ceramides were identified by simultaneous GLC analysis of fatty acid methyl esters derived from the corresponding ceramides on a 3% Silar 10C column. When the fatty acid composition of intact egg yolk ceramides determined by the HPLC method was quantitatively compared with the composition of fatty acid methyl esters determined by the GLC method, a good correlation was found, except for some minor components, as shown in Table 1. Therefore, the HPLC analysis of intact ceramides and the GLC analysis of fatty acid methyl esters derived from these ceramides are complementary for the accurate identification and quantitation of fatty acid moiety. When the purified tri-*p*-nitrobenzoyl derivatives of egg yolk nonhydroxy ceramides derived from sphingomyelins were subjected to the HPLC analysis, all the peaks in Figure 5a were readily identified by comparison with those of reference ceramides in Figure 4, except for a small, unknown peak (labeled 16:0 LCB 18:0) eluting immediately after a major peak (labeled 16:0) corresponding to N-palmitoyl D-sphingosine. The quantitative analysis of peak areas revealed that the peak area ratio of the major peak to the unknown peak was 97:3. Since the composition of long-chain bases of the same egg yolk ceramides had been characterized by GLC as their TMS derivatives and found to consist of 97% D-sphingosine and 3% D-dihydrosphingosine in our previous studies (7), the unknown peak was suspected to be the peak corresponding to the tri-*p*-nitrobenzoyl derivative of N-palmitoyl D-dihydrosphingosine based on its relative peak area ratio and retention time.

In order to characterize the unknown peak, the authentic N-palmitoyl D-sphingosine was completely hydrogenated with platinum oxide and the resulting N-palmitoyl D-dihydrosphingosine was derivatized with *p*-nitrobenzoyl

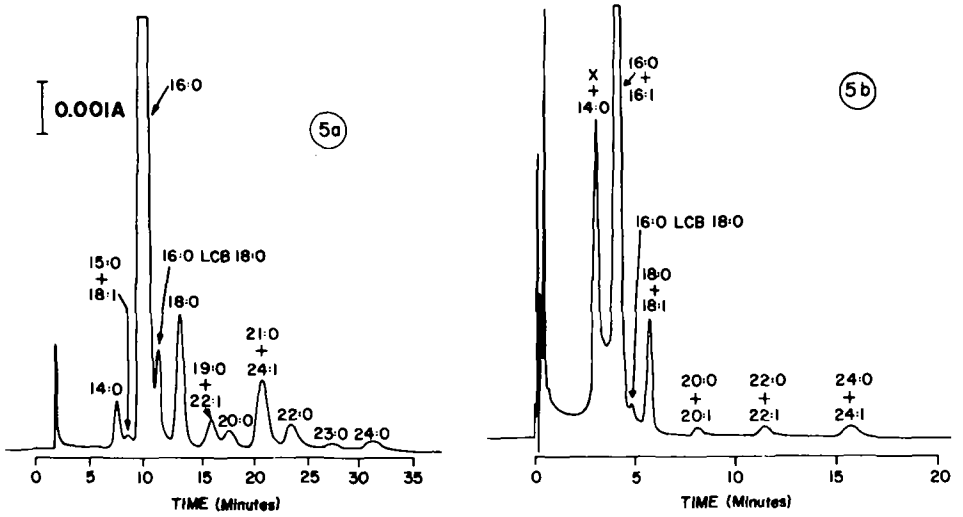

FIG. 5. (a) The high performance liquid chromatogram of tri-*p*-nitrobenzoyl derivatives of nonhydroxy ceramides derived from egg yolk sphingomyelins. The chromatographic conditions are the same as in Fig. 4 except that the eluting solvent is 2% chloroform in methanol. (b) The gas liquid chromatogram of trimethylsilyl ether derivatives of nonhydroxy ceramides derived from egg yolk sphingomyelins. The chromatographic conditions are given in the text: column temperature, 320 C; nitrogen flow rate, 35 ml/min. The abbreviations for fatty acid moiety are the same as those of Fig. 4. The long-chain base of the ceramides is D-sphingosine, except the peak, 16:0 LCB 18:0, in which the long chain base is D-dihydrosphingosine.

TABLE 1

Fatty Acid Composition of Nonhydroxy Ceramides Derived from Bovine Brain Cerebrosides and Egg Yolk Sphingomyelins

Fatty acid moiety	Bovine brain ceramides[a]	Egg yolk ceramides[b]	
	HPLC (%)	HPLC (%)	GLC (%)[c]
14:0	–	1.4	1.0
15:0 + 18:1	0.5	0.5	0.3
16:0	0.4	82.3	84.3
17:0 + 20:1	1.2	–	–
18:0	8.5	5.9	5.2
19:0 + 22:1	1.1	1.7	0.7
20:0	1.6	1.0	1.0
21:0 + 24:1	24.2	4.5	4.7
22:0 + 25:1	14.0	1.7	1.5
23:0 + 26:1	11.9	0.3	0.6
24:0	30.0	0.7	0.7
25:0	5.0	–	–
26:0	1.5	–	–
Total	100.0	100.0	100.0

[a]Derived from nonhydroxy cerebrosides.

[b]Derived from sphingomyelins by phospholipase C.

[c]Fatty acid methyl esters derived from nonhydroxy ceramides.

chloride at 60 C for 3 hr. When the tri-*p*-nitrobenzoyl derivative of N-palmitoyl D-dihydrosphingosine was purified by preparative TLC and injected with that of N-palmitoyl D-sphingosine into a 10-μ LiChrosorb RP18 column, the peak corresponding to N-palmitoyl D-dihydrosphingosine was eluted immediately after the peak corresponding to N-palmitoyl D-sphingosine. The observed elution patterns of these derivatives were theoretically expected on a reverse-phase column because N-palmitoyl D-dihydrosphingosine would be more hydrophobic than N-palmitoyl D-sphingosine and also have a longer retention time. When the tri-*p*-nitrobenzoyl derivative of N-palmitoyl D-dihydrosphingosine was injected with those of egg yolk nonhydroxy ceramides, the peak corresponding to N-palmitoyl D-dihydrosphingosine chromatographed with the unknown peak in Figure 5a.

When the tri-*p*-nitrobenzoyl derivative of N-palmitoyl D-dihydrosphingosine was injected with that of either N-heptadecanoyl or N-eicosenoyl D-sphingosine, the peak corresponding to N-palmitoyl D-dihydrosphingosine was eluted before the peak corresponding to either N-heptadecanoyl or N-eicosenoyl D-sphingosine. Furthermore, the GLC analysis of fatty acid moiety of the egg yolk nonhydroxy ceramides revealed that no detectable amount of heptadecanoic or eicosenoic acid was present as shown in Table 1. These results eliminated the possibility that N-heptadecanoyl or N-eicosenoyl D-sphingosine might be responsible for the presence of the unknown peak.

The presence of N-palmitoyl D-dihydrosphingosine in egg yolk ceramides derived from sphingomyelins was further supported by the HPLC analysis of their purified di- and mono-*p*-nitrobenzoyl derivatives formed at room temperature. When these derivatives were analyzed by HPLC on the reverse-phase column, there was always an unknown peak which was eluted immediately after the major peak corresponding to N-palmitoyl D-sphingosine; the peak area ratio of N-palmitoyl D-sphingosine to the derivative was 97:3. The constant peak area ratio of these 2 peaks before and after TLC purification observed with mono-, di- and tri-*p*-nitrobenzoyl derivatives strongly suggested that the presence of the small peak was not due to an impurity formed during the derivatization or purification processes. Although the conclusive proof for the presence of N-palmitoyl D-dihydrosphingosine in egg yolk nonhydroxy ceramides requires the MS analysis of the unknown peak, the experiments just described make it possible to assign its structure as N-palmitoyl D-dihydrosphingosine with considerable certainty. Interesting is that Jungalwala et al. (11) also described the HPLC resolution of molecular species of intact bovine brain sphingomyelins containing N-stearoyl D-sphingosine and N-stearoyl D-dihydrosphingosine on a reverse-phase column.

However, the resolution of peaks corresponding to N-acyl D-dihydrosphingosine from those corresponding to N-acyl D-sphingosine

was not evident when bovine brain nonhydroxy ceramides were derivatized and analyzed by HPLC, presumably because bovine brain ceramides consisted of more complex fatty acids than egg yolk ceramides and peaks corresponding to ceramides containing D-dihydrosphingosine overlapped those corresponding to ceramides containing D-sphingosine. In agreement with our result, Yahara et al. (6) also reported that they were unable to resolve the long-chain base moiety of benzoylated sphingolipids derived from calf brain, even though they achieved excellent resolution of fatty acyl group of these derivatives on a 5-μ Spherisorb ODS column with methanol/acetonitrile mixture. Although the accurate determination of minor molecular species of ceramides from tissues necessitates the use of MS along with GLC or HPLC, the proper choice of a reverse-phase HPLC column (10 μ LiChrosorb RP18) and the inexpensive solvent system consisting of 2-10% chloroform in methanol allows one to separate benzoyl and *p*-nitrobenzoyl derivatives of ceramides by isocratic elution mainly according to their fatty acyl group and, in some cases, their long-chain base moiety. Therefore, the HPLC method described in this paper is recommended for the routine analysis of molecular species of ceramides.

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Potential Bile Acid Metabolites: IV. Inversion of 7α-Hydroxyl; Ursodeoxycholic Acid[1]

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ABSTRACT

A new method for inversion of chenodeoxycholic to ursodeoxycholic acid which does not involve an intermediary ketone is described. Methyl chenodeoxycholate-3-cathylate-7-mesylate undergoes a mild reaction with potassium superoxide-18-crown ether-6 in DMSO to give ursodeoxycholic acid in good yield.

INTRODUCTION

Recent use (1-3) of potassium superoxide-crown ether for inverting alcohols suggested that a direct inversion of a 7α-hydroxy acid to its epimer without passing through an intermediary ketone (4-6) might be feasible. This paper is a report of the successful application of the method, as exemplified by conversion of chenodeoxycholic (3α,7α) to ursodeoxycholic (3α,7β) acid.

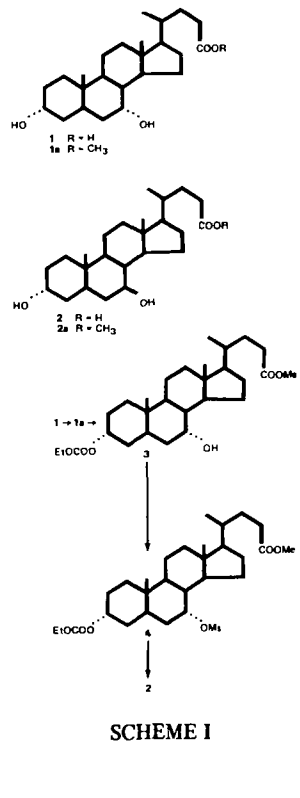
DISCUSSION

Methyl chenodeoxycholate-3-cathylate-7-mesylate (Scheme I, 4) treated with potassium superoxide and a crown ether in dimethyl sulfoxide gave ursodeoxycholic acid (2). The cathylate mesylate 4 was prepared from chenodeoxycholic acid without crystallization of the intermediates, methyl ester 1a and methyl ester 3-cathylate 3. Crystalline 4 from *i*-propyl ether is a solvate which is stable at room temperature and melts sharply when placed on a melting point block at a temperature near its melting point, but decomposes when dried in vacuum at 78 C, and slowly even at room temperature when evacuated. However, 4 solvate undergoes the inversion reaction smoothly.

In the inversion reaction, the medium is sufficiently alkaline to cause simultaneous hydrolysis of the ethoxycarbonyloxy group at C-3 and the methyl ester of C-24, resulting in the formation of the dihydroxy carboxylate salt. Extraction of the aqueous saline product with benzene completely removes the less polar contaminants so that subsequent acidification leaves the bile acid, which is extracted with ethyl acetate (If the product of the inversion reaction after treatment with saline is first acidified before extraction with methylene chloride [or ethyl acetate], the organic extracts contain nonpolar contaminants, mainly DMSO, crown ether and dimethylsulfone, which are difficult to remove even by column chromatography.) Crystallization from ethyl acetate/hexane affords ursodeoxycholic acid (2) in excellent yield (56% overall from chenodeoxycholic acid [1]).

EXPERIMENTAL

Melting points were determined on an electric micro-hotstage and are uncorrected. Infrared spectra were obtained on a Perkin-



SCHEME I

[1]Paper 3 of the series: Iida, T., and Chang, F.C. (1981) J. Org. Chem. 46, 2786.

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Elmer 137 spectrophotometer. NMR spectra were determined on a Perkin-Elmer R-32 instrument, with deuteriochloroform containing Me_4Si as solvent. Solvents were removed by distillation at 50 C under reduced pressure.

Methyl Chenodeoxycholate-3-Cathylate (3)

This was prepared by the method (7) of Fieser and Rajogopalan by treatment of methyl chenodeoxycholate with ethyl chloroformate. The cathylate 3 crystallized from aqueous methanol melted at 97.5-100.0 C (lit. [8] 136 C [from methanol]); NMR δ 0.67 (3 H, s, C-18 Me), 0.91 (3 H, s, C-19 Me), 1.28 (3 N, t, J=7 Hz -OCOCH$_2$CH$_3$), 3.64 (3 H, s, COOMe), 3.82 (1 H, m, C-7 CHOH), 4.14 (2 H, q, J=7 Hz, -OCOCH$_2$CH$_3$), 4.44 (1 H, brm, C-3 CHOCOCH$_2$CH$_3$).

Anal. calcd for $C_{20}H_{46}O_6 \cdot 0.5H_2O$: C, 68.97; H, 9.71. Found: C, 68.87; H, 9.38.

Methyl Chenodeoxycholate-3-Cathylate-7-Mesylate (4)

Chenodeoxycholic acid (1), 6.5 g, dissolved in 60 ml of methanol, was treated with 1.5 ml of conc. HCl and allowed to stand overnight at room temperature. Upon evaporation of the solvent, the residue was dissolved in methylene chloride, shaken with $NaHCO_3$ solution, washed with water to neutrality, then evaporated to dryness. After azeotropic distillation in benzene, the residual oil (1a) was dissolved in 60 ml of dioxane containing 12 ml of pyridine. To the stirred solution, 12 ml of ethyl chloroformate was gradually added. After standing at room temperature for 2 hr, the reaction mixture was diluted with H_2O and extracted with ether (60 ml, 2X). The combined ether solution was washed successively with ice-cold dilute HCl and H_2O to neutrality, evaporated to dryness, then azeotropically distilled in benzene. To the residual oil (3) redissolved in 50 ml of pyridine and magnetically stirred, 6.5 ml of methanesulfonyl chloride was slowly added by drops. After overnight standing at room temperature, the dark pyridine solution was stirred, very gradually diluted with ice chips, and extracted with methylene chloride (60 ml, 2X). The combined CH_2Cl_2 extract was washed successively with cold, dilute HCl, $NaHCO_3$ and H_2O, decolorized with Norite and evaporated to an oil, which upon addition of isopropyl ether, crystallized. Recrystallization from *i*-propyl ether afforded 5.57 g (60%) of 4 solvated with 1/3 mol of the ether, as prismatic needles, mp 107.5-108.0 C; NMR ($CDCl_3$) δ 0.67 (3 H, s, C-18 Me), 0.95 (3 H, s, C-19 Me), 1.30 (3 H, t, J-7 Hz, -OCOCH$_2$CH$_3$), 3.00 (3 H, s, -SO$_2$-CH$_3$), 3.64 (3 H, s, -COOMe), 4.17 (2 H, q, J=7 Hz, -OCOCH$_2$CH$_3$), 4.42 (1 H, brm, C-3 CHOCOCH$_2$CH$_3$), 4.89 (1 H, m, C-7 CHOSO$_2$-CH$_3$).

Anal. calcd for $C_{29}H_{48}O_8S \cdot 0.33\ (C_3H_7)_2O$: C, 63.03; H, 8.99. Found: C, 63.09; H, 8.72.

Ursodeoxycholic Acid (2)

To powdered KO_2 (1.2 g, Ventron Co. product, powdered in a dry box before use) in 90 ml of DMSO (dried in 4A molecular sieves) stirred under N_2 for 10 min, was added 0.75 g of 18-crown-6 (Aldrich Chemical Co. product 18,665-1). Stirring under N_2 was continued for 5 min and throughout the subsequent reaction, as 2.25 g of the cathylate mesylate 4, after azeotropic distillation (benzene) and dissolved in 8 ml of DMSO, was added. The reaction was monitored by TLC (hexane/EtOAc/HOAc; 50:48:2, v/v/v). After 2 hr, the reaction flask was immersed in an ice bath, and with stirring continued, 120 ml of saturated sodium chloride solution was added. The mixture was extracted with benzene (100 ml, 2X). The lower (aqueous) layer was cooled in an ice bath, acidified with 3 N HCl to neutrality, and extracted with EtOAc (100 ml, 2X). The EtOAc extract washed with water and subsequently dried (Drierite), upon evaporation, yielded a residue which crystallized from EtOAc/hexane as colorless tufts of needles, 1.38 g (87%), mp 199.5-200.5 C. Crystallized from ethyl acetate, the mp was 203.4-205.0 C (lit. 196-197 C [4] and 201-202 C [5]). IR (KBr): 1709 (COOH); 1050, 1010 cm^{-1} (-C-OH). NMR ($CDCl_3$-DMSO-d_6, 9:1), ref. 9: δ 0.69 (3 H, s, C-18 Me), 0.96 (3 H, s, C-19 Me), 3.62 (2 H, br m, C-3 and C-7 CHOH).

Anal. calcd for $C_{24}H_{40}O_4$: C, 73.43; H, 10.27. Found: C, 73.73; H, 10.63.

A second crop of crystals weighed 0.09 g (6%), mp 196-198 C.

Methyl Ursodeoxycholate (2a)

Esterification by overnight standing of the acid 2 in methanol containing conc. HCl and processed in the usual manner yielded the methyl ester 2a which crystallized from aqueous ethanol as fine colorless needles, mp 157-159 C (lit. [10] 152 C). IR ($CHCl_3$): 1721 (-COOMe); 1042, 1007 cm^{-1} (-COH). NMR ($CDCl_3$): δ 0.69 (3 H, s, C-18 Me), 0.94 (3 H, s, C-19 Me), 3.55 (2 H, br m, C-3 and C-7 CHOH), 3.65 (3 H, s, COOMe).

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COMMUNICATIONS

Production of Prostaglandins in Homogenates of Kidney Medullae and Cortices of Spontaneously Hypertensive Rats Fed Menhaden Oil

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ABSTRACT

Menhaden oil (MO), whose polyunsaturated fatty acids consist mainly of (n-3) fatty acids, was fed to spontaneously hypertensive rats to determine the effect of (n-3) fatty acid on the in vitro production of prostaglandins produced from arachidonic acid (20:4[n-6]). Capacity to form PGE_2 and $PGF_{2\alpha}$ was impaired in homogenates of kidney medullae and cortices from rats fed the MO diet compared to rats fed the control diet. The lower amounts of diene prostaglandins produced corresponded to the decrease in the amount of 20:4 (n-6) in the tissue. Possibly changes produced in tissue lipids by dietary fatty acids affect prostaglandin production by reducing the availability of substrate in tissue lipids.

INTRODUCTION

Interest in metabolism of (n-3) fatty acids has been stimulated by reports that link the low incidence of cardiovascular disease in Greenland Eskimos with their dietary intake of marine fish oil (1). The polyunsaturated fats of fish oils are predominantly from the (n-3) family and when ingested replace (n-6) fatty acids in tissue lipids. This effect has been demonstrated in plasma and platelets from Greenland Eskimos (2,3), in subjects fed mackerel (4), and in tissues of laboratory animals fed fish oil (5,6).

Depression in platelet aggregation, which results in a bleeding tendency, is thought to palliate the development of cardiovascular disease (7). Apparently, eicosapentaenoic acid (20:5[n-3]) is the dietary fatty acid in the marine oil that lowers platelet responsiveness in the Eskimos. In the platelet, 20:5(n-3) may exert its effect by competing with 20:4(n-6) for the cyclooxygenase enzyme (8) thereby lowering the formation of proaggregatory 20:4(n-6) metabolites. Triene prostaglandins produced from 20:5(n-3) may be formed by some tissues in vitro (9-11), but evidence for in vivo production is lacking.

Studies in our laboratory have focused on the relationships among dietary intake of polyunsaturated fatty acids, prostaglandin metabolism, and control of blood pressure. We found that a diet deficient in essential fatty acids exacerbated the development of hypertension in spontaneously hypertensive rats (SHR) (12). We also found that in vitro production of PGE_2 and $PGF_{2\alpha}$ in kidney medullae of SHR and the normotensive controls (Kyoto-Wistar, WKY) was decreased because of the deficiency in (n-6) fatty acids. These results suggested that the amount of 20:4(n-6) available in tissue lipids might be an important determinant of amount and type of diene prostaglandins formed.

In 1974, Okamoto et al. (13) introduced a substrain of the SHR (SHR/SP) that was more hypertensive than the SHR and subject to strokes after the age of 6 months. Since hypertension is one of the most important risk factors in the development of cerebro-and cardiovascular diseases, we have studied the effects of a diet containing fish oil on blood pressure and prostaglandin metabolism in the SHR and SHR/SP. The effects of the diet on blood pressure appeared in a preliminary report (14). We now report the effects of an MO diet on the potential for production of prostaglandins in kidneys of SHR, SHR/SP and WKY.

MATERIALS AND METHODS

Male weanling rats were fed purified diets containing either 5% corn oil (CO) or 1% corn

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oil plus 4% menhaden oil (MO) (Zapata Haynie Corp., Reedville, VA) as the source of fat (15). Corn oil was added to the MO diet in order to avoid a deficiency of (n-6) fatty acids that can produce an increase in blood pressure in the SHR (12). The antioxidant butylated hydroxy toluene was added to both diets (0.02%). The animals, 10/group, were maintained on the diets for 22 weeks. SHR and WKY were obtained from Taconic Farms, Germantown, NY. Dr. Carl Hansen, NIH, Bethesda, MD, kindly furnished the SHR/SP. At the termination of the experiment, kidneys were rapidly removed from anesthetized animals. The medullae and cortices were immediately excised, weighed, homogenized, and incubated for 10 min at 37 C under air in phosphate buffer (0.01 M, pH 7.2). Deuterated internal standards were then added and the pH adjusted to 3.5 with 2 M citric acid. Prostaglandins were extracted with ethyl acetate and derivatized by the method of Nicosia and Galli (16). PGE_2 and $PGF_{2\alpha}$ were determined by selected ion monitoring with a Finnigan 3200 F GC-MS system with a PROMIN attachment (12). Lipids were extracted from the kidneys and fatty acid methyl esters were prepared as described previously (15).

RESULTS AND DISCUSSION

In vitro production of PGE_2 and $PGF_{2\alpha}$ by homogenates of kidney medullae and cortices were significantly lower for rats fed the MO rather than the CO diet (Table 1). Because of the high activity of catabolizing enzymes (17) and lower activity of the synthetase enzyme (18) in the cortex of rat kidney, amounts of prostaglandins in the cortical homogenates were lower than those in the medullary homogenates. Our data does not indicate a strain difference in catabolism as described by Pace-Asciak (19). He reported that kidneys of SHR had lower activity of 15-hydroxyprostaglandin dehydrogenase compared to that in normotensive controls. However, incubation conditions differed and this might explain why the results do not agree.

When SHR and WKY previously were fed diets deficient in (n-6) fatty acids, the results indicated that the in vitro production of $PGF_{2\alpha}$ by kidney medullae was significantly higher for SHR than for WKY (12). Our present data from older animals (15 vs 25 weeks) did not confirm these data. Also, in contrast to the earlier experiment, the MO diet contained 1% corn oil and thus was not lacking in (n-6) fatty acids. Pace-Asciak (19) reported that the decrease of prostaglandin catabolism in the kidney of SHR compared to the normotensive controls was age-related. Possibly in SHR, the activity of the 9-ketoreductase, which converts PGE_2 and $PGF_{2\alpha}$, changes with age. The level of 20:4-(n-6) was significantly lower in medullary total lipids for rats fed the MO instead of the CO diet (Table 2). A similar decrease was seen in the level of 20:4(n-6) in the cortical lipids for rats fed the MO diet. These decreases in 20:4(n-6) corresponded to the depression of PGE_2 and $PGF_{2\alpha}$ production in the homogenates from kidneys of rats fed the MO instead of the CO

TABLE 1

Levels of PGE_2 and $PGF_{2\alpha}$ Produced by Kidney Medullae and Cortices from Rats Fed Corn Oil and Menhaden Oil Diets

Strain[a]	PGE_2 (ng/mg tissue)		$PGF_{2\alpha}$ (ng/mg tissue)	
	CO[b]	MO	CO	MO
		Medullae		
SHR	18.8 ± 0.6[c,d]	9.7 ± 0.4	10.8 ± 1.0	5.7 ± 0.6
SHR/SP	17.9 ± 0.7	9.3 ± 0.6	10.3 ± 0.7	5.3 ± 0.3
WKY	18.1 ± 0.7	8.3 ± 0.5	10.6 ± 0.5	4.9 ± 0.3
		Cortices		
SHR	0.25 ± 0.05[c,d]	0.12 ± 0.02	2.89 ± 0.98	0.34 ± 0.09
SHR/SP	0.25 ± 0.03	0.07 ± 0.02	1.44 ± 0.38	0.19 ± 0.07
WKY	0.28 ± 0.05	0.12 ± 0.03	4.10 ± 1.22	0.29 ± 0.09

[a]SHR = spontaneously hypertensive rat, SHP/SP = stroke-prone spontaneously hypertensive rat, WKY = Kyoto Wistar rat.

[b]CO = 5% corn oil diet, MO = 4% menhaden oil + 1% corn oil diets.

[c]Mean ± SEM.

[d]Significant difference by diet for all strains, $p<0.001$.

diet. The amount of 20:4(n-6) was decreased by 44% in the kidney medulla whereas diene prostaglandin production dropped by 50%. However, the depression of diene production was greater in the cortical homogenate than what would be expected from the reduction in amounts of 20:4(n-6), e.g., in cortical tissue from SHR fed MO diene, prostaglandin production dropped by 85% whereas the amount of 20:4(n-6) fell by 57%. The level of linoleic acid (18:2[n-6]) was also lowered as the (n-3) fatty acids replaced (n-6) fatty acids in the tissue glycerolipids of rats fed the MO diet.

Our results demonstrating that (n-6) fatty acids were replaced with (n-3) fatty acids in tissue lipids and the subsequent effect on diene prostaglandin production are similar to those reported by others (4,20). Siess et al. (4) showed that the composition of platelet fatty acids from human subjects fed a mackerel diet for 1 week reflected the composition of the ingested lipid and that, in platelet phospholipids, the (n-3) fatty acids increased whereas (n-6) fatty acids decreased. Radioimmunoassay of thromboxane B_2 showed decreased production of this proaggregant that correlated with the increase in 20:5(n-3) in plasma and platelet lipids. Both 20:4(n-6) and 20:5(n-3) were released from platelet phospholipids during aggregation. Hwang and Carroll (20) fed varying levels of linolenic acids (18:3[n-3]) to rats and found an inverse relationship between dietary intake of 18:3(n-3) and levels of prostaglandins in serum as determined by radioimmunoassay.

At this time, we do not have the experimental evidence to state whether the (n-3) fatty acids are directly inhibiting the metabolism of 20:4 during the incubations. We can say with certainty, however, that 20:4(n-6) levels were decreased when rats fed (n-3) fatty acids and that the ability of the kidney homogenates to produce diene prostaglandins was diminished. We do have evidence that PGE_3 was produced during incubations of the medullary homogenates of rats fed MO in a proportionate amount of PGE_2 relative to the ratios of these 2 fatty acids in the tissue lipids (A. Ferretti, N.W. Schoene and V.P. Flanagan, Lipids 16:800 [1981]).

In vitro incubations of exogenous 20:5(n-3) with platelets, seminal vesicles and aortic microsomes (9-11) have produced evidence for the formation of various triene prostaglandins and verified the fact that 20:5(n-3) is a poor substrate for the cyclooxygenase (8). Urinary prostaglandins are an indication of production of these compounds in the kidney (21). We could not, however, detect triene prostaglandins in the urine of rats fed the MO diet, possibly because the analytical methodology lacked sensitivity. Thus, we cannot yet dismiss the possibility of in vivo production of the triene prostaglandins.

TABLE 2

Concentration of (n-6) and (n-3) Fatty Acids in Kidney Medullae from Rats Fed Corn Oil and Menhaden Oil Diets

Fatty acid (μg/mg tissue)	Diet treatment	
	CO[a]	MO
	Medullae	
18:2(n-6)	0.88 ± 0.04[b]	0.55 ± 0.02
20:4(n-6)	2.66 ± 0.14	1.50 ± 0.08
20:5(n-3)	ND[c]	0.43 ± 0.03
22:5(n-3)	ND	0.38 ± 0.04
22:6(n-3)	Trace[d]	0.43 ± 0.05
	Cortices	
18:2(n-6)	5.37 ± 0.51[b]	3.50 ± 0.21
20:4(n-6)	12.04 ± 0.84	5.12 ± 0.25
20:5(n-3)	ND[c]	2.40 ± 0.23
22:5(n-3)	ND	0.40 ± 0.03
22:6(n-3)	Trace[d]	1.53 ± 0.11

[a]Data as described in Table 1.

[b]Mean ± SEM. Concentrations of polyunsaturated acids are significantly different between diets ($p < 0.001$).

[c]None detected.

[d]Less than 1% of total fatty acids.

Extrapolation from in vitro and ex vivo experiments to physiological conditions is always difficult. The study of the intricate relationships among dietary fatty acids, lipid metabolism and prostaglandin production requires further experiments that should take this caveat into consideration.

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Identification of Arachidonic Acid in Gulf of Mexico Shrimp and Degree of Biosynthesis in *Penaeus setiferus*[1]

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ABSTRACT

An icosatetraenoic fatty acid, though to be all *cis*-5,8,11,14-icosatetraenoic acid (arachidonic acid), was isolated from shrimp total lipid. The acid was subjected to partial reduction with hydrazine hydrate, with subsequent isolation of the monoenoic reaction products which were shown to be *cis* in structure. These were then cleaved by periodate-permanganate oxidation and the resulting mono- and dicarboxylic acids were converted to methyl esters. Identification of the resulting mono- and dicarboxylic acids indicated that the original icosatetraenoic acid had the all *cis*-5,8,11,14 pattern of double bonds, and it was thus identified as arachidonic acid. Experiments were also performed to study the synthesis of arachidonic acid 20:4(5,8,11,14) from linoleic acid, 18:2(9,12) in microsomes prepared from shrimp hepatopancreas and tail muscle tissue. Each step of the pathway 18:2(9,12)→ 18:3(6,9,12) →20:3(8,11,14) →20:4(5,8,11,14) was assayed separately, and the level of activity of each enzyme was expressed as percentage conversion of substrate to product. It was found that, in each step of the sequence, the enzyme activity in the shrimp tissue was very low compared to the activity found in rats. These and previous observations seem to indicate that the arachidonic acid in shrimp tissue originates mostly in the diet.

INTRODUCTION

It has become increasingly evident that several major aspects of fatty acid metabolism in some marine organisms are quite different from those of land mammals. For example, while the ω6 polyenes are most important in maintaining growth and preventing symptoms of essential fatty acids (EFA) deficiency in rats, the ω3 polyenes seem to be more necessary for efficient growth and survival in cold-blooded marine animals, including shrimp (1-4). (In view of the ambiguity of the "ω" denomination for double bond position in the fatty acid chain, this nomenclature is only used in reference to biological properties of the fatty acids.) Our analyses of shrimp fatty acids (5) showed the presence of relatively high levels of ω3 polyenes, as would be expected, but they also evidenced the consistent presence of 6-9% of a fatty acid tentatively identified as arachidonic acid. This was curious, in view of the fact that only the ω3 fatty acids seem to be essential for shrimp. The positive identification and biosynthesis of this fatty acid in shrimp are the subjects of the work presented here.

EXPERIMENTAL PROCEDURES AND RESULTS

Identification of Arachidonic Acid in Shrimp

Gulf of Mexico penaeid shrimp were obtained from various sources, their heads and exoskeletons were removed, and the remaining organs and tail muscles were extracted for lipids according to Folch et al. (6). The fatty acids of the resulting lipid mixtures were converted to fatty acid methyl esters (FAME) and analyzed by gas liquid chromatography (GLC) as described previously (7) using siliconized polyethyleneglycol succinate (EGSS-X, Applied Science, State College, PA) as liquid phase. This procedure will be designated as EGSS-X GLC in the remainder of the paper. GLC analysis on EGSS-X of the FAME from shrimp total lipid revealed a peak with a retention time relative to 18:1 of 3.0. This peak was tentatively identified as corresponding to 20:4(5,8,11,14) and/or 20:3(11,14,17) with an area comprising 9% of the total chromatogram. FAME were subjected to repeated urea complexation according to Schlenk and Holman (8). By this treatment, the 4 double-bond fraction was enriched to a level of 14%. This increase was relatively small because of technique limitations and the fact that the shrimp lipids contained relatively large amounts of 20:5 and 22:6, which were also enriched by treatment with urea. Because 20:4(5,8,11,14) and 20:3(11,14,17) could not be resolved by EGSS-X GLC, it was necessary to first separate them by argentation thin layer chromatography (Ag-TLC) and then isolate the 20:4 acid by preparative GLC. This was done as follows: The tetraene fraction was isolated from the enriched FAME by Ag-TLC, using 9.3% $AgNO_3$ on Silica Gel G (Merck,

[1]Work performed by M.L. Lilly in partial fulfillment of the requirements for the Master of Science Degree.

Darmstadt, Germany) and hexane/ethyl ether/acetic acid/methanol (93:4:2:1, v/v) as developing solvent (9). The tetraene band was scraped from each plate, and the methyl esters eluted from the gel by addition of equal vol of 20% NaCl and methanol, followed by extraction with several portions of hexane. The 20:4 peak was collected from the tetraene fraction by preparative GLC on a Varian Aerograph Gas Chromatograph Model A-90-P (Varian, Palo Alto, CA) equipped with a 6 ft × 1/4 in. id column of 3% (w/w) SE-30 on Varaport-30 100-120 mesh (Varian) using the collection system described by Wood and Reiser (10). The major component collected was found to contain 2-3% impurities but it chromatographed on EGSS-X GLC at the position of 20:4(5,8,11,14). None of the procedures we used actually defined what percentage of the GLC peak in question was 20:4 and what percentage, if any, was 20:3. To determine this, an aliquot of total FAME was applied to Ag-TLC and the triene and tetraene bands were analyzed individually by quantitative EGSS-X GLC using methyl oleate as an internal standard. It was found that the peak contained a minute amount of 20:3 but was, in fact, 99% 20:4. It was clear at this point, then, that the peak in question was primarily a 20-carbon fatty acid containing 4 double bonds. The next step was to identify the positions of these 4 double bonds to determine if the acid was 20:4(5,8,11,14) or some other 20:4 isomer.

Identification of the Double Bond Positions

This was achieved by partially reducing the 20:4 acid and then cleaving the resulting monoenes at the positions of their double bonds. After saponification with 0.5 N NaOH in methanol, partial reduction of the 20:4 free fatty acid was accomplished with a 10% (v/v) methanolic solution of hydrazine hydrate (J.T. Baker, Phillipsburg, NJ) according to Privett and Nickell (11). Preliminary experiments with authentic arachidonic acid indicated that the maximal yield of monoenes was obtained by heating for 7 hr at 35-37 C with constant, vigorous stirring. The reduction of 20:4 from shrimp was done under these conditions. The reaction products were methylated, and the monoene fraction was collected by Ag-TLC on Silica Gel G impregnated with 6% $AgNO_3$ using chloroform as developing solvent. Hydrazine was used as a partial reductant because it does not cause any migration of double bonds within the chain, and it does not cause *cis-trans* isomerization of the bonds (11). It has been reported, however, that the reduction of polyunsaturated fatty acids is faster at the terminal and proximal double bonds (12). The reduction process of 20:4 was gradual and depended on the reaction temperature and vigor of stirring, as monitored by EGSS-X GLC. The products were a mixture of unreacted 20:4, 20-carbon trienes, dienes and monoenes, and 20:0. Figure 1 shows a typical chromatogram of the reaction products. The 20:4 from shrimp yielded ca. 36% monoenes. A maximal yield of monoenes was needed so that there would be enough material for the following oxidative cleavage. The monoene products theoretically consisted of 4 isomers (with respect to double bond position along the chain) because the original acid had 4 double bonds. The monoenes were separated from the other products by an Ag-TLC

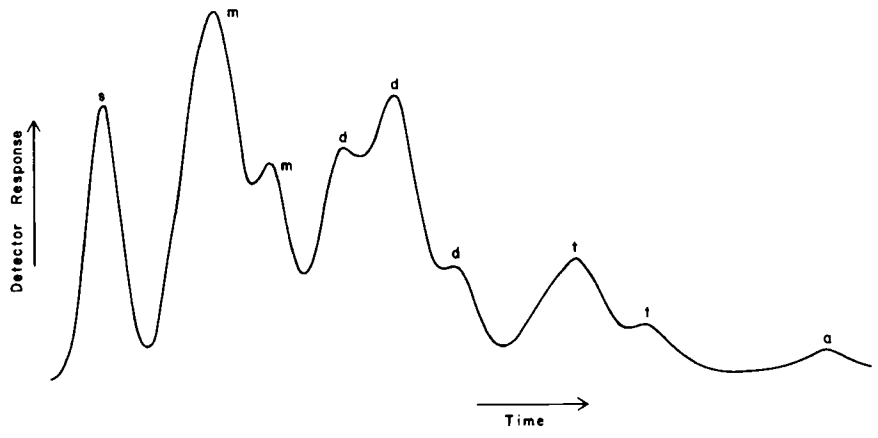

FIG. 1. Gas liquid chromatographic analysis of products obtained from partial reduction of 20:4(5,8,11,14) with hydrazine. The reaction was done at 36 C for 4 hr. s=20:0; m=C_{20} monoenes; d=C_{20} dienes; t=C_{20} trienes; a=unreacted arachidonic acid.

technique that also allows for the detection of *trans*-monoenes possibly formed during the hydrazine reaction if *trans* double bonds were present in the original acid. No *trans*-monoenes were detected in this step and therefore the original tetraenoic acid was considered to have the all *cis* configuration.

The 4 monoenes were oxidized with permanganate-periodate according to Von Rudloff (13) and Albro and Dittmer (14). The mono- and dicarboxylic acid products were methylated, hydrogenated according to Farquhar et al. (15) and the products analyzed by EGSS-X GLC. For comparison, an aliquot of authentic arachidonic acid (99% pure, Nu-Chek-Prep, Elysian, MN) was submitted to similar treatment, starting with the partial hydrogenation with hydrazine. The final chromatograms of the products from the shrimp fatty acid and from arachidonic acid had apparent similarities. The final hydrogenation step helped to enhance these similarities. The data had to be considered only from a qualitative standpoint due to the fact that the monoenes were produced from the original acid in different amounts. The final chromatograms, both from the shrimp sample and from the arachidonic acid standard, showed the presence of some minor peaks that could not be identified or, if assigned a possible identity, could not be combined so as to define any double bond position other than those found in arachidonic acid. In summary, the results presented here indicate that the 20:4 fatty acid isolated from shrimp was, indeed, all *cis*-5,8,11,14-icosatetraenoic acid. Somewhat similar procedures have been used by Light to identify arachidonate in the gorgonian *Plexauro homomalla* (16).

The question remains as to the source and function of the arachidonic acid in shrimp. It is possible that the arachidonic acid had been obtained by the shrimp intact from the diet. Alternatively, the shrimp could have synthesized the acid by elongation and desaturation of linoleate, which is common in mammals. Consequently, experiments were undertaken to determine if shrimp had the enzymatic capability to convert linoleate to arachidonate.

Conversion of Linoleate to Arachidonate by Shrimp Microsomes

From the literature, as well as our own data, there was a strong indication that very little, if any, enzyme activity would be obtained in the shrimp tissue. For this reason, it was considered necessary to first test an assay system on rat liver, so that low enzyme activity could not be blamed on faulty methodology. The assay procedures used were essentially those of Bernert and Sprecher (17), with the following modifications: (a) incubation time was 20 min for both desaturation and elongation assays; (b) the elongation assay was performed using [2-^{14}C] malonyl CoA (45.6 mCi/mmol) instead of labeled 18:3(6,9,12). Unlabeled malonyl CoA was added to the labeled malonyl CoA to achieve the desired concentration. The control incubations were terminated by boiling the enzyme preparation. The treatment of the reaction products of both desaturation and elongation assays differed from that of Bernert and Sprecher (17) and is described later. A known aliquot of the FAME from each incubation tube was separated on 9.3% Ag-TLC as previously described. The reaction mixtures were run by TLC on silica gel against appropriate standards, such that the substrate and product bands could be identified. In most cases, these 2 bands were collected, and the remainder of the lane containing the sample was collected in several bands as well. The lipids were eluted from each band as described previously, and the hexane phases were transferred to glass scintillation vials. After evaporation of most of the hexane by nitrogen flushing, the vials were counted in a Packard Tri-Carb Model 3375-Liquid Scintillation Spectrometer (Packard, Downers Grove, IL) with either Insta-gel (Packard) or Bray's solution (18) as scintillation cocktail. Protein was determined by the biuret method (19). The enzyme activity was determined by comparing the dpm (after correcting for efficiency) present in the product TLC band to the total dpm of substrate added to the incubation. The enzyme activity was expressed as "% conversion." In each case, the activity value represented the minimal activity, as it was not possible to recover 100% of the radioactivity originally added to the incubation tube. The assays were first performed on rat liver until reproducible enzyme activity was obtained. In liver microsomes of chow-fed rats, percentage conversions of 9.3 and 13 were obtained for the first and second desaturations, respectively, and 6.5 for the elongation step. Afterwards, the same procedures were applied to shrimp hepatopancreas and tail muscle. Gulf of Mexico white shrimp (*Penaeus setiferus*) were obtained from several bait camps near Dickinson, Texas. They were transported to College Station in aerated sea water and then kept in artificial sea water for no more than 4 days prior to the experiments. The shrimp were killed by removal of the tail fan, the legs and the front portion of the cephalothorax. An incision was made along the entire length of the dorsal side of each animal and the hepatopancreata and gastric mills were removed. The exoskeleton

and remaining head parts were removed from the tail muscles. Hepatopancreas and tail muscle are 2 likely sites for desaturation and elongation. The hepatopancreas is considered to be the counterpart of the mammalian liver. Recently, Heinen and Dandrifosse (20) have demonstrated the existence of a Δ^9 desaturase (stearate → oleate) activity in the muscle microsomes of 2 aquatic crustaceans.

The levels of enzymatic activity obtained in the shrimp microsomes were: 18:2(9,12) $\xrightarrow{0.2\%}$ 18:3(6,9,12) $\xrightarrow{0.1\%}$ 20:3(8,11,14) $\xrightarrow{0.2\%}$ 20:4(5,8,11,14).

DISCUSSION

The unequivocal identification of arachidonic acid in shrimp tissue opens questions about the origin and metabolic fate of this fatty acid in members of the aquatic ecosystem. Arachidonic acid has been reported in fish and many other aquatic organisms, but unequivocal identifications such as that performed by Light (16) and the one reported here are very few. Ackman (21) had discussed the use of GLC on open-tubular columns for the resolution of arachidonic acid from fatty acids overlapping with it in packed columns, and has shown that arachidonic acid is present in substantial amounts in a nonpenaeid shrimp (22) as well as in several other aquatic invertebrates.

What is the origin of shrimp's arachidonic acid? When rats are fed a diet rich in 18:2(9,12) or 18:3(9,12,15), the tissues exhibit a slight increase of these acids over previous levels. Instead, the most significant increase is seen in the C_{20} and C_{22} polyenes which are synthesized from these precursors by elongation and desaturation (23). When shrimp were fed high levels of 18:2(9,12) or 18:3(9,12,15) (refs. 5, 24,25), the tissue levels of these C_{18} acids increased markedly whereas the levels of the C_{20} and C_{22} polyenes of the $\omega6$ and $\omega3$ families remained unchanged. It appeared that the dietary 18:2 and 18:3 were deposited in the tissues without modification. Kanazawa et al. (26) have confirmed the apparent inability of shrimp to convert labeled 18:3(9,12,15) to the longer chain, more unsaturated counterparts. The results reported here for the conversion of 18:2(9,12) into arachidonic acid (0.2% conversion for each of the 2 desaturation steps, 0.1% conversion for the elongation step) are very low and certainly much smaller than the values found by Bernert and Sprecher in rats fed no fat (17), and our own values for fat-fed rats. These results confirm previous indications, already discussed, of the low degree of conversion of linoleate to arachidonate in shrimp.

The presence of about 9% arachidonic acid in shrimp lipids is still puzzling and so is the function that its presence may serve. If shrimp do not control their tissue levels of arachidonic acid by its endogenous synthesis, it is probable that these levels may be a reflection of the amount of arachidonic acid in the shrimp diet. Because shrimp are omnivorous organisms, it would be difficult to determine the exact source of arachidonic acid. Many species of algae have been found to contain significant levels of arachidonic acid (27-29), so it is possible that shrimp obtain the acid from this source, either directly or through the food chain. The function of arachidonic acid in shrimp is even more obscure. As the $\omega3$ fatty acids have been shown to be more important for growth in shrimp, it is probable that the role of arachidonic acid as an EFA and/or a precursor for prostaglandins is minimal in these animals. It is likely, however, that the membranes of shrimp tissues require arachidonic acid for maintaining permeability and integrity for enzyme action, ion transport and other membrane-associated functions.

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Comparison of Phospholipid Profiles of Primary Adenocarcinoma in the Lung and Other Organs

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ABSTRACT

Phospholipid profiles, particularly molecular structure of phosphatidylcholine, of human primary lung adenocarcinoma were compared with those of the histologically same type of carcinoma from other organs in order to search for a possibility that differentiates between primary and metastatic tumors in the lung. The saturated class, mainly containing palmitic acid at both positions, accounted for 20.8% of phosphatidylcholine in lung adenocarcinoma, whereas it accounted for only 6-10.7% in the adenocarcinoma of other organs. Adenocarcinoma of organs other than the lung had specific characteristics of unsaturated molecular classes of phosphatidylcholine for each organ. Stomach tumor had high contents of dienes and tetraenes with particularly high proportion of arachidonic acid at the 2-position. Breast tumor had a high content of monoenes, containing palmitoleic acid at the 2-position. Adenocarcinomas of rectum, colon and thyroid contained more dienes compared to lung adenocarcinoma.

INTRODUCTION

Tumor cells have been reported to have ultrastructural and biochemical abnormalities of their cell membranes (1-3). Because phospholipids are specific components of the cell membrane, much attention has been given to the lipids of tumor cells (4-7). Although recently, a number of studies on the lipid analyses of tumor tissues, mainly in experimental tumors (4,5), has been reported, only a few reports have been on the phospholipid analysis of carcinoma tissues of human material (6,7).

Our previous study revealed that one of the most characteristic features in the phospholipids of human lung carcinoma is that they contain 17-20% of the saturated classes in phosphatidylcholine (PC), predominantly the dipalmitoyl species (8). This characteristic in the content of saturated PC was almost the same among histologically different types of lung carcinoma such as adenocarcinoma and squamous-cell and small-cell carcinoma.

In this study, the phospholipid profiles, especially the content of saturated PC, in primary lung adenocarcinoma were compared with those of histologically similar carcinomas from other organs, i.e., in the rectum, colon, stomach, thyroid and breast.

MATERIALS AND METHODS

Primary adenocarcinoma tissues in various organs were obtained at the time of operation in the Sapporo Medical College Hospital and from the related Hospitals in Sapporo, Japan. It was confirmed by histological examination that all the carcinoma tissues were adenocarcinomas. Each adenocarcinoma in the various organs was confirmed clinically and pathologically to be a primary tumor. The patients with these adenocarcinomas in various organs had had no special chemotherapy or radiation before the operation which might have conceivably altered lipid composition.

The sites of origin and number of primary adenocarcinomas examined in this study are: lung, 7; rectum, 6; colon, 4; stomach, 7; thyroid, 3; and breast, 4, respectively.

Analytical procedures for lipids were generally the same as described in our previous paper (8), except for the analysis of phospholipid composition. Individual phospholipids were separated by 2-dimensional thin layer chromatography (TLC) with a 0.25-mm layer of Silica Gel G plates prepared with 0.4 M boric acid. The solvent systems used were: chloroform/methanol/water/conc ammonium hydroxide (70:30:3.2, v/v) for the y dimension and chloroform/methanol/water (65:35:5, v/v) for the x dimension (9). Each spot was identified by cochromatography with known samples of rat liver and lung phospholipids which were isolated in our laboratory. After development, the spots on the plates were detected by charring. Individual spots were scraped off and analyzed for phosphorus (10).

RESULTS

The contents of phospholipid and total

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TABLE 1

Lipid Content of Adenocarcinoma of Various Organs

	Lipid content (μg/mg protein)					
	Lung (n=7)	Rectum (n=6)	Colon (n=4)	Stomach (n=7)	Thyroid (n=3)	Breast (n=4)
Cholesterol	47.7 ± 5.5	52.8 ± 15.7	39.3 ± 6.9	43.7 ± 7.1	32.9 ± 9.8[a]	41.9 ± 4.5
Phospholipid	128.9 ± 20.1	144.3 ± 18.7	127.4 ± 12.5	134.4 ± 23.4	108.5 ± 19.5	122.1 ± 14.6

Values are means ± SD (n).

[a]$p < 0.05$ compared to values for lung.

TABLE 2

Phospholipid Composition of Adenocarcinoma of Various Organs

	Phospholipid composition (mol %)					
	Lung (n=7)	Rectum (n=6)	Colon (n=3)	Stomach (n=7)	Thyroid (n=3)	Breast (n=4)
Lysophosphatidylcholine	2.0 ± 0.8	1.8 ± 0.9	1.2 ± 0.5	1.5 ± 0.6	1.9 ± 0.7	1.5 ± 0.5
Phosphatidylinositol	5.1 ± 1.2	6.1 ± 1.0	6.0 ± 0.7	5.5 ± 1.4	5.3 ± 0.7	6.0 ± 1.2
Phosphatidylserine	5.7 ± 1.3	9.2 ± 0.8[a]	8.6 ± 0.7[b]	11.6 ± 2.3[a]	4.7 ± 0.4	5.8 ± 1.1
Sphingomyelin-I	5.6 ± 0.8	6.6 ± 1.3	7.1 ± 1.0	7.9 ± 1.8	7.5 ± 0.8	7.0 ± 1.8
Sphingomyelin-II	7.2 ± 1.8	6.9 ± 1.7	5.3 ± 1.8	7.4 ± 2.4	9.1 ± 1.0	4.0 ± 1.1
Phosphatidylcholine	46.5 ± 4.7	39.8 ± 2.5	42.4 ± 1.4	39.5 ± 5.3	44.3 ± 2.8	47.2 ± 5.0
Phosphatidylethanolamine	22.8 ± 2.0	22.9 ± 3.3	20.9 ± 1.7	20.1 ± 2.3	24.2 ± 2.9	23.3 ± 1.1
Cardiolipin	3.8 ± 1.1	3.7 ± 0.8	3.7 ± 0.7	4.0 ± 1.4	3.8 ± 0.9	3.2 ± 0.4
Lyso-bis-phosphatidic acid	1.4 ± 0.4	1.9 ± 0.9	2.0 ± 1.0	2.0 ± 0.9	0.6 ± 0.3	0.8 ± 0.2

Values are means ± SD (n).

[a]$p < 0.001$ compared to values for lung.

[b]$p < 0.01$ compared to values for lung.

cholesterol/mg protein in adenocarcinoma from various organs examined in this study are shown in Table 1. The amounts of phospholipid and total cholesterol were almost the same among various sites of the adenocarcinoma.

Table 2 indicates the phospholipid composition of lung adenocarcinoma in comparison with those of various adenocarcinomas of other origins. The primary adenocarcinomas of the lung contained only negligible amounts of phosphatidylglycerol. This finding suggests that none of the primary lung adenocarcinomas examined in this study was of Type II cell origin, as it is established that Type II cells contain relatively large amounts of phosphatidylglycerol (ca. 7-9% of total phospholipid) (11). There were some differences in the composition of the phospholipids among these adenocarcinomas compared to that of lung adenocarcinoma. The phospholipid profile of lung adenocarcinoma was similar to those of adenocarcinomas originating in the thyroid and breast, whereas it differed from those of adenocarcinomas originating in the alimentary tract, such as rectum, colon and stomach. In comparison to the lung adenocarcinoma, the alimentary tract adenocarcinoma contained significantly more phosphatidylserine. The adenocarcinoma originating in the rectum and stomach, however, contained a relatively lower content of PC, the main phospholipid of these adenocarcinomas, although the values were not statistically significant.

As seen in Table 3, palmitic acid at the 1- and 2-positions of PC in lung adenocarcinoma was ca. 64 and 22%, respectively. PC in the adenocarcinomas from sites other than lung, however, contained much less palmitic acid at both positions. Compared to the lung adenocarcinoma, a significant decrease in palmitic acid and a concomitant increase in stearic acid at the 1-position were observed in all other adenocarcinomas, although palmitic acid was still the most abundant fatty acid at the 1-position.

Significant decreases in palmitic acid at the 2-position were observed in all adenocarcinomas other than the lung adenocarcinoma. It should be noted, however, that they still contained about 9-13% palmitic acid at the 2-position.

TABLE 3

Fatty Acid Composition of Phosphatidylcholine of Adenocarcinoma of Various Organs

	Fatty acid composition (mol %)											
	Lung (n=7)		Rectum (n=6)		Colon (n=4)		Stomach (n=7)		Thyroid (n=2)		Breast (n=4)	
	1-Position	2-Position	1-Position	2-Position	1-Position	2-Position	1-Position	2-Position	1-Position	2-Position	1-Position	2-Position
14:0	1.5 ± 0.5	1.8 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.4	0.2 ± 0.1	0.8 ± 0.5	2.3 ± 1.0	1.3 ± 0.4
16:0 (ald)	1.1 ± 0.3	0.6 ± 0.2	1.1 ± 0.7	–	1.3 ± 0.4	–	1.2 ± 0.4	–	0.2 ± 0.1	–	1.1 ± 0.1	–
16:0	63.9 ± 4.8	22.3 ± 1.5	40.1 ± 6.2[a]	13.3 ± 1.3[a]	44.8 ± 2.1[a]	11.3 ± 2.1[a]	46.7 ± 7.2[a]	11.0 ± 3.9[a]	47.7 ± 2.5	9.1 ± 0.2	49.9 ± 1.5[a]	10.1 ± 1.8[a]
16:1	0.6 ± 0.2	5.6 ± 1.9	0.4 ± 0.1	2.3 ± 1.7[b]	0.6 ± 0.1	3.4 ± 0.9	0.5 ± 0.4	1.4 ± 0.7	0.2 ± 0.1	0.7 ± 0.4	1.1 ± 0.9	12.8 ± 2.7[a]
18:0	20.3 ± 2.9	0.5 ± 0.1	40.1 ± 7.6[a]	1.4 ± 0.4	30.5 ± 7.7[b]	0.8 ± 0.4	35.4 ± 7.1[a]	1.4 ± 0.6	36.7 ± 6.9	1.3 ± 0.7	26.2 ± 6.3	0.3 ± 0.1
18:1	11.1 ± 1.8	33.2 ± 5.5	14.8 ± 0.8	33.5 ± 5.8	18.3 ± 3.3[b]	39.1 ± 5.0	14.2 ± 3.0	26.6 ± 2.0	12.6 ± 4.3	38.6 ± 9.2	17.4 ± 3.8[b]	44.9 ± 5.3[b]
18:2	1.6 ± 0.3	15.5 ± 4.8	1.9 ± 0.9	20.6 ± 5.2	2.7 ± 0.4	20.4 ± 2.4	1.7 ± 0.6	22.8 ± 3.9[b]	2.6 ± 0.7	28.2 ± 6.3	1.2 ± 0.4	12.7 ± 1.4
20:1	0.2 ± 0.1	0.2 ± 0.1	0.6 ± 0.3	0.3 ± 0.1	1.2 ± 0.6	0.2 ± 0.1	0.9 ± 0.4	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	1.0 ± 0.3	0.9 ± 0.1
20:2	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	1.2 ± 0.1	0.6 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	–	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.4
20:3	–	3.0 ± 0.3	–	4.9 ± 1.0	–	3.3 ± 1.1	–	3.3 ± 0.6	–	4.7 ± 0.7	–	4.4 ± 1.3
20:4	–	13.0 ± 2.8	–	17.8 ± 5.2	–	12.6 ± 2.6	–	23.1 ± 3.8[a]	–	14.1 ± 2.0	–	10.2 ± 2.5
22:5	–	0.5 ± 0.1	–	1.6 ± 0.4	–	1.0 ± 0.3	–	1.6 ± 0.6	–	0.3 ± 0.1	–	1.3 ± 0.3
22:6	–	2.4 ± 0.4	–	3.1 ± 1.4	–	2.9 ± 1.3	–	3.2 ± 0.7	–	2.0 ± 0.9	–	2.8 ± 0.5

Values are means ± SD (n).

[a] $p < 0.001$ compared to values for lung.

[b] $p < 0.01$ compared to values for lung.

Concomitant with this was an increase in the percentages of the unsaturated fatty acids of the 2-position in the adenocarcinoma from origins other than the lung. Interesting was that they had the characteristic profiles of the unsaturated fatty acids of the organs of their origin. Compared to the lung adenocarcinoma, stomach adenocarcinoma contained more linoleic acid and arachidonic acid, breast adenocarcinoma contained more palmitoleic acid and oleic acid and thyroid adenocarcinoma contained more oleic acid. Moreover, adenocarcinoma of rectum and colon contained relatively more oleic acid than those of lung origin, although the values were not statistically significant.

In reflecting on their fatty acid profiles, significant differences in the molecular classes of PC were found among adenocarcinoma originating in various organs (Table 4). Compared to lung adenocarcinoma, the most striking feature in adenocarcinoma of other organs was a marked decrease in the saturated classes. The saturated classes of PC in lung adenocarcinoma accounted for 20.8%, whereas they accounted for only 6-10.7% in adenocarcinoma of other organs, On the other hand, adenocarcinoma of other origins contained many more unsaturated classes. Furthermore, they each had specific characteristics of their origin as noted in their fatty acid patterns. Compared to lung adenocarcinoma, stomach adenocarcinoma contained more dienes and tetraenes. Similarly, breast adenocarcinoma contained more monoenes. Adenocarcinoma of the rectum, colon and thyroid also contained more dienes, which seem to be mixtures of saturated-dienoic and monoenoic-monoenoic classes.

PC of lung adenocarcinoma did not seem to contain 1-alkyl or 1-alkenyl type, because almost all the PC was hydrolyzed by mild alkaline treatment. PC of other adenocarcinomas examined did not seem to contain at least the 1-alkenyl type, as only small peaks of dimethylacetals were found to be on the gas chromatograms.

DISCUSSION

Our results clearly demonstrate that there are marked differences between the phospholipid profiles of primary lung adenocarcinoma and those of other sites of origin. The most notable characteristic in the phospholipid profile of lung adenocarcinoma compared with those of other origins was the high content of the saturated species of PC, predominantly those of dipalmitoyl type. Previously, we disclosed that there are 2 pools of saturated PC in the lung, i.e., the membrane pool, containing 2/3 of the saturated PC, and the surface-active pool, which contains the remaining saturated PC and which functions as a pulmonary surfactant (8). It seems probable that the saturated PC in lung adenocarcinoma may be derived from the membrane pool, but not from the surface-active pool, because the lung adenocarcinomas examined in this study did not seem to be of alveolar Type II cell origin, as suggested on the basis of the negligible amount of phosphatidylglycerol in lung adenocarcinoma. It should be noted that the adenocarcinomas of other sites of origin did not contain such high levels of saturated PC, although they still contained 6-11% of the saturated species as membrane constituents. It is known that many tissues other than lung also contain different amounts of saturated PC (12,13). It is uncertain, however, whether the differences in the saturated PC content found in various adenocarcinoma reflect differences in its content in the mother tissues, or are the result of carci-

TABLE 4

Molecular Class Composition of Phosphatidylcholine of Adenocarcinoma of Various Organs

	Molecular class composition (mol %)					
	Lung (n=7)	Rectum (n=4)	Colon (n=3)	Stomach (n=5)	Thyroid (n=2)	Breast (n=3)
Saturates	20.8 ± 3.1	10.7 ± 2.1[a]	9.0 ± 1.8[a]	9.5 ± 0.7[a]	6.0 ± 1.4	8.6 ± 1.6[a]
Monoenes	34.5 ± 2.8	34.2 ± 2.4	41.3 ± 6.0	31.3 ± 1.9	41.2 ± 3.1	51.2 ± 3.5[a]
Dienes	18.9 ± 3.7	29.9 ± 4.5[b]	23.2 ± 2.2[b]	25.5 ± 3.0[b]	30.3 ± 2.4	17.2 ± 4.3
Trienes	3.6 ± 1.0	5.1 ± 2.2	5.6 ± 2.0	5.4 ± 1.8	3.1 ± 0.4	5.9 ± 1.1
Tetraenes	12.9 ± 3.3	12.4 ± 3.4	10.6 ± 1.2	20.4 ± 2.2[b]	10.6 ± 0.6	9.1 ± 1.8
Polyenes	9.2 ± 4.5	7.6 ± 2.2	10.2 ± 2.8	7.8 ± 3.3	8.8 ± 2.4	11.0 ± 3.5

Values are means ± SD (n).

[a]$p < 0.01$ compared to values for lung.

[b]$p < 0.001$ compared to values for lung.

nomatous changes.

This study also revealed that adenocarcinomas of organs other than the lung each had organ-specific characteristics in their profiles of PC molecular species. Stomach adenocarcinoma had high levels of dienes and tetraenes, with an especially high percentage of arachidonate at the 2-position compared to other adenocarcinoma; the characteristic feature of breast adenocarcinoma, however, was a high monoene content, particularly of the molecular species having palmitoleic acid at the 2-position. In comparison to lung adenocarcinoma, adenocarcinomas of the rectum, colon and thyroid contained more dienes.

In the lung, the distinction between a metastatic and a new primary tumor can usually be made by correlating the gross and microscopic findings and by comparing the histological appearance of the lung lesion with that of the primary tumor. However, in some cases, it has been difficult and occasionally virtually impossible for the pathologist to differentiate between a metastatic and a new primary carcinoma. In this respect, it will be interesting if the characteristic features in the phospholipid profiles of the primary lung adenocarcinoma compared to those of adenocarcinomas from various origins could be useful for the differentiation between a metastatic and a primary adenocarcinoma in the lung. This possibility is currently under investigation in our laboratory.

ACKNOWLEDGMENTS

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Intestinal Cholesterol Uptake from Phospholipid Vesicles and from Simple and Mixed Micelles

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ABSTRACT

This study was undertaken in vitro to examine the rat jejunal uptake of cholesterol from phospholipid vesicles and from mixed bile salt micelles, under conditions of low effective resistance of the intestinal unstirred water layer. Cholesterol uptake, J_d, occurred from vesicles only when the cholesterol:phospholipid ratio was high. The addition of phospholipid (PL) to micelles comprising 20 mM taurodeoxycholic acid (TDC) extended the concentration of cholesterol, beyond which the relationship between cholesterol concentration and uptake remained linear. When the concentration of cholesterol in the bulk phase was held constant and the concentration of TDC or of PL added to the TDC was increased, there was a decline in cholesterol uptake; this effect was masked when the concentration of TDC was high, or when higher concentrations of PL were added to the mixed micelle. When increasing concentrations of palmitic acid were added to mixed micelles composed of cholesterol, TDC and PL, the uptake of cholesterol decreased; in contrast, cholesterol uptake progressively increased when palmitic acid was added to simple TDC micelles. The results suggest that the mechanism responsible for cholesterol uptake may vary, depending on the nature of the constituents of the micelle, and it is proposed that PL inhibits the intestinal uptake of cholesterol by altering the partitioning of cholesterol out of the micelle.

INTRODUCTION

Lipid absorption varies with the modification of the constituents of the micelle (1-3). The phospholipid phosphatidylcholine (PC) is a component of bile which facilitates the solubilization of cholesterol, and luminal PC has an important role in the total absorption of fat (4-6). Phospholipids have a variable effect on the absorption of bile acids and fatty acids (1,2, 7-15), and it is unknown how phospholipids depress cholesterol absorption. The intracellular process of esterification of absorbed fatty acids is unchanged (11,16,17). PC increases the size and molecular weight (MW) of bile salt micelles (18,19) and the larger micelles might diffuse more slowly across the intestinal unstirred water layer, thereby leading to a lower rate of cholesterol uptake. There is, however, no correlation between the weight of pure bile salt or surfactant micelles and cholesterol influx, and the addition of fatty acid to micelles increases the influx rate of cholesterol only from bile salts and not from surfactants (20). Techniques are now available to reduce the effective resistance of the intestinal unstirred water layer (21, 22), and it now becomes possible to minimize any potential effect of micellar expansion on lipid uptake, and to thereby determine if the inhibitory effect of phospholipids on cholesterol uptake persists when the effective resistance of the unstirred layer is low.

PC also reduces the monomer activity of the lipids present in mixed micellar solutions, although the extent of this effect is small (23-26). Perhaps phospholipids interact with the cholesterol-containing bile salt micelles, leading to interference with access of the lipid to the intestinal membrane (27). This effect of phospholipids on lipid uptake is of particular relevance because phospholipid vesicles are being examined as targeted-carrier molecules of potential therapeutic interest (28-36).

Accordingly, this study was undertaken in vitro to examine the rat jejunal uptake of cholesterol from phospholipid vesicles and from mixed bile salt micelles under conditions of low effective resistance of the intestinal unstirred water layer.

METHODS OF PROCEDURE

Chemicals

Unlabeled and 4-^{14}C-labeled cholesterol were all greater than 99% pure as supplied by Applied Science Laboratories Inc., State College, PA; unlabeled taurodeoxycholic acid (TDC) and egg PC were from Sigma Chemical Corp., St. Louis, MO, and were greater than 99% pure. The specific activity (sp act) of the cholesterol was 50-60 mCi/mmol; [G-^{3}H]-dextran with an approximate MW of 15,000 was obtained from New England Nuclear Corp., Boston, MA, and was used as a nonpermeant marker of adherent mucosal fluid volume. All other compounds were of reagent grade and were obtained from Fisher Scientific Co.

Preparation of Simple Bile Salt Micellar Solutions

The technique used to prepare the micellar

solutions has been published (37). Briefly, an appropriate amount of both the ^{14}C-labeled and unlabeled cholesterol probe molecule was dissolved in an extract volume of chloroform/methanol (2:1, v/v) in an incubation beaker. The chloroform/methanol phase was evaporated and 63 ml of taurodeoxycholate solution in Krebs-bicarbonate (KRB) buffer (with Ca^{++} omitted) at pH 7.4 was added to a beaker and the solution was stirred with a magnetic bar for 2 hr at 37 C. The solution was then further diluted by the addition of 63 ml of KRB buffer to give a final vol of 126 ml of incubation solution to which the mucosa was exposed. The final taurodeoxycholate concentration was varied from 1.25 to 20 mM and the final cholesterol concentration varied from 0.025 to 0.2 mM. The solutions were sonicated for 2 hr at 4 C and centrifuged, as described later. The solutions were then gassed with 95% O_2/5% CO_2 for 2 hr at 37 C and, if necessary, the pH was readjusted to 7.4. A tracer amount of the radiolabeled volume marker [G-^3H] dextran was then added and the solution was ready to be used for determination of tissue uptake rates.

Preparation of Vesicles and Phospholipid-Containing Mixed Bile Salt Micelles

PC and cholesterol were solubilized together in chloroform. The chloroform was removed by evaporation under nitrogen. The lipid was then dissolved in benzene. This solution was frozen in dry ice/acetone and lyophylized using a high-vacuum pump. The dry lipid was then suspended in KRB, and sonicated for 45 min in a temperature-controlled cell under nitrogen, using a Branson 375 W sonicator with a standard titanium probe and a power output of 65 W. The phospholipid vesicle preparations were sonicated at 4 C. Unsonicated vesicles were prepared by the suspension of dried egg PC and cholesterol in KRB, followed by 20 min of vigorous agitation on a Vortex mixer.

In some experiments, mixed micelles were prepared by dissolving TDC, PC, palmitic acid and cholesterol in benzene; the molar ratio of these 4 components was varied among experiments. The [^{14}C] cholesterol was added, and the lipid mixture was dried on a rotary evaporator at 37 C, under vacuum for ca. 3 hr. An appropriate vol of calcium-free KRB was added. The solutions were allowed to stand at room temperature for 1 hr, and then the suspension was sonicated for 2 hr at 4 C using a 19-mm titanium probe at 1.5 A on a MSE 150 W sonicator. The suspension was kept at 4 C for 1 hr longer and then centrifuged in an MSE Superspeed 50 centrifuge at 10,000 × g for 2 hr. The pellet was resuspended in an appropriate vol of KRB to yield the desired concentration of TDC, PC, palmitic acid and cholesterol. Just prior to experimentation, the sonicates were centrifuged for 10 min at 3,000 × g in order to remove any titanium particles shed by the sonicator, [^3H] dextran was added, and the rate of uptake of cholesterol into the jejunum of rats was determined as outlined later.

Tissue Preparation

The technique used to measure rates of intestinal uptake has been published. Briefly, as described in detail elsewhere (21,22,38), 150-175-g female Wistar rats were sacrificed and a 15-cm length of proximal jejunum was rapidly removed, rinsed with 50 ml of cold saline, opened along the mesenteric border, and the mucosal surface was carefully washed with a stream of cold saline from a syringe to remove visible mucus and detritus. Circular pieces of intestine, 1 cm in diam, were cut from the segment using a sharpened steel punch, mounted as a flat sheet in incubation chambers, and clamped between 2 plastic plates so that the serosal surface was 0.5 cm in diam. To the serosal compartment of the disc chambers was added 1.2 ml of KRB buffer, and each chamber was then placed in a beaker containing KRB buffer at 4 C and constantly oxygenated by a stream of 5% CO_2 in oxygen until it was used in the various experiments. The tissue was then transferred to beakers containing oxygenated KRB buffer at 37 C for a preincubation of 30 min; then they were transferred to other beakers for specific experiments. The preincubation solutions were mixed at identical stirring rates with circular magnetic bars, and the stirring rates were precisely adjusted using a strobe light; stirring rates are reported as the revolutions/min (rpm) at which the stirring bar was driven. The bulk phase was stirred at 600 rpm in most experiments to reduce and minimize the effective resistance of the unstirred layer.

Determination of Rates of Uptake of Cholesterol

After preincubation in KRB for 30 min (22, 37), the tissues were transferred to other beakers containing [^3H] dextran and various concentrations of ^{14}C-labeled cholesterol in oxygenated KRB buffer at 37 C. Preliminary experiments had demonstrated that the rate of cholesterol uptake was constant between 4 and 8 min, and a 6-min incubation period was selected. After incubation for 6 min, the experiment was terminated by removing the chamber and quickly rinsing the jejunal tissue in cold saline for ca. 5 sec. The exposed mucosal tissue was then cut out of the chamber with a circular

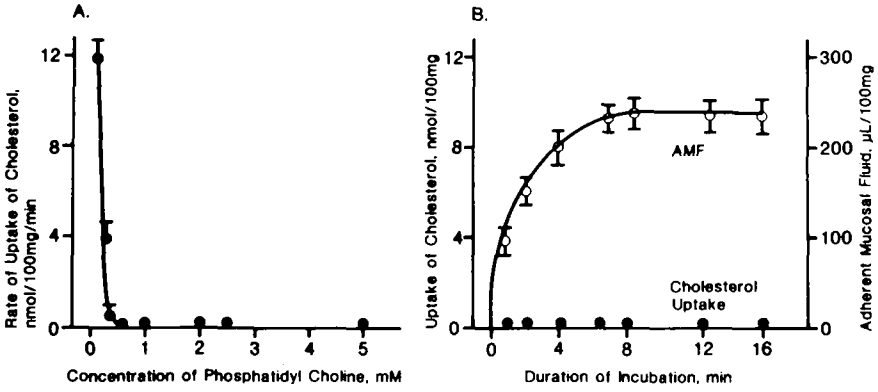

FIG. 1. Effect of varying concentrations of PC and varying durations of incubation on the rate of uptake of vesicle-entrapped cholesterol into rat jejunal discs. In panel A, the concentration of cholesterol was 0.2 mM, and the concentration of PC was varied from 0.05 to 5 mM. In panel B, the concentration of cholesterol was 0.2 mM and the concentration of PC was 2 mM. The bulk phase was stirred at 600 rpm. Each point represents the mean ± SEM of the results of 9-12 animals.

steel punch, blotted on filter paper, and placed in a tared counting vial. The tissue was dried overnight in an oven at 75 C and the dry wt was determined. The sample was then saponified with 0.75 ml of 0.75 N NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the 2 isotopes (21,22). The rate of uptake, J_d, was calculated after correcting the total tissue ^{14}C radioactivity for the mass of the probe molecules present in the adherent mucosal fluid; these uptake rates were expressed as nmol of cholesterol taken up into the mucosa/min/100 mg dry wt of tissue. The average tissue dry wt was ca. 1.5 mg. The statistical significance of the difference between any 2 means was determined using Student's t-test.

RESULTS

Effect of Varying Concentrations of PC and Varying Durations of Incubation on the Rate of Uptake of Vesicle-Entrapped Cholesterol into Rat Jejunal Discs

The bulk phase was stirred to 600 rpm to reduce the effective resistance of the unstirred layer and tissues were incubated for 6 min; the concentration of added cholesterol was 0.2 mM and the initial concentration of added PC was 0.05 mM. Under these conditions, the rate of cholesterol uptake was 12 mmol/100 mg/min (Fig. 1A). However, the rate of cholesterol uptake decreased rapidly to zero as the concentration of added PC was increased to 0.5 mM, and no cholesterol was taken up as the concentration of added PC was further increased to 5 mM.

Then, the duration of incubation was varied from 1 to 16 min, and the test solutions contained 0.2 mM cholesterol and 2 mM PC. The vol of the adherent mucosal fluid (AMF) was estimated from the [^{3}H]dextran space. This value reached a constant in 6 min, and incubation for longer periods was not associated with any further change in AMF; no cholesterol was taken up at any duration of incubation up to 16 min (Fig. 1B).

Effect of Varying Concentrations of Cholesterol on the Rate of Uptake of Cholesterol into Rat Jejunal Discs, Using Vesicles, Simple Micelles and Mixed Micelles

When the concentration of added PC was held constant at 2 mM, and the concentration of added cholesterol was increased from 0.05 to 1.0 mM, no cholesterol was absorbed from the vesicles (Fig. 2). In contrast, cholesterol was readily absorbed from micellar solutions: when the concentration of TDC was 20 mM, a linear relationship was noted between cholesterol uptake and cholesterol concentrations of 0.05-0.4 mM (Fig. 2). At higher concentrations of cholesterol, the solutions losts their optical clarity, and the uptake of cholesterol then reached a plateau. When the concentration of TDC was 20 mM and PC was 2.0 mM, a linear relationship was again noted between cholesterol concentration and the rate of cholesterol uptake from the mixed micelles (Fig. 2); the rate of cholesterol uptake then plateaued at concentrations of cholesterol above 1 mM. When using concentrations of cholesterol between 0.05 and 0.4 mM, the rate of cholesterol

uptake was similar whether 2.0 mM PC was present or absent. While the rate of cholesterol uptake from a 20-mM TDC solution reached a plateau beyond 0.4 mM cholesterol, a plateau in the rate of cholesterol uptake was achieved at 1 mM when the 20-mM TDC solution contained 2 mM PC.

Effect of Stirring the Bulk Phase and Varying Concentrations of Phospholipid on the Rate of Jejunal Uptake of Cholesterol from Micellar Solutions

When the bulk phase was unstirred, the addition of PC had little effect on the uptake of 0.2 mM cholesterol from a 5 mM TDC solution (Fig. 3). In contrast, when the bulk phase was stirred at 600 rpm, the progressive addition of PC was associated with a decline in the rate of uptake of cholesterol (Fig. 3).

Effect of Varying Concentrations of Palmitic Acid on the Rate of Uptake of Cholesterol from Micellar Solutions with and without PC

When the bulk phase was stirred at 600 rpm and the solution contained 0.2 mM cholesterol and 20 mM TDC, the uptake of cholesterol was increased by adding varying concentrations of palmitic acid (Fig. 4). In contrast, the rate of uptake of cholesterol decreased when palmitic acid was added to a mixed micellar solution containing 0.2 mM cholesterol, 20 mM TDC and 2.0 mM PC.

Effect of Varying Concentrations of TDC on the Rate of Uptake of Cholesterol from Simple and Mixed Micelles

As the concentration of TDC was increased from 2.5 to 20 mM, there was a decline in the rate of uptake of 0.2 mM cholesterol (Fig. 5A); similar results have been reported previously (37,38). The rate of uptake of cholesterol was lower when PC was added to the micellar solution (Fig. 5B). When the concentration of added PC was 1.0 mM, there was a decline in cholesterol uptake as the concentration of TDC was increased (Fig. 5B). On the other hand, when the solution contained 0.2 mM cholesterol, 2.0 or 5.0 mM PC, and the concentration of TDC was increased, cholesterol uptake initially rose slightly, then declined.

DISCUSSION

The current model of the mechanism of cholesterol uptake from the bile salt micelle is one in which cholesterol partitions out of the micelle in which it is solubilized into an aqueous phase, from which uptake of the cholesterol molecule proceeds into the intestinal membrane (39-41). The experimental support

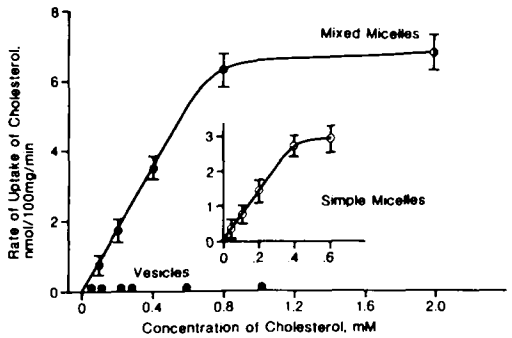

FIG. 2. Effect of varying concentrations of cholesterol on the rate of uptake of cholesterol into discs of rat jejunum, using vesicles, simple micelles and mixed micelles. The simple micelle solution contained 20 mM TDC, and the concentration of cholesterol was varied from 0.05 to 0.6 mM. The mixed micelles were prepared with 20 mM TDC and 2.0 mM PC. The vesicles were comprised of 2 mM PC and varying concentrations of cholesterol. The bulk phase was stirred at 600 rpm. Each point represents the mean ± SEM of the results of 9-12 animals.

for this model is derived from the reciprocal decline in cholesterol uptake noted with increasing the concentration of PC (Fig. 1), with increasing the concentration of PC in the presence of TDC (Fig. 3A), with increasing the concentration of palmitic acid in the presence of TDC and PC (Fig. 4), with increasing the concentration of TDC (Fig. 5A), or with increasing the concentration of TDC in the presence of 1 mM PC (Fig. 5B).

The second possible model for the uptake of cholesterol is one in which the cholesterol partitions directly from the micelle into the lipid membrane, without an intervening aqueous phase (37). If this model were correct, then the rate of cholesterol uptake would increase as the concentration of the micellar components increased. Such an increase in cholesterol uptake was observed as the concentration of palmitic acid was increased in the presence of 20 mM TDC (Fig. 4), and as the concentration of TDC was initially increased in micelles containing 2.0 mM or 5.0 mM PC (Fig. 5B). Thus, it is possible that the mechanism of uptake of cholesterol from the micelle may be influenced by the composition of the micelle: under some conditions, the uptake of cholesterol may occur through an obligatory aqueous phase, whereas under other conditions, the uptake of cholesterol into the membrane may proceed directly from the lipid phase of the micelle.

The rate of cholesterol uptake may be influenced by the stirring of the bulk phase or by the presence of varying concentrations of

phospholipid, fatty acid, or bile acid (Figs. 1-6). The amount of bile acid, fatty acid and phospholipid present in the proximal intestine may vary in a number of clinical conditions such as bacterial overgrowth syndrome, ileal dysfunction syndrome, or pancreatic insufficiency. These conditions may be associated with fat malabsorption, and one possible mechanism of the steatorrhea highlighted by these studies is the influence of micellar composition on the different mechanisms of lipid uptake. If the rate of absorption of cholesterol is found to be altered in response to certain manipulations, such as the modification of the diet (40) or the administration of drugs (41), then this altered rate of cholesterol uptake may be due to a change in the ratio of the various constituents of the micelle, leading to a different mechanism of lipid uptake from the micelle, rather than necessarily being caused by a change in the permeability properties of the intestinal brush border membrane.

Although phospholipid vesicles are gaining attention as targeted drug carriers (28-36), they prove to be poor carriers for the delivery of cholesterol to the intestinal membrane: cholesterol is taken up from phospholipid vesicles only when the ratio of cholesterol:PC is high

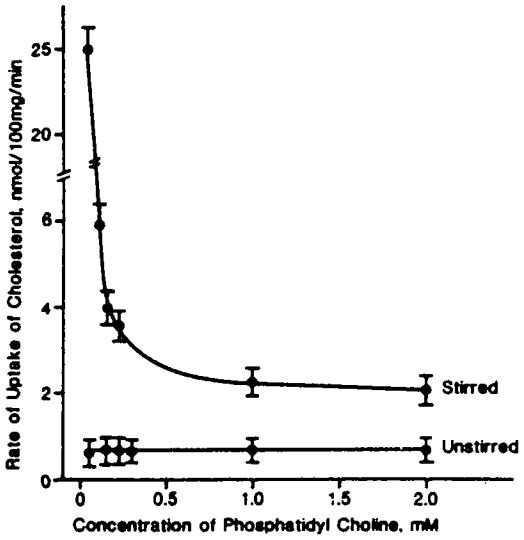

FIG. 3. Effect of stirring the bulk phase and varying concentrations of phospholipid on the rate of uptake of cholesterol from micellar solutions. The solutions were prepared with 5 mM TDC. In those experiments in which the bulk phase was "stirred," the magnetic stirrer revolved at 600 rpm; in the "unstirred" condition, there was no mixing of the bulk phase (0 rpm). Each point represents the mean ± SEM of the results of 9-12 animals.

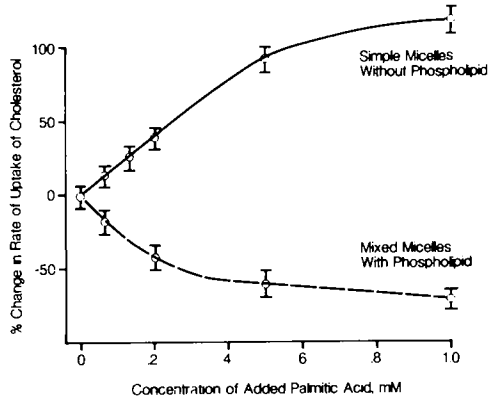

FIG. 4. Effect of varying concentrations of palmitic acid on the rate of uptake of cholesterol from micellar solutions with and without PC. The solutions of "simple micelles" contained 0.2 mM cholesterol plus 20 mM TDC; "mixed micelles" were prepared with 2 mM PC. The bulk phase was stirred at 600 rpm. Each point represents the mean ± SEM of the results of 9-12 animals.

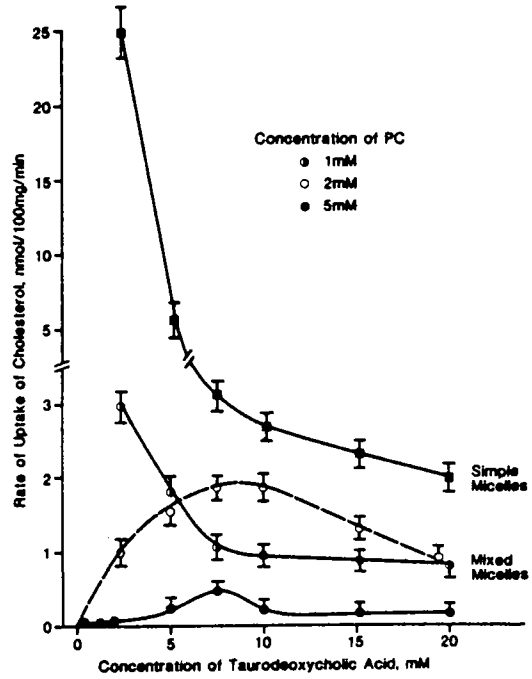

FIG. 5. Effect of varying concentration of TDC on the rate of uptake of cholesterol from simple and mixed micelles. The concentration of cholesterol was 0.2 mM, and the "simple micelles" were prepared using varying concentrations of TDC; the "mixed micelles" were prepared using 1, 2 or 5 mM PC. The bulk phase was stirred at 600 rpm. Each point represents the mean ± SEM of the results of 9-12 animals.

(Fig. 1A). The only advantage of the addition of PC on cholesterol uptake is the maintenance of the linear rate of uptake beyond those concentrations of cholesterol which are not normally solubilized by a simple bile salt micelle (Fig. 2).

It has been recognized over the past decade that, under some conditions, phospholipids may impair the rate of uptake of cholesterol into the intestine, but the mechanism responsible for this effect has not been elucidated (9-17). The addition of PC to a cholesterol/TDC solution may expand the micelle and thereby retard its diffusion across the unstirred water layer (18,19); arguing against this being the only mechanism of the phospholipid effect are the observations that the addition of phospholipid expands the micelle by only a small amount (18-20,42); adding palmitic acid enhances rather than reduces cholesterol uptake (Fig. 4) and the inhibitory effect of PC on cholesterol uptake is clearly present when the bulk phase is stirred to reduce the effective thickness of the unstirred layer (Figs. 1, 3-5).

Some exchange of membrane cholesterol for bulk phase cholesterol may have occurred, and depending on the magnitude of this exchange, the amount of [^{14}C]cholesterol present in the tissue may have been thereby influenced. If PC reduced the possible exchange between unlabeled membrane cholesterol and labeled bulk phase cholesterol, the addition of phospholipid would have led to the appearance of reduced cholesterol uptake, as shown in Figures 1A, 3, 4 and 5. When the bulk phase contained 0.2 mM cholesterol, ca. 0.05 nmol cholesterol was taken up into the average 1.5-mg sample of jejunum, and this represented less than 0.0001% of the 25,200 nmol of labeled cholesterol in the bulk phase. The amount of cholesterol in the rat jejunal brush border membrane is small (Yakymyshyn and Thomson, unpublished observations), and direct measurements must now be performed to determine what proportion of the membrane cholesterol is available for exchange, and how this exchange process may be influenced by phospholipids.

A further explanation for the inhibitory effect of PC on cholesterol uptake is an impairment of the partitioning of the cholesterol out of the micelle. Indeed, as the ratio of cholesterol:PC was lowered, cholesterol uptake declined (Figs. 1, 3 and 5). PC does reduce the monomer activity of the lipids present in mixed micellar solutions, although the extent is small (23-26) and these results cannot be directly applied to these studies. The possibility of phospholipids interacting with the intestinal membrane or with the cholesterol-containing bile salt must now be directly tested.

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Mitochondrial Hydroxylation of the Cyclohexane Ring as a Result of β-Oxidation Blockade of a Cyclohexyl Substituted Fatty Acid

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ABSTRACT

Among the urinary metabolites of dodecylcyclohexane or cyclohexylacetic acid, the glycine conjugate of 1-hydroxy-cyclohexylacetic acid was identified and its origin studied, using cyclohexylacetic acid as the starting molecule, as it results from β-oxidation of cyclohexyldodecanoic acid produced by terminal oxidation of the alkyl chain of the cycloparaffin. Three hypotheses were tested: (a) hydroxylation by the liver microsomal mixed-function oxidases involved in detoxication mechanisms; (b) hydroxylation by a cyt. P_{450}-containing mitochondrial hydroxylase; and (c) β-oxidation blockade after the reaction catalyzed by enoyl-CoA-hydratase. Liver microsomal or mitochondrial fractions were prepared and incubated in the presence of [^{14}C]cyclohexylacetic acid, glucose-6-phosphate dehydrogenase and a NADPH-producing system. On the other hand, mitochondria were incubated in a suitable respiratory medium with or without cofactors required for ATP production. The reaction products were extracted and analyzed by thin layer radiochromatography and radio gas chromatography. Evidence is given that hydroxylation of cyclohexylacetic acid in position 1 is a mitochondrial step requiring activation in the acyl-CoA form and results from β-oxidation blockade, the cyclohexane ring hindering hydroxyacyl-CoA-dehydrogenase action.

INTRODUCTION

Oxidation of cyclic hydrocarbons by microorganisms has received considerable attention, but little is known about the biotransformations of the nonbenzoid hydrocarbons in higher animals. Studies in mammals have been conducted only with cyclohexane (1) and *n*-alkyl cyclohexane compounds (2,3). It has been observed, in the rabbit, that urinary metabolites of cyclohexane and methylcyclohexane are cyclohexanol, cyclohexanediols and methylcyclohexanols in the form of glucuronides.

When studying the fate of dodecylcyclohexane in rats, evidence was obtained that, after terminal oxidation of the alkyl chain (4), elimination of this naphthenic hydrocarbon occurred as cyclohexenylaceturic acid (5). Two possibilities for the formation of such a cyclohexane compound were considered, i.e., aromatization of the cyclohexane ring, and dehydration of cyclohexylacetic acid hydroxylated in position 1 or 2.

This report describes the identification in rat urine of 1-hydroxycyclohexylacetic acid in the glycine conjugated form, as well as the subcellular mechanism involved in the hydroxylation process of the cyclohexane ring.

MATERIALS AND METHODS

[^{3}H]Dodecylcyclohexane (116 mCi/mmol) was prepared as previously described (3). [Carboxy-^{14}C]cyclohexylacetic acid (60 mCi/mmol) was obtained by catalytic hydrogenation of [^{14}C]phenylacetic acid (Radiochemical Center, Amersham) using the same method.

The purity of the labeled molecules was checked by thin layer chromatography (TLC) using Silica Gel G plates (Merck, Darmstadt, Germany) and hexane as developing solvent or Polygram® polyamid-6 sheets (Macherey Nagel Co., Duren, Germany) using a hexane/toluene/methanol/acetic acid (30:15:4:1, by vol) mixture. Chemical purity of dodecylcyclohexane was tested by gas liquid chromatography (GLC) on a 1/4 in. x 1.5 m glass column packed with 3% Dexsil 300 on 80/100 Chromosorb W AW DMCS, and that of cyclohexylacetic acid using a 3 m stainless steel column packed with 10% DEGS-PS on 80/100 Supelcoport. Purities were better than 99%.

[^{14}C]Phenylaceturic acid was synthesized by reaction of glycine ethyl ester (Fluka A.G., Buchs, Switzerland) and [^{14}C]phenylacetylchloride obtained by action of thionylchloride on [^{14}C]phenylacetic acid.

1-Hydroxycyclohexylacetic acid was synthesized directly in its ethyl ester form by the Reformatsky reaction of cyclohexanone with zinc and ethyl bromoacetate (6). Ethyl-1-hydroxycyclohexylacetate was distilled under low pressure, then purified by preparative TLC on Silica Gel G (Merck), using hexane/ether/acetic acid (30:10:1, by vol) as developing solvent. The structure was authenticated by electron-impact mass spectrometry (MS); the

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spectra were recorded on a Hewlett Packard HP 5992 instrument.

in vivo Studies

Male Wistar rats (200-250 g) in metabolism cages were maintained on a semisynthetic diet. A single dose of [^{3}H]dodecylcyclohexane (200 mg) or [^{14}C]cyclohexylacetic acid (100 mg) was administered by stomach tube as a solution in corn oil, after which urine was collected for 48 hr. Urine was processed as in a previous study by Tulliez and Peleran (5): acidified urine (pH 2) was extracted with ethyl acetate; ethyl esters of the extracts were prepared, then analyzed directly by GLC on a cyanopropyl phenyl silicone column (1/4 in. × 2.5 m, 3% OV-225 on 100/120 mesh Chromosorb W AW DMCS) or after derivatization. Acetylation was performed with acetic anhydride in pyridine (4:1, v/v) at 35 C; trimethylsilyl esters were prepared by heating at 70 C for 15 min with the Trisil "TBT" mixture (Pierce, Rockford, IL).

Analysis by radio gas chromatography (RGC) was performed with a Perkin Elmer reactor (RGC 170) connected to a Packard GLC instrument. Tritium gas or $^{14}CO_2$ was measured with a proportional counting tube after hydrogenative cracking of the ^{3}H-labeled compounds or combustion of the ^{14}C-labeled ones. A Model 852 (Packard Instruments) gas fraction collector was used to collect fractions that emerged from the gas chromatograph.

in vitro Assays

Microsomes were prepared from the livers of male Wistar rats (150-200 g) according to the method of Remmer et al. (7). The microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.4. Protein concentration was determined by the procedure of Gornall et al. (8). Hydroxylase activity was assayed in a mixture (H_1) containing 12 mg microsomal protein, 7.5 μmol glucose-6-phosphate, 2.6 units glucose-6-phosphate dehydrogenase, 20 μmol $MgCl_2$, 25 μmol nicotinamide, 3 μmol NADP and 0.1 M potassium phosphate buffer, pH 7.4, in a total vol of 5 ml. Five nmol of [^{14}C]cyclohexylacetic acid in 10 μl ethanol was added and the mixtures were shaken at 37 C for 30 min.

Mitochondria were prepared using the method of Hogeboom (9) in ice-cold 0.25 M sucrose solution containing 1 mM EGTA. Hydroxylase assay incubation mixture H_2 was composed of 50 μl mitochondria (2 mg protein/incubate) in 1 ml 0.25 M sucrose and 1 mM EGTA, adjusted to pH 7.4 with 0.2 M Tris and containing 1 μmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate dehydrogenase, 1 μmol $MgCl_2$, 50 nmol NADP and 5 nmol [^{14}C]cyclohexylacetic acid (300,000 dpm). β-Oxidation steps were studied as follows: 50 μl of mitochondrial fraction were placed in a 1-ml medium (E) containing 24 μmol glycylglycine, 9.6 μmol $MgCl_2$, 60 μmol KCl and 87 μmol sucrose to which cofactors (C), composed of an ATP generator system (20 μl 0.2 M Pi, 10 μl M succinate and 5 μmol ADP) were added where indicated. Unless otherwise stated, mitochondria incubations were done aerobically with shaking at 30 C for 30 min.

The effect of exogenous ATP added in the incubation medium was studied, and inhibition of acyl-CoA synthetase by carboxyatractyloside was used to provide the requirement of an ATP-dependent step in the metabolism of cyclohexyl acetic acid.

In every case, incubations were stopped with perchloric acid and the reaction products extracted with ethyl acetate, then separated by TLC on Silica Gel G using benzene/dioxane/acetic acid (45:12:2, by vol) as developing solvent. Hydroxylation rates were calculated from TLC radioactivity scans.

RESULTS

Identification of 1-Hydroxycyclohexaneacetic Acid in Urines

RGC analysis of ethyl esters prepared from ethyl acetate extracts (Fig. 1) indicated 3 radioactive peaks; the same results were obtained whether [^{14}C]cyclohexylacetic acid or [^{3}H]dodecylcyclohexane was administered to rats. Nevertheless, the relative proportions among the 3 peaks differed.

Peak I was cyclohexeneaceturic acid as previously observed by Tulliez and Peleran (5). Retention time of peak III was analogous to that of the synthetic [^{14}C]phenylaceturic acid ethyl ester; the identity was confirmed by electron impact GC-MS analysis that exhibited the molecular ion at m/e 221 and a major fragment at m/e 91 that corresponds to the aromatic structure $[C_6H_5\text{-}CH_2]^+$. Thus, evidence for aromatization of the cyclohexane ring is given.

Peak II showed the same retention time as hippuric acid; the corresponding fraction was trapped using the gas fraction collector. After hydrolysis of the amide bond, ethyl acetate extraction and ethylation of the extract, RGC analysis indicated that no labeling was associated with benzoic acid; it was entirely due to a product, the polarity of which was modified by silylation but not by acetylation, suggesting

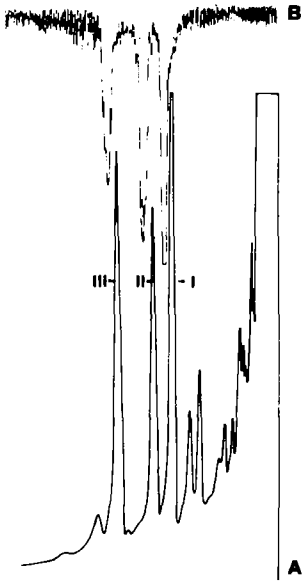

FIG. 1. RGC analysis of urinary extracts from [^{14}C]cyclohexylacetic-acid-treated rats: (A) flame ionization detection (GLC); (B) radioactivity. I = cyclohexeneaceturic acid; II = hippuric acid + unknown metabolite; and III = phenylaceturic acid.

the presence of tertiary OH group.

This hypothesis was confirmed by GC-MS. The mass spectrum was similar to that of the synthetic 1-hydroxycyclohexylacetic acid; it indicates (Fig. 2) a molecular ion at m/e 186 and shows a loss of 18 mass units at 168, characteristic of the dehydration of the molecule. A peak at m/e 157 confirms the presence of an ethyl ester (m-29), whereas the peak at m/e 81 reveals the cyclohexene structure occurring after dehydration. The peak at m/e 130 corresponds to the loss of C_4H_8 from the cyclohexane ring that is not observed in the mass spectra of cyclohexylacetic acid hydroxylated in position 2, 3 or 4.

in vitro Assays

The following were investigated in order to determine the pathway for the formation of 1-hydroxy-cyclohexylacetic acid: (a) hydroxylation by a liver microsomal mixed function oxidase; (b) hydroxylation by a cyt. P_{450}-containing mitochondrial hydroxylase analogous to those involved in steroid hydroxylations; and (c) β-oxidation blockade due to impossibility of dehydrogenation of the 1-hydroxy-cyclohexylacetyl-CoA.

Incubations of the liver microsomal fraction in medium H_1 did not lead to any hydroxylation of cyclohexylacetic acid.

Figure 3 indicates results obtained with the mitochondrial fraction; in medium H_2, as well as in medium E, the hydroxylation rate was in the 5% range, whereas when cofactors were added (E + C), 80% of the cyclohexylacetic acid was transformed into a hydroxylation product which was identified mainly as 1-hydroxy-cyclohexylacetic acid by RGC analysis under the previous conditions as well as by TLC on silica gel using benzene/dioxane/acetic acid (45:12:2, by vol) as developing solvent. Under these conditions, the R_f of 1-hydroxy-cyclohexylacetic acid was 0.47 whereas lower R_f values were obtained with cyclohexylacetic hydroxylated in position 2, 3 or 4. Moreover, traces of cyclohexene-acetic acid and glycine conjugates were observed.

Figure 4 summarizes results of an experiment in which, before incorporation of [^{14}C]-cyclohexylacetic acid in the flasks, mitochondria were preincubated at 30 C for a 15-min period in medium E, to which succinate was added, or in medium H_2. The complete absence of hydroxylation in medium H_2 and

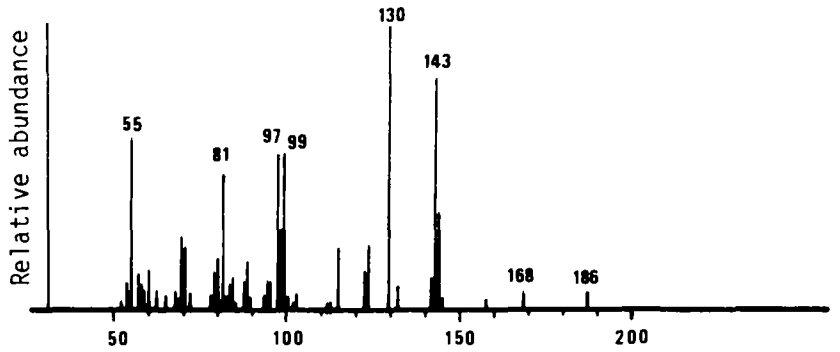

FIG. 2. Mass spectrum of 1-hydroxycyclohexylacetic acid in the ethyl ester form, after hydrolysis of the amide bond of the glycine conjugate.

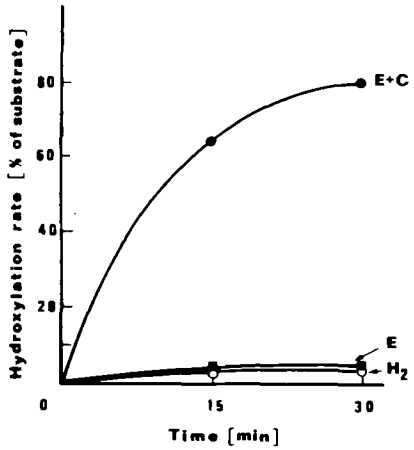

FIG. 3. Hydroxylation of cyclohexylacetic acid in position 1 by rat liver mitochondria. H_2 = incubation medium (mitochondrial hydroxylation assay); E = respiratory medium for mitochondria; C = cofactors (Pi, succinate, ADP).

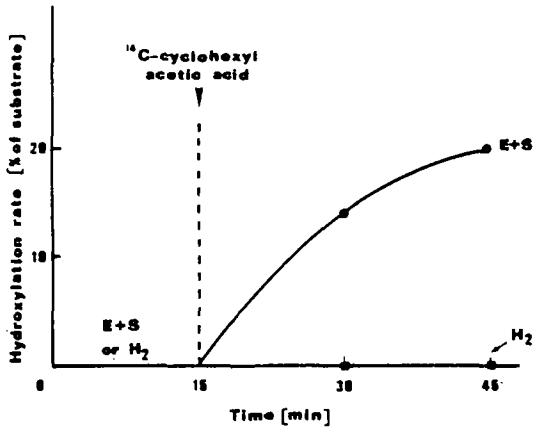

FIG. 4. Mitochondrial hydroxylation of cyclohexylacetic acid after a 15-min preincubation period without substrate. E, H_2: same as in Fig. 3; S: succinate.

the 20% hydroxylation rate in medium E + succinate revealed the necessity for ATP; the weak hydroxylation observed on Figure 3 in medium H_2 or E may correspond to use of ATP present in the mitochondrial suspension. The requirement for activation of cyclohexylacetic acid in the presence of ATP was confirmed by addition in medium E of increasing quantities (200-1,000 nmol) of ATP (Fig. 5), and by the strong decrease in hydroxylation rate observed when carboxyatractyloside (2 nmol/mg protein) was added.

When the carboxyl group of [^{14}C] cyclohexylacetic acid was protected as the methyl ester, no hydroxylation occurred in medium E + C with mitochondria, confirming the requirement for an activation to cyclohexylacetyl-CoA to make β-oxidation, as well as aromatization, possible.

[^{14}C] 1-Hydroxycyclohexylacetic acid isolated by TLC after incubation of mitochondria in medium E + C was placed into flasks, in which incubation of mitochondria was performed in medium E + C to which 10 μmol of glycine was added; thus, the classic conditions for studying conjugation or aromatization as described by Gatley and Sheratt (10) or Mitoma et al. (11) were fulfilled, and the only product obtained was glycine conjugate, i.e., the 1-hydroxycyclohexylaceturic acid.

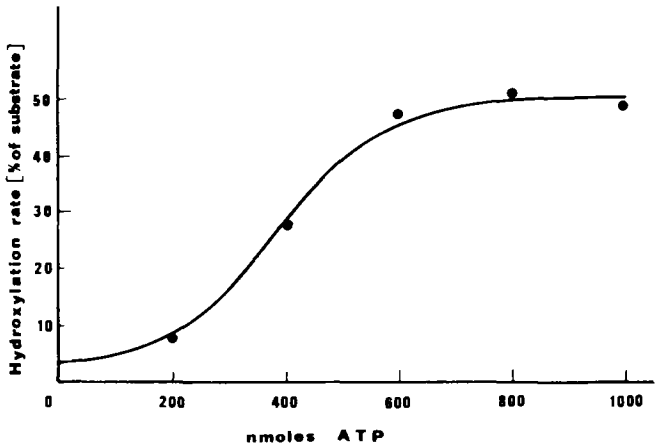

FIG. 5. Effects of increasing quantities of ATP in incubation medium on hydroxylation rate of cyclohexylacetic acid.

DISCUSSION

Microsomal mixed-function oxidases of rat liver seem inefficient for cyclohexylacetic acid hydroxylation in position 1; however, Frommer et al. (12) showed that 1-methylcyclohexanol was an important product of the hydroxylating reaction that occurs when incubating rabbit liver microsomes with methylcyclohexane, whereas it was not detected in urine of rabbits receiving this substituted cycloparaffin (2).

It is possible that the functional group of cyclohexylacetic acid does not allow fixation of the substrate into the active center of cyt. P_{450}; nevertheless, no hydroxylation occurred when the methyl ester of cyclohexylacetic acid was incubated with microsomes in medium H_1.

It also seems that other cyt. P_{450}-catalyzed hydroxylations, such as those performed by hydroxylases localized in liver mitochondria, were ineffective on cyclohexylacetic acid hydroxylation. However, one cannot exclude the existence of cyt. P_{450}-enzymes with higher degrees of specificity for substrates like cyclohexyl-substituted fatty acids.

This investigation describes cyclohexylacetic acid hydroxylation in the 1-position, as the result of activation in the form of the CoA ester followed by β-oxidation blockade after intervention of enoyl-CoA hydratase, the cyclohexane ring hindering the action of hydroxyacyl CoA dehydrogenase. This is fortified by the results of Brewster et al. (13,14), who did not observe any hydroxylated metabolite when studying the biotransformations of [^{14}C] cyclohexane carboxylic acid in the rat, whether they analyzed the urinary metabolites or the perfusate of the isolated liver; moreover, when [^{14}C] cyclohexane carboxylate is incubated in the conditions described in "materials and methods," no hydroxylation of the cyclohexane ring, even at the C_1 position, occurred (J. Tulliez and E. Durand, unpublished data). Thus, the possibility of a monooxygenase requiring the CoA ester as substrate seems to be excluded in the formation of 1-hydroxycyclohexane acetic acid.

This hydroxylation is a mitochondrial reaction, as are glycine conjugation and aromatization. These are reactions involved in the formation of metabolites previously identified and underlined by the presence of cyclohexeneacetic acid and cyclohexeneaceturic and phenylaceturic acids, after incubation of cyclohexylacetic acid in medium E + C. It is interesting to note that, if incubations were conducted under aromatization conditions, dehydrogenation of the cyclohexane ring was observed when cyclohexylacetic acid was the substrate, whereas with 1-hydroxycyclohexylacetic acid, the only product obtained was the glycine conjugate of the unchanged substrate. These data, associated with the results of Beer et al. (15) and Babior and Bloch (16) who observed that none of the 7 possible monohydroxycyclohexane carboxylates was aromatized and that dehydrogenation of cyclohexane carboxylic acid to benzoic acid was by way of cyclohexene-1-carboxyl CoA, indicate that aromatization takes place by direct dehydrogenation, rather than by hydroxylation followed by dehydration.

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In vitro Conversion of Saturated to Monounsaturated Fatty Acid by Ehrlich Ascites Cells

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ABSTRACT

In this paper, evidence is presented on the capacity of Ehrlich ascites cells to synthesize in vitro monounsaturated fatty acids from radioactive palmitate. Localization of the double bond was determined by ozonolysis and subsequent reduction of the ozonides to aldesters followed by gas liquid chromatography. These results proved that Ehrlich ascites cells have a Δ9 desaturase that catalyzes the biosynthesis of palmitoleic acid from palmitic acid and oleic and vaccenic acid via elongation-desaturation and desaturation-elongation, respectively, using palmitic acid as substrate. Furthermore, it is shown that, as in the hepatic cells, Δ9 desaturase enzyme activity of the tumoral cells is associated with the endoplasmic reticulum. The electron transport components involved in the desaturase system, i.e., NADH-cytochrome b_5 reductase and NADH-cytochrome c reductase, were also measured. The activities of these enzymes do not appear to be rate-limiting in the desaturase activity of these tumoral cells.

INTRODUCTION

It is well established that there are significant alterations in the fatty acid composition of tumor membranes. The replacement of polyunsaturated fatty acids in cellular phospholipids from neoplastic tissues by monounsaturated fatty acids has been demonstrated (1-3).

Wood and Healy (4) had claimed that Ehrlich ascites cells are capable of elongating palmitoyl-CoA but are incapable of desaturating the stearoyl-CoA produced in vivo by these cells. Apparently, there is no discrepancy between lack of desaturation and high levels of octadecenoic fatty acids in Ehrlich ascites cells, because the fatty acids accumulated are derived by these cells from the ascites plasma (5).

Nevertheless, it is important to take into account that cultured cells preferentially use preformed fatty acids from the medium, but when they grow in a lipid-free medium, are capable of synthesizing unsaturated fatty acids from simple precursors (6). These experiments were performed in order to prove the presence of a Δ9 desaturase system in the endoplasmic reticulum of Ehrlich ascites cells, including 2 enzymes from the microsomal electron transport chain associated with the desaturase activity: NADH-cytochrome b_5 reductase and NADH-cytochrome c reductase.

MATERIALS AND METHODS

[1-^{14}C] Palmitic acid (58 mci/mmol) was purchased from the Radiochemical Centre, Amersham, England. Pentex bovine albumin, fatty acid poor, was purchased from Miles Laboratories, Kankakee, IL.

Animals and Diet

Weanling Swiss mice were fed a Purina chow diet and water ad libitum before being used. Experiments were done with groups of 5 male Swiss mice, 12 g in weight. Animals were fed ad libitum a control diet as previously described (7) for 5 weeks prior to intraperitoneal (ip) implantation of Ehrlich ascites cells. The experimental diet was continued during tumor growth and the cells were harvested 8 days after implantation.

Cells

We used one hyperdiploid strain of the Ehrlich ascites cells carcinoma which shows the following characteristics: mean survival time 15 days and mean generation time 38 hr. Transplantation was achieved by ip injection of 0.2 ml of a sterile suspension in isotonic NaCl containing ca. 2.0×10^7 cells.

The mice were sacrificed by cervical fracture and the tumor cells were drained after injection of 1 ml of heparinized NaCl solution into the peritoneal cavity. The cells were separated by sedimentation at 4 C for 20 min at 400 × g. After washing and resedimentation 2 or 3 times, the cells were resuspended in a modified Hank solution (8). Cell counting was done with a hemocytometer. Cell integrity was estimated by 10% trypan blue exclusion. Viability was usually greater than 96%. Livers from host animals were separated, homogenized and the microsomes isolated by differential centrifuga-

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tion as previously described (9). The microsomal protein was estimated by the biuret method (10).

Assay for in vitro Desaturation in Host Liver Microsomes

[1-^{14}C] Palmitic acid was diluted to a specific activity (sp act) of about 1.7 mCi/mmol with the corresponding unlabeled pure fatty acid. The assay conditions were: 5 mg of microsomal protein was incubated in an open tube with 100 nmol of the diluted, labeled fatty acid in a Dubnoff shaker at 37 C for 30 min in a total vol of 1.5 ml of 0.15 M KCl; 0.25 M sucrose containing, in μmol: 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 fatty acids esterified (11). The conversion of saturated to monounsaturated fatty acid was measured by thin layer chromatography (TLC) of the fatty acid methyl esters on $AgNO_3$-impregnated silica gel plates as described (12). The areas containing labeled methyl esters were scraped off and counted directly in vials in a Packard Scintillation Spectrometer. Other monounsaturated fractions were analyzed by gas liquid chromatography (GLC), collecting the $^{14}CO_2$ at the exit chimney of the detector.

Assay for in vitro Desaturation in Intact Ehrlich Ascites Cells

The incubation of intact Ehrlich ascites cells was performed in 25-ml siliconized Erlenmeyer flasks in a total vol of 4 ml of modified Hank solution (8), containing 1 μCi of ^{14}C palmitic acid/albumin solution (13) and ca. 5×10^7 cells. The Erlenmeyer flasks were incubated in a Dubnoff shaker at 37 C at ca. 100 oscillations/min for 1 hr. All incubations were in duplicate. At the end of the incubation period, the flasks were placed in ice and their contents transferred to centrifuge tubes and centrifuged for 5 min at 3,000 x g. The cellular pellets were washed 3 times and extracted by the method of Folch-Pi et al. (14). The nonreacted free fatty acids were eliminated from the lipid extract by Elovson's method (15). The conversion of saturated to monounsaturated fatty acids was measured as already described.

Preparation of Smooth Membranes

The microsomal fraction from Ehrlich ascites cells obtained from groups of 20 mice was purified from free ribosomes by gradient centrifugation according to Holtzman et al. (16). Smooth microsomes were incubated in vitro as described for liver microsomes.

Determination of Structure of Monounsaturated Fatty Acids via Reductive Ozonolysis

The ozonization was done on the monoethylenic fatty acid fraction separated by Ag-TLC with a microozonizator, Supelco, Inc., according to Beroza and Bierl (17). The radioactivity was measured in the $^{14}CO_2$ effluent from the gas chromatograph according to Blank et al. (18).

Assay for the Microsomal Transport Chain Enzymes

NADH-cytochrome b_5 reductase was assayed at 25 C by measuring the NADH-ferricyanide reductase activity of the enzyme according to Strittmatter (19). NADH-cytochrome c reductase was determined at 550 nm as described by Rogers and Strittmatter (20).

RESULTS AND DISCUSSION

Results from in vitro experiments have shown that intact Ehrlich ascites cells are capable of desaturating saturated fatty acids. A conversion was found of 4.2% ± 1.4 [^{14}C]-palmitic acid to radioactive monoethylenic fatty acids. The analyses by GLC of the monoene fatty acid fraction separated by Ag-TLC shows that only hexadecenoic acid, 16:1, and octadecenoic acid, 18:1, were radioactive.

Further analyses of the saturated fatty acid fraction show that 91.7% of the total radioactivity remains in the palmitic acid, whereas only 4.1% of the label was found in the stearic acid (Table 1), indicating that this acid was produced by chain elongation of its radioactive predecessor as described previously by Wood and Healy (4).

The position of the double bond in the labeled products of the monoene fraction, determined by ozonolysis and reduction of

TABLE 1

Radioactivity from [1-^{14}C] Palmitic Acid Incorporated into Saturated and Monounsaturated Fatty Acid from Total Lipids of Ehrlich Ascites Cells (%)

Fatty acid	Distribution of radioactivity (%)
16:0 (Palmitic acid)	91.7[a]
18:0 (Stearic acid)	4.1
16:1Δ9 (Palmitoleic acid)	1.9
18:1Δ9 (Oleic acid)	0.8
18:1Δ11 (Vaccenic acid)	1.5

[a]Values are means of 2 pooled samples of total lipids from Ehrlich ascites cells.

the ozonide, shows that the aldesters of C_9 and C_{11} were produced during the molecular cleavage (Fig. 1). According to the radioactivity incorporated into 16:1 and 18:1, we can deduce that 2 isomers, oleic acid (18:1Δ9) and vaccenic acid (18:1Δ11) were present in the 18:1 fraction, as shown in Table 1.

Apparently, under the conditions of these experiments, desaturation of 16:0 → 16:1Δ9 preceded chain elongation 16:1Δ9 → 18:1Δ11, and chain elongation 16:0 → 18:0 preceded chain desaturation 18:0 → 18:1Δ9. The route of vaccenic acid biosynthesis in these cells appears to be the same as that reported in rat liver by Holloway and Wakil (21) and in HTC cells (22) by Wiegand and Wood.

The presence of the enzyme responsible for the Δ9 desaturase activity found in intact Ehrlich ascites cells was also analyzed in the microsomal fraction of the tumoral cells.

The electron microscope observations of microsome preparations from Ehrlich ascites cells showed that this subcellular fraction was rich in free ribosomes and lacking in membrane structures. Because desaturase enzyme activity is associated in mammalian cells with these membrane structures (23), it was necessary to isolate them in order to assay the Δ9 desaturating system.

The presence of a Δ9 desaturase was confirmed in 2 preparations of smooth endoplasmic reticulum from Ehrlich ascites cells. These results indicated that the sp act of the enzyme was 0.04 nmol/min/mg protein in this subcellular fraction (Table 1), contrasting with the value of 0.12 ± 0.02 nmol/min/mg protein corresponding to the microsomal fraction from host liver.

It is well known that the microsomal desaturase activity is associated with the electron transport chain (21-25). It has been reported that microsomal electron transport may be impaired in some cancer cells (2,26). The values obtained from the assay of the enzymes involved in the electron flux show an activity of 0.063 μmol/min/mg protein for NADH-cytochrome c reductase and 0.44 μmol/min/mg protein for NADH-cytochrome b_5 reductase as shown in Table 2. Electron transport does not appear to be rate-limiting in the Δ9 desaturase reaction in Ehrlich ascites cells.

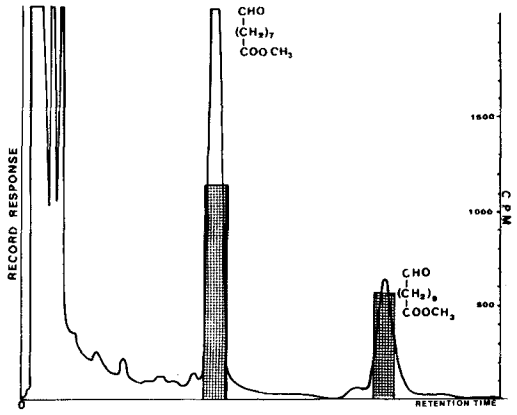

FIG. 1. Gas liquid chromatographic analysis and distribution of ^{14}C activity of the methyl esters of ozonolysis products of the methyl esters of monounsaturated fatty acids. Analyses were performed on glass column packed with 15% DEGS at 170 C.

In early experiments, Wood and Healy (4) studied the distribution of radioactivity in saturated and monoethylenic fatty acids incorporated into lipid fractions of Ehrlich ascites cells incubated in the peritoneal cavity of Swiss mice with labeled palmitoyl-CoA. We assume that 16:0-CoA is hydrolyzed to 16:0 before being taken up by the cells.

Despite these authors' conclusion that saturated fatty acids are not desaturated to the corresponding monoenoic acids, their results indicate that cells did exhibit a small amount of desaturase activity. Probably they arrived at this conclusion because, under the conditions of their in vivo experiments, it is not possible

TABLE 2

In vitro Activity of Fatty Acid Desaturase, NADH-Cytochrome b_5 Reductase and NADH-Cytochrome c Reductase from Smooth Endoplasmic Reticulum of Ehrlich Ascites Cells

Enzyme	Fatty acid desaturase	NADH-cytochrome b_5 reductase	NADH-cytochrome c reductase
	16:0 → 16:1 nmol/min/mg protein	μmol/min/mg protein	μmol/min/mg protein
	0.04[a]	0.44	0.06

[a]Values are means of 2 preparations of smooth endoplasmic reticulum from Ehrlich ascites cells.

to deduce to what extent the labeled palmitic acid is taken up by the host liver, elongated and desaturated, incorporated into lipoproteins and recirculated to the peritoneal cavity.

From the results of the present experiments, we can conclude that Ehrlich ascites cells have a Δ9 desaturase enzyme activity associated with the endoplasmic reticulum. The low capacity of these cells to desaturate palmitic acid to monounsaturated fatty acids could be the consequence of at least 2 contributing factors, i.e., the low content of endoplasmic reticulum in Ehrlich ascites cells associated with a poor Δ9 desaturase enzyme activity in this subcellular fraction.

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Studies of Biosynthesis of Waxes by Developing Jojoba Seed: III. Biosynthesis of Wax Esters from Acyl-CoA and Long Chain Alcohols

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ABSTRACT

Acyl-CoA:alcohol transacylase activity was demonstrated in cell-free homogenates of developing jojoba seeds. The optimal pH was 8.0-8.1. Under optimal conditions, wax formation had a nearly linear relationship with extract concentration; the time course of wax formation was also linear up to 30 min. *cis*-11-Eicosenol was the most effective alcohol substrate whereas tetradecanol, octadecanol, dodecanol, *cis*-9-octadecanol, and *cis*-13-eicosenol gave progressively lower activities. Either saturated or unsaturated acyl-CoA with 18 or 20 C-atoms had similar activity. The enzyme was fairly stable at 0 C, less stable at RT and labile above 30 C. Differential centrifugation showed that the 12,000 × g fat pad was the most active in wax formation; to maximize the activity, a 12,000 × g supernatant appeared to be necessary. This factor in the supernatant was thermolabile and nondialyzable.

INTRODUCTION

Seeds of the jojoba shrub, *Simmondsia chinensis*, are unique in accumulating large amounts of liquid wax ester (defined as the oxygen esters of long-chain fatty acids and alcohols) as their major storage reserve (1). Recent investigations with fresh tissue slices of the maturing seed and with homogenates of these tissues have provided the general scheme for the biosynthesis of these wax esters (2,3). The results of these studies show that oleic acid, synthesized in a compartment from C_2 and C_3 precursors, is transported to another compartment where it is converted to oleoyl CoA, elongated to eicosenoyl CoA and docosenoyl CoA; these are then reduced to their corresponding alcohols and finally, both the acyl CoA and the alcohols combined to form C_{40}, C_{42} and C_{44} wax esters.

This paper presents data that describe in more detail the enzyme acyl CoA:alcohol transacylase that catalyzes the reaction by which acyl CoA react with primary long-chain alcohols to form wax esters.

MATERIALS AND METHODS

Materials

Developing jojoba seeds of undetermined age were the generous gift of Dr. D.M. Yermanos, University of California, Riverside. Seeds, harvested on June 16, 1980, were frozen 3 days after harvesting and stored at -10 C until used. The shells and the seed coats were removed from the thawed seeds, and the cotyledons were gently homogenized in a chilled pestle and mortar with 2 vol of chilled grinding medium containing 0.6 M sucrose and 0.5% (w/v) defatted bovine serum albumin (BSA) in 0.15 M Tricine, pH 7.5. The resulting homogenate was filtered through 44-μ^2 nylon cloth to remove the coarser debris and was either frozen or used directly.

^{14}C-Labeled acyl-Coenzyme A was prepared according to Jaworski (4) or Bishop and Hajra (5). Coenzyme A (lithium salt) was obtained from Sigma Chemical Co., St. Louis, MO, and [1-^{14}C] fatty acids were from New England Nuclear, Boston, MA. The specific activities (sp act) of the synthesized acyl-CoA were: [1-^{14}C] *cis*-11-eicosenoyl CoA, 3.09 Ci/mol; [1-^{14}C] eicosanoyl-CoA, 10 Ci/mol; [1-^{14}C] *cis*-13-docosenoyl-CoA, 4.82 Ci/mol; [1-^{14}C] stearoyl-CoA, 56.5 Ci/mol and [1-^{14}C] oleoyl-CoA, 57 Ci/mol. Nonradioactive eicosanoyl-CoA was prepared according to Bishop and Hajra (5). [1-^{14}C] Dodecanol (sp act 55.7 Ci/mol) was purchased from ICN Pharmaceuticals Inc., Irvine, CA. All nonlabeled alcohols were obtained from Applied Science Laboratory Inc., State College, PA; BSA, ATP, UTP, GTP, CTP, AMP, ADP, NADPH and NADH were purchased from Sigma.

Incubation and Assay Methods

For the assay of wax biosynthesis, 50 μl of the cell-free homogenate (1.4 mg protein/50 μl) was added to the reaction mixture in a total vol of 1 ml containing the following, except when otherwise stated: 0.5 μmol nonlabeled dodecanol dissolved in ethyleneglycol-monomethyl ester (this solvent had no effect on wax-forming activity of jojoba homogenate

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at a concentration of 10% v/v, but was inhibitory at higher concentrations), 0.1 μmol ATP, 0.2 mmol Tris HCl (pH 8.0), 5 μmol $MgCl_2$ and 50,000 cpm of either [1-^{14}C]eicosanoyl-CoA (7.2 nmol) or [1-^{14}C]*cis*-11-eicosenoyl-CoA (1.8 nmol). The incubation was done at 30 C for 20 min and terminated by the addition of 0.1 ml glacial acetic acid. Lipid was extracted essentially by the method of Hara and Radin (6). Three ml of petroleum ether (bp 30-60 C)/isopropanol, 3:2 (v/v) and 3 ml of 6.7% (w/v) aqueous Na_2SO_4 were added to the terminated incubation mixture and vortexed for 30 sec. After the mixture separated into 2 phases, the lipid phase was removed and another 5 ml of petroleum ether was added, vortexed, allowed to stand and the lipid layer removed again. The partition of wax, free fatty acid, free long-chain alcohol and acyl-CoA between the 2 phases in the presence of all the components of the incubation mixture had been ascertained by thin layer chromatography (TLC) techniques using jojoba wax and [1-^{14}C]-18:0 free acid, [1-^{14}C]12:0 alcohol and [1-^{14}C]20:0 CoA, respectively. More than 95% of the wax, free fatty acid and free fatty alcohol was in the lipid phase and more than 95% of the acyl-CoA was in the aqueous phase.

The lipid phase obtained from each reaction mixture was evaporated under nitrogen at room temp. to dryness. The residue was taken up with benzene, spotted on a Silica Gel G TLC plate and chromatographed with petroleum ether/diethyl ether/glacial acetic acid (80:20:1, v/v/v). The chromatogram was visualized under UV light (360 nm) after spraying with 0.1% 2.7-dichlorofluorescein in methanol. The quenching effect of this dye at the amount being sprayed onto the plate was less than 3%. The R_f values were: wax, 0.85; an unknown band, 0.67; free fatty acid, 0.54; free fatty alcohol, 0.24; and polar lipid, the origin. The wax band and/or other bands were scraped and counted as described previously (2). An aliquot of the aqueous layer was counted when necessary for the determination of acyl-CoA.

Ethanolysis of the wax and the subsequent radio-GLC of the products of ethanolysis were essentially as described previously (2). Protein was determined according to Lowry et al. (7).

RESULTS

Characteristics of the Enzymatic Esterification of Fatty Alcohol by Acyl-CoA

The optimal pH determined with 0.2 M Tris of different pH values was 8.0-8.1. The salt effects of NaCl and KCl at concentrations up to 20 mM at pH 8.0 were studied but no marked stimulation or inhibition of the system was noted.

It has been reported that homogenization of the jojoba cotyledons in the presence of defatted BSA (5 mg ml^{-1}) had a stimulating effect (3). Moreover, defatted BSA was able to increase the incorporation of acyl-CoA into wax in the homogenate of jojoba cotyledons and these observations were confirmed, in part, in the present investigation. Homogenization with 5 mg ml^{-1} BSA increased the wax-forming activity about 2-fold, but further addition of BSA appeared to be inhibitory. Because BSA also inhibited thioesterase activity, the observed stimulating effect in the presence of BSA in the grinding medium could be attributed to decreased hydrolysis of acyl-CoA, one of the substrates of the reaction.

As ATP, GTP, CTP and UTP at concentrations of 1 or 2 mM were shown to increase wax formation from acyl-CoA and fatty alcohol by about 10%, ATP was added routinely to reaction mixtures. AMP and ADP had no effect.

Under optimal conditions, wax formation had nearly a linear relationship with extract concentration. No wax formation occurred when both acyl-CoA and alcohol were incuubated in the absence of the extract. In the presence of 1.3 mg jojoba seed protein (50 μl of the cell-free homogenate), the time course for enzymatic wax formation was linear within 30 min but decreased thereafter. This decrease may be related to the thermolability of the

TABLE 1

Specificity of Alcohols in the Presence of Ethyleneglycolmonomethyl ether[a]

Alcohol	Wax formed/20 min (nmol)
Decanol	0.12
Dodecanol	0.46
Tetradecanol	0.53
Hexadecanol	0.28
Octadecanol	0.22
cis-9-Octadecenol	0.46
Eicosanol[b]	0.08
cis-13-Eicosenol	0.32
cis-11-Eicosenol	0.55
Docosanol[c]	0.03

[a]All alcohols were dissolved in ethyleneglycolmonomethyl ether (5 mM) and 0.1 ml of each solution was added to the reaction mixture to give a final conc of 0.5 mM alcohol (except as noted). Final vol was 1 ml.

[b]Conc of 20:0 alcohol in the reaction mixture was 0.4 mM.

[c]Conc of 22:0 alcohol in the reaction mixture was 0.1 mM.

enzyme as described later.

The alcohol specificity of the enzyme is shown in Table 1. *cis*-11-Eicosenol, which is the endogenous alcohol in jojoba wax, gave the highest activity whereas tetradecanol, *cis*-9-octadecenol, dodecanol and *cis*-13-eicosenol gave progressively lower activities. The specificity of this enzyme is similar to that of the long-chain alcohol oxidase that catabolizes fatty alcohol during germination of the jojoba seedling (8). The other alcohols tested had low activities. It is interesting that the physiological substrate *cis*-11-eicosenol gave highest activity whereas its positional isomer, *cis*-13-eicosenol and its saturated analog, eicosanol, were far less effective as substrates. A substrate concentration curve (Fig. 1) with nonlabeled dodecanol showed that 0.5 mM alcohol in the incubation mixture gave the highest activity. Although activity dropped gradually with higher concentration, interpretation is difficult because of the low solubility of fatty alcohols.

Either saturated or unsaturated acyl-CoA with 18 or 20 C-atoms were similar in activity. Three acyl CoA were tested for specificity of the reaction. At equal molar concentrations (6.3 μM), stearoyl-CoA, eicosanoyl-CoA and *cis*-11-eicosenoyl CoA were incorporated into wax esters at the following rates (nmol/20 m), respectively: 0.96, 0.76 and 0.93. Figure 2 indicates that, with ^{14}C-labeled eicosanoyl-CoA as substrate, the acyl-CoA:alcohol transacylase was not saturated, even at concentrations as high as 100 μM which is many times higher than the critical micelle concentration of acyl-CoA ($\sim$ 3 μM). Presumably, the formation of the oxygen ester used both monomers and micelles of acyl-CoA although, without information concerning the possible interaction of binding protein(s), further interpretation of these data would be premature.

During the course of incubation, the amount of wax formed was correlated with the amount of acyl-CoA used (Fig. 3). Minor amounts of polar lipid, free fatty acid and free fatty alcohol also were formed during the course of the experiment (1 hr).

To determine whether acyl-CoA and alcohol were directly incorporated into the wax formed during incubation, or whether the added acyl-CoA was first converted into the corresponding alcohol and then incorporated into wax, an experiment was performed using either labeled acyl-CoA or labeled alcohol as susbstrate. The labeled wax was subjected to ethanolysis and the resulting products analyzed by radio-GLC. The data clearly showed that, under the conditions of the experiments, label from [^{14}C] acyl-CoA was exclusively in the acyl moiety and

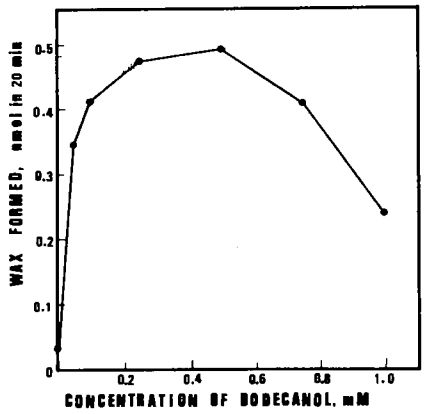

FIG. 1. Effect of dodecanol concentration on the formation of wax from [1-^{14}C] eicosanoyl-CoA. Dodecanol was dissolved in ethyleneglycolmonomethyl ether. Incubation conditions were the same as described in Materials and Methods.

label from [^{14}C] alcohol was correspondingly in the alcohol moiety. Even when NADH and NADPH were added, no label from [^{14}C] acyl-CoA was found in the alcohol moiety of the hydrolyzed wax.

The stability of the activity at 0 C, room temperature (25 C) and higher temperatures has been studied. The extract seemed to be fairly stable at 0 C, but less stable at room temp. At 4 C, the enzyme activity of the homogenate obtained by grinding in the presence of BSA was fairly stable for about 2-3 hr, but not beyond this time limit. PVP40 (polyvinylpyrrolidone) did not stabilize the activity; in fact, it exerted some inhibitory effect on even the initial activity of the extract. At tem-

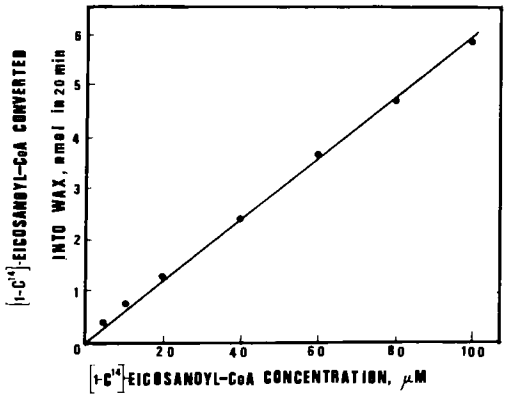

FIG. 2. Effect of the concentration of [1-^{14}C]-eicosanoyl-CoA on its conversion to wax. Incubation conditions are given in Materials and Methods.

peratures above 30 C, the enzyme was thermolabile. At 50 C, the activity was completely lost within 10 min.

Several attempts were made to employ mixed micelles of long-chain alcohols with dicaproyl phosphatidylcholine. These micelles were ineffective as carriers of the alcohols.

Differential Centrifugation Studies

Freshly prepared homogenate was centrifuged at 500 x g for 10 min and the resulting supernatant was centrifuged at 12,000 x g for 30 min; the floating fat pad, the supernatant and the pellet were collected separately. The pellet and the fat pad were resuspended in the grinding medium, the volume of which corresponded to the original volume of the homogenate. These fractions were assayed for wax formation separately or in different combinations. The results are summarized in Table 2. When the fractions from the 500 x g centrifugation were assayed, the 500 x g pellet was essentially inactive whereas the supernatant was the most active. The 12,000 x g centrifugation of the 500 x g supernatant yielded a pellet (P_2), a supernatant fraction (S_2) and a fat pad (F). The data in Table 2 and Figure 4 indicate that the wax-forming activity was located exclusively in the fat pad, but to maximize the activity, S_2 and F had to be recombined. With a constant amount of fat pad (25 μg protein, corresponding to 50 μl homogenate) and different amounts of S_2 (from 0.34 to 2.7 mg protein, corresponding to 50 to 400 μl homogenate), a factor in S_2, capable of stimulating the wax-forming activity of F, is thermolabile (Fig. 5). The factor is also nondialyzable. S_2 showed slight wax-forming activity only at relatively higher concentrations, presumably because of contamination of a small amount of fat pad. The function of the S_2 fraction is now under investigation.

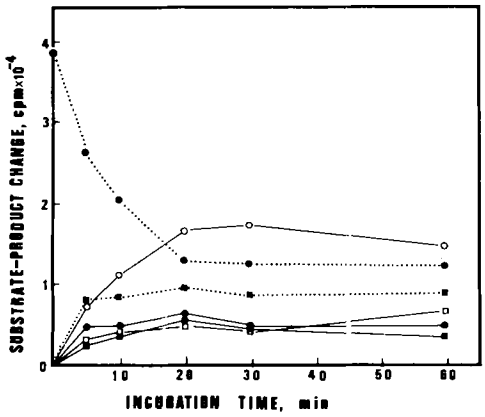

FIG. 3. Time course of the disappearance of [1-^{14}C] 11-eicosenoyl-CoA and its incorporation into labeled products as a function of time. Incubation conditions are given in Materials and Methods. (●····●) acyl-CoA, (○——○) wax, (□····□) free fatty acid, (●——●) polar lipids, (□——□) free alcohol, (■——■) unknown compound(s).

TABLE 2

Fractions Obtained by Differential Centrifugation of Jojoba Cotyledon Homogenate[a]

Centrifugation conditions	Fraction assayed	Wax formed/20 min (nmol)
–	Original homogenate	0.320
500 X g/10 min	P_1[b]	0.011
	S_1	0.205
	$S_1 + P_1$	0.270
12,000 X g/30 min	F	0.220
	S_2	0.007
	P_2	0.012
	$S_2 + P_2$	0.021
	$F + S_2$	0.253
	$F + P_2$	0.169
	$F + S_2 + P_2$	0.237
	$F + S_2 + P_2 + P_1$	0.246
	F (boiled)	0.006
	S (boiled)	0.011
	F (boiled) + S_2	0.008
	F + S_2 (boiled)	0.200

[a]Incubation conditions as described in Methods. The amount of original homogenate used was 50 μl and the amount of different fractions used, separately or in combination, was corresponding to 50 μl original homogenate.

[b]P=pellet; S=supernatant; F=fat pad.

DISCUSSION

Wax esters are widespread in the plant kingdom. Although they are localized on the surface of epidermal cells of the leaf, they are presumably synthesized in the epidermal cells and extruded onto the surface (9). These compounds are, however, not observed to be synthesized in the parenchymal cells of the leaf. Some lower plants do synthesize large amounts of wax esters. The system in *Euglena gracilis* has been examined in some detail (10) and both a microsomal NADH-dependent acyl-CoA reductase and an acyl-CoA:alcohol transacylase have been identified in these cells.

The synthesis of storage wax esters in the developing jojoba seed is, however, unique in the higher plant kingdom. Moreover, jojoba seeds have no capacity to form triacylglycerols. In fact, it is the only species in the phyla Anthophyta that has these biosynthetic properties. Upon germination, these storage wax esters are completely mobilized by a series of enzymes that are again quite unique to these tissues. Wax is hydrolyzed by an enzyme on the wax body membrane (11). The resulting fatty alcohol is oxidized to fatty acid by a fatty alcohol oxidase and a fatty aldehyde dehydrogenase (8). Both fatty acids are then efficiently converted to sugars by the enzymes of the β-oxidation cycle, the glyoxylate cycle and gluconeogenesis (12).

In general, 3 possible mechanisms have been suggested for the synthesis of wax esters: (a) the direct esterification of a free fatty acid and a fatty alcohol by an esterase, (b) direct transfer of an acyl group from phospholipids to fatty alcohol, and (c) the reaction of an acyl-CoA and a fatty alcohol catalyzed by the enzyme acyl-CoA:alcohol transacylase (9). Because free fatty acids do not serve as substrates for wax ester synthesis (3) and because, as indicated in Figure 3, there does not appear to be a correlation between wax ester synthesis and phosphatidylcholine use. The first 2 possible mechanisms probably do not play a role in wax ester biosynthesis in the jojoba cotyledons.

The data presented in this paper, however, identify an acyl-CoA:alcohol transacylase that may be associated with membranes. The picture is complicated by the nature of the substrates that are used. Because of the low solubility properties of long-chain alcohols, even simple kinetic analyses are difficult. Further work must therefore be directed toward (a) solubilization of the transacylase, and (b) an explanation for the physical nature of long-chain alcohols serving as substrates for the transacylase. A number of proteins have now been

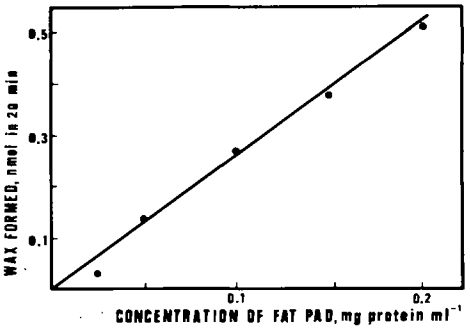

FIG. 4. Formation of wax from [1-^{14}C]eicosanoyl-CoA as a function of the concentration of fat pad obtained by 12,000 X g centrifugation of the homogenate. Incubation conditions were as described in Materials and Methods.

isolated from animal tissues that have specific binding properties. For example, it is well documented that there exists in animal tissues a number of soluble binding proteins specific for either retinol or retinoic acid (13). Moreover, a sterol carrier protein has been isolated from a number of tissues that specifically bind steroids and, in fact, stimulate the synthesis of sterols in animal tissue (14). Because the possibility exists that the protein factor in S_2 is one of this type, the S_2 fraction is now under

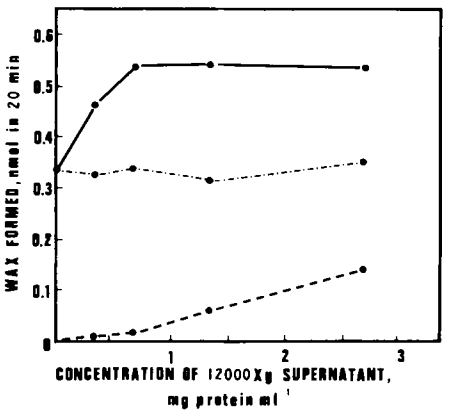

FIG. 5. Stimulation of 12,000 X g supernatant (S_2) on wax formation catalyzed by 12,000 X g fat pad (F). Incubation conditions were as described in Materials and Methods. The amount of fat pad added in each incubation, except those without fat pad, was 50 μl (containing 25 μg protein), corresponding to 50 μl original homogenate). The amounts of S_2 added were indicated on the abscissa corresponding to 50-400 μl of the original homogenate). (●---●) F with different amount of unboiled S_2, (●-·-●) F with different amount of S_2 which had been boiled for 15 min, and (●——●) unboiled S_2 only.

investigation. Once these problems are resolved, we shall be able to understand more fully the systems involved in the formation of the unique jojoba wax esters.

ACKNOWLEDGMENTS

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Hypolipidemic Effects of Clofibrate and Selected Chroman Analogs in Fasted Rats: I. Chow-Fed Animals

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ABSTRACT

The hypolipidemic properties of ethyl 6-chlorochroman-2-carboxylate (II), ethyl 6-phenylchroman-2-carboxylate (III) and ethyl 6-cyclohexylchroman-2-carboxylate (IV) were compared to clofibrate (I) in fasted normolipidemic rats. The chroman analog II, like its parent compound, clofibrate, reduced serum and α-lipoprotein cholesterol concentrations. Although analog III had no effect on serum cholesterol, it caused a slight elevation of α-lipoprotein cholesterol concentration. Serum free cholesterol was increased and LCAT activity was reduced in clofibrate-treated rats. The hypolipidemic agents had no consistent effect on liver lipid concentrations and liver microsomal HMG-CoA reductase activity. In addition, we have shown that drug efficacies varied directly with seasonal variations in serum lipid concentrations.

INTRODUCTION

A series of cyclic chroman analogs of clofibrate (I) were synthesized in our laboratory in order to develop more active and selective hypolipidemic agents. Hypolipidemic properties of chroman analogs were previously evaluated using nonfasted normolipidemic (1,2) and Triton WR-1339 induced hyperlipidemic (3) rat models. In these experiments, the chroman analogs were shown to possess both hypotriglyceridemic and hypocholesterolemic effects. These single-dose studies of the drugs were limited to observations of total serum lipid concentrations with no measurement of lipid distribution changes among lipoprotein classes. This information is necessary because epidemiological and case-controlled studies with human subjects have shown that LDL-CH is atherogenic and HDL-CH is antiatherogenic (4). Thus, comparative effects of clofibrate and structurally related chroman analogs on serum lipids in these lipoprotein classes in the normolipemic rat is the major objective of this communication.

We report here the dose-dependent effects of clofibrate (I), a chlorochroman analog (II) of similar lipophilicity, as well as the more lipophilic phenyl-(III) and cyclohexyl-(IV) chroman esters (Fig. 1) on serum total and lipoprotein (LP) lipid composition in fasted normolipidemic Sprague-Dawley rats. Liver and serum enzyme parameters also were monitored. Studies on different doses of clofibrate were done to find the minimal effective dose to be used for comparison of hypolipidemic activity

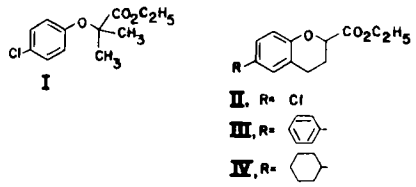

FIG. 1. Chemical structures of clofibrate (I), 6-chlorochroman-2-carboxylate (II), 6-phenylchroman-2-carboxylate (III) and 6-cyclohexylchroman-2-carboxylate (IV).

of the analogs. In these experiments, clofibrate served as a positive internal control to monitor and statistically correct for potential seasonal variations in analyte concentrations.

MATERIALS AND METHODS

Chemicals

Clofibrate was a gift from Ayerst Laboratories, Rouses Point, NY. Ethyl 6-chloro-, phenyl- and cyclohexyl- chroman-2-carboxylates were synthesized as described previously (3,5).

Animals

Male Sprague-Dawley rats (180-210 g) were housed in groups of 6 and given Purina rat chow and water ad lib. Rats were maintained in a vivarium at 25-26 C on an alternate 12-hr light and dark cycle and were acclimatized to the housing conditions for 1 week before drug treatment.

Experimental Design

Groups (n = 6) of rats received various doses of clofibrate (0.1, 0.2, 0.4 and 0.6 mmol/kg/day in 0.25% methyl cellulose) or 0.25% methyl cellulose orally twice daily for 7 consecutive days. In all experiments, drugs were given in a vol of 1.0 ml/100 g body wt. Following these initial dose-response studies, groups of animals were treated with vehicle or 0.2 and 0.4 mmol/kg/day of compound twice daily.

All rats were fasted 16-18 hr before blood collection. Blood was drawn (orbital plexus) from rats under light ether anesthesia on 1 day before (day -1) and after 4 days (day +4) of drug treatment. After 7 days (day +7) of drug treatment, blood was collected by exsanguination from the abdominal aorta. Livers were immediately excised, rinsed in ice-cold 0.1 M TRIS-HCl buffer, pH 7.2 (containing 1.15% KCl, w/v), blotted, weighed, minced and homogenized in the same buffer using a glass homogenizer equipped with a Teflon pestle. Liver microsomes were isolated as described previously (1).

Liver Assays

Microsomal 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34) activity was assayed by the method of Huber et al. (6), and protein content was determined by the procedure of Lowry et al. (7). CH and TG were extracted from the liver homogenates (25%, w/v) by the method of Abell et al. (8) and analyzed by the method of Parekh and Jung (9) and Soloni (10), respectively.

Serum Assays

All blood samples were placed on ice and, after clotting, serum was separated by centrifugation at 2,000 x g for 10 min. The serum samples were kept at 4 C and analyzed within 48 hr. The serum total CH was measured by the enzymatic method of Allain et al. (11) with A-Gent cholesterol reagent (Abbott Laboratories, Diagnostics Division, Chicago, IL) on an Abbott Bichromatic analyzer (ABA-100) equipped with a 1:51 syringe-plate. Serum total TG concentrations were measured with A-Gent triglyceride reagent (Abbott Labs) on an ABA-100 according to the method of Sampson et al. (12).

α-Lipoprotein (LP)-CH (high density LP-CH) was determined by an enzymatic method after electrophoretic separation of LP on agarose film by a modification of the method of Newman and Jenny (13). Serum samples (2-3 μl) were applied to each well of the agarose plate, and electrophoresis was done using 3-(N-morpholino)-2-hydroxypropane sulfonic acid buffer, pH 7.8 (Corning Medical, Palo Alto, CA).

Following electrophoretic separation of pre-β-("VLDL"), β-("LDL") and α-("HDL") LP as described later, TG in these particles were stained by a procedure using the colorimetric method of Stavropoulos and Crouch (14). Two to 3 μl of rat serum was applied to each well of the agarose plate, and electrophoresis was done at 90 V for 25 min with Universal barbital buffer, pH 8.6. Following electrophoresis, plates were overlaid with reconstituted (0.5 ml) Triglyceride Reagent (Dow Chemical Co., Indianapolis, IN). Plates were incubated at 37 C (15 min) and room temperature (15 min) for maximal color development and were scanned using the Corning Model 720 densitometer. The relative percentages of pre-β-, β- and α-LP-TG, and a cathodically migrating band were determined. LP-TG concentrations were computed by multiplying serum total TG with the relative percentage of LP. The TG concentration of different LP obtained by this method was shown to be linear to a TG concentration of 700 mg/dl.

Serum Free Cholesterol and Lecithin:Cholesterol Acyltransferase Activity (LCAT)

LCAT (EC 2.3.1.43) activity was assayed according to the method of Glomset and Wright (15). Serum free CH was measured by the enzymatic method of Nagasaki and Akanuma (16) which is a modification of the method of Allain et al. (11). Serum free CH was expressed as a percentage of serum total CH.

Statistical Evaluation

Because these experiments were designed to measure the performance trend over a period of time, the 2-way analysis of variance (ANOVA) with repeated measurements design was selected to analyze each variable. Seasonal and individual biological variations were observed over an 8-month period. To minimize error and normalize data for comparative analysis, each animal was used as its own control whenever analytes were measured on day -1, day +4 and day +7, and all values were expressed as a percentage of day -1. These percentages were used to compare results among the variously treated groups of animals. When measurements were only made on day +7, analyte concentrations were expressed in absolute values. Additionally, post hoc analysis was performed using the Newman-Keuls procedure at $p<0.05$.

RESULTS

Determination of Appropriate Clofibrate Dosage Regimens

Clofibrate treatment at the dose level 0.1 mmol/kg/day x 2 had no significant effect on serum total CH and α-LP-CH at any stage of the experimental period whereas higher doses (0.2, 0.4, and 0.6 mmol/kg/day x 2) produced significant hypocholesterolemic effects (Fig. 2, panels A and C). At the higher doses, serum total CH and α-LP-CH concentrations were lowered to a similar degree after 4 and 7 days of treatment. By contrast, serum total TG concentrations were unaffected by clofibrate treatment. For these reasons, comparative effects of clofibrate and all analogs were examined at the 0.2 and 0.4 mmol/kg/day x 2 dose levels.

Seasonal Variations of Serum Lipid Concentrations

During this 8-month study (April-Nov.) of clofibrate and analogs, we observed that parallel changes occurred in serum total CH, TG and α-LP-CH concentrations of animals at day -1 (Fig. 3). To assess whether drug-induced effects were dependent on initial lipid levels at day -1, we examined the serum total and α-LP-CH concentration lowering effects of clofibrate during this time period (Fig. 4, panels A and B). From these data, the magnitude of the hypocholesterolemic effect of clofibrate (days +4 and +7) was found to vary directly with initial serum total- and α-LP-CH concentrations throughout the time period (Fig. 4, panels A and B).

Serum Total Cholesterol

In chow-fed rats, the mean serum CH concentration was 63.3 ± 0.8 mg/dl (± SEM; n = 129). Following clofibrate treatment, serum total CH concentrations were significantly lowered on days +4 and +7 in 3 out of 4 experiments (Table 1). In those experiments which showed a significant serum CH lowering effect by clofibrate at day +4, mean decreases of 30 and 50% were observed at 0.2 and 0.4 mmol/kg/day x 2, respectively. Only chlorochroman II produced a significant decrease in serum total CH concentrations on day +4 at both dose levels (Table 1). This hypocholesterolemic effect was absent on day +7.

α-Lipoprotein Cholesterol

The mean concentration of α-LP-CH was 40.7 ± 0.7 mg/dl (± SEM; n = 129), which accounts for 65% of the serum total CH. In general, changes in α-LP-CH concentrations

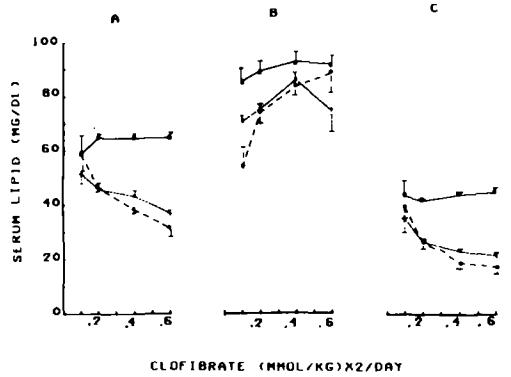

FIG. 2. Effects of oral administration of clofibrate on the serum total CH (panel A), serum total TG (panel B) and α-LP-CH (panel C) concentrations in drug-treated, fasted male Sprague-Dawley rats at day +4 (●----●) and +7 (●----●) compared to control serum lipid concentrations at day -1 (□——□). Data points are $\bar{X}$ ± SEM of 4 experiments (n = 23-24).

produced by clofibrate treatment were similar to those noted for serum total CH. By day +4, clofibrate reduced α-LP-CH by 35 and 58% at the 0.2 and 0.4 mmol/kg/day doses, respectively (Table 1). In 3 out of 4 experiments, clofibrate showed a dose-dependent α-LP-CH lowering effect only at day +4. On day +7, the percentage decrease of α-LP-CH at the 2 doses was 33 and 42%, respectively. Chlorochroman II significantly lowered α-LP-CH concentration only at day +4 of treatment whereas phenylchroman III increased α-LP-CH concentration at day +7 (Table 1).

Triglycerides

Treatment with clofibrate or chroman analogs caused no hypotriglyceridemic effect. However, when the amount of TG in each of the serum lipoprotein fractions was estimated

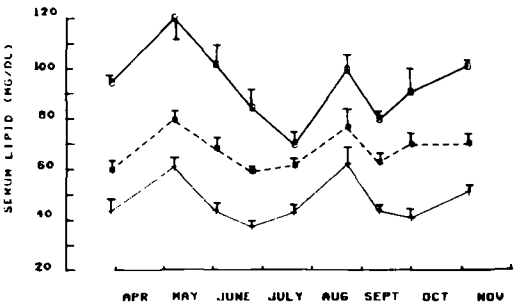

FIG. 3. Temporal changes in serum total TG (□——□), CH (*– – –*) and α-LP-CH (+——+) concentrations of normal, fasted male Sprague-Dawley rats. Each data point represents $\bar{X}$ ± SEM (n = 5-6).

as percentage of the serum total TG, a shift in the distribution of TG from the pre-β- to the β-position in the serum electrophoretic pattern was observed in clofibrate-treated rats. This change in the distribution pattern was also evident when densitometric scans from the control group were matched with the clofibrate-treated group. Results are expressed as a percentage of the day -1 value to minimize variation (Fig. 5).

α-LP-Cholesterol:α-LP Triglyceride Ratio

The α-LP-CH-to-α-LP-TG ratio for clofibrate-treated rats (0.4 mmol/kg/day x 2 at day +4) was significantly reduced by 67% (Fig. 6). Only chlorochroman II showed a similar effect (47% decrease, $t = 3.46$, $p<0.005$) at the same dose on day +4.

Serum Free Cholesterol and LCAT Activity

Clofibrate treatment (0.4 mmol/kg/day x 2) significantly decreased the serum LCAT activity and elevated serum free CH concentrations on day +7. Cyclohexylchroman IV also significantly lowered serum LCAT activity but did not affect serum free CH concentration (Table 2). By contrast, chlorochroman II did not modify these indices of CH metabolism.

Effects of Drugs on Liver Parameters

Liver/body wt ratios were significantly increased by clofibrate treatment for 7 days (at both doses). In general, clofibrate treatment had no significant effect on microsomal protein or liver CH and TG concentrations (Table 2). In one experiment, clofibrate at the 0.4-mmol dose increased liver TG and liver microsomal protein concentration. Effects of clofibrate on the activity of HMG-CoA reductase also were less consistent, although this drug reduced the activity of HMG-CoA reductase in 2 out of 4 experiments. Except for cyclohexylchroman IV, which decreased microsomal protein concentration at both doses, none of the analogs had a significant effect on any of these liver parameters.

DISCUSSION

Despite seasonal variations, we have shown that clofibrate treatment casued a consistent and dose-dependent lowering of serum total CH concentrations in fasted normolipidemic rats. Among the chroman analogs, only chloro-

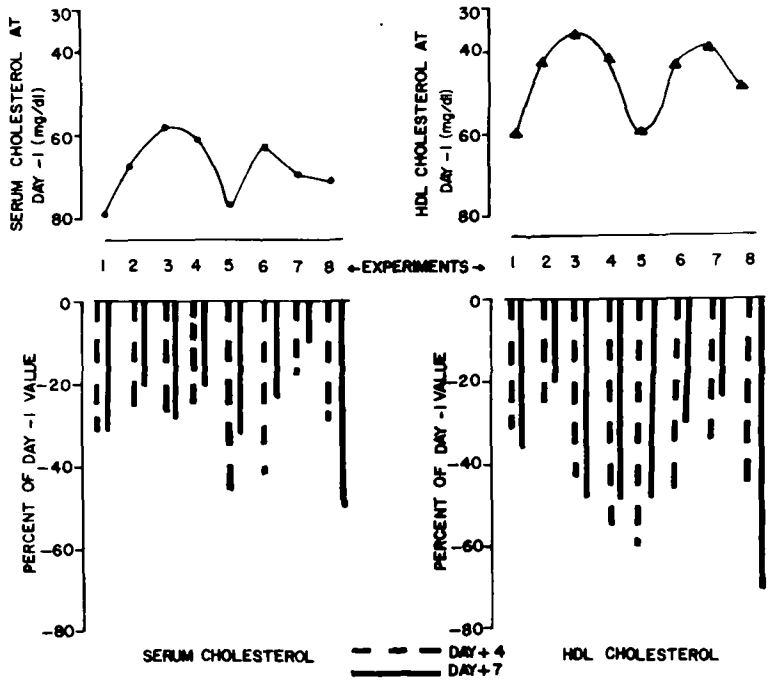

FIG. 4. Relationship between effects of clofibrate (panels A & B, bottom) and initial concentrations of serum total CH (panel A, top) and α-LP-CH (panel B, top) of fasted Sprague-Dawley rats. Data points 1-8 represent the mean value (n = 5-6) of replicate experiments conducted over an 8-month period.

TABLE 1

Serum Total Cholesterol and α-LP-Cholesterol in Male Sprague-Dawley Rats (expressed as % of day -1 value ± SD)[a]

	Serum total cholesterol		α-LP-cholesterol	
	Day +4	Day +7	Day +4	Day +7
Exp. 1				
Control	88.6 ± 7.4	89.1 ± 7.5	84.6 ± 9.1	87.1 ± 10.9
CPIB 0.2	58.1 ± 12.1[b]	57.5 ± 10.7[b]	54.8 ± 11.5[b]	51.9 ± 17.7[b]
CPIB 0.4	45.6 ± 5.8[c]	52.7 ± 8.2[c]	38.6 ± 7.1[c]	44.9 ± 9.1
Exp. 2				
Control	95.3 ± 8.5	90.9 ± 12.1	90.0 ± 6.1	84.0 ± 8.9
CPIB 0.2	69.4 ± 10.9[b,c]	70.4 ± 9.4[b,c]	61.4 ± 11.1[b,c]	64.5 ± 7.9[b]
CPIB 0.4	46.9 ± 6.5 [b,d,e]	66.5 ± 7.3[b,d]	30.7 ± 5.6[b,d]	58.9 ± 7.0[b,d]
Ph Chroman 0.2	98.8 ± 7.0	96.3 ± 8.5	100 ± 6.7	94.3 ± 9.8
Ph Chroman 0.4	103 ± 12.5[b]	101 ± 12.5[b]	102 ± 14.0[b]	98.0 ± 8.8[b,f]
Exp. 3				
Control	101 ± 6.4	91.2 ± 8.0	113 ± 10.1	107 ± 8.3
CPIB 0.2	73.7 ± 9.5[c]	62.6 ± 5.3[c]	71.5 ± 13.3	60.0 ± 12.7[c]
CPIB 0.4	60.9 ± 5.2[d]	58.6 ± 7.7[d]	52.8 ± 11.5[d]	51.1 ± 13.1[d]
Cx Chroman 0.2	104 ± 6.1[b]	102 ± 14.4[b]	106 ± 9.1[b]	103 ± 13.1[b]
Cx Chroman 0.4	105 ± 11.1	102 ± 5.5	107 ± 13.4	103 ± 11.1
Exp. 4				
Control	90.5 ± 11.8	87.8 ± 13.5	91.3 ± 11.9	95.8 ± 9.9
CPIB 0.2	74.2 ± 10.1[c]	77.8 ± 11.5	59.0 ± 12.0[c]	73.3 ± 17.4
CPIB 0.4	72.8 ± 11.1[d]	82.4 ± 17.4	39.7 ± 5.1[d]	60.8 ± 10.6[d]
Cl Chroman 0.2	74.3 ± 10.6	77.7 ± 12.3	80.1 ± 8.0	92.9 ± 9.6
Cl Chroman 0.4	65.4 ± 11.0[f]	83.2 ± 6.8	64.8 ± 11.4[f]	92.0 ± 7.8

[a]n = 6 unless otherwise noted.

[b]n = 5.

[c]$p<0.01$—control significantly different from CPIB 0.2.

[d]$p<0.01$—control significantly different from CPIB 0.4.

[e]$p<0.01$—CPIB 0.2 significantly different from CPIB 0.4.

[f]$p<0.05$—control significantly different from analog 0.4.

The standard error term used to calculate the critical values is 4.41.

chroman II showed the hypocholesterolemic effect. Chlorochroman II previously was shown to be hypotriglyceridemic and hypocholesterolemic in nonfasted normolipidemic and hyperlipidemic rats (3,17,18), but in the current study, II, like clofibrate, exhibited only a hypocholesterolemic effect. While clofibrate treatment significantly reduced the activity of liver HMG-CoA reductase, the chroman II treatment showed no significant alteration in the activity of this enzyme. Goldberg et al. (1) previously showed that the hypocholesterolemic effects of clofibrate and chroman analogs could be related to a concomitant reduction in the activity of liver microsomal HMG-CoA reductase. However, the results of the present study with clofibrate and chlorochroman II showed no such relationship between these parameters. Similarly, Cavestri et al. (2) found no relationship between the hypocholesterolemic action of clofibrate-related drugs and their effect on liver HMG-CoA reductase activity. Moreover, a positive correlation between the CH lowering effect of clofibrate and inhibition of CH synthesis at the step catalyzed by HMG-CoA reductase has only been observed in nonfasted rats receiving the drug incorporated in the diet (19,20) or receiving the drug at preselected times (21,22). In other studies, inhibition of liver microsomal HMG-CoA reductase activity in vitro was related to the presence of the drug at high concentration (>1 mM) (23). Because our experiments were done with fasted rats, the available plasma and liver drug concentrations likely were reduced significantly 16-18 hr following the last dose. Failure to detect inhibition of liver HMG-CoA reductase activity by chronic clofibrate (and possibly chlorochroman II) administration may be related to the predicted low drug levels in liver. This explanation gains further support because (a) the plasma $t_{1/2}$ for clofibrate in rat is 8-12 hr (24,25), (b) serum lipids after clofibrate treatment decreased in nonfasted rats, but did not change in 24-hr fasted animals with the drug withdrawn at the same time (26) and (c) serum clofibric acid concentrations are reduced

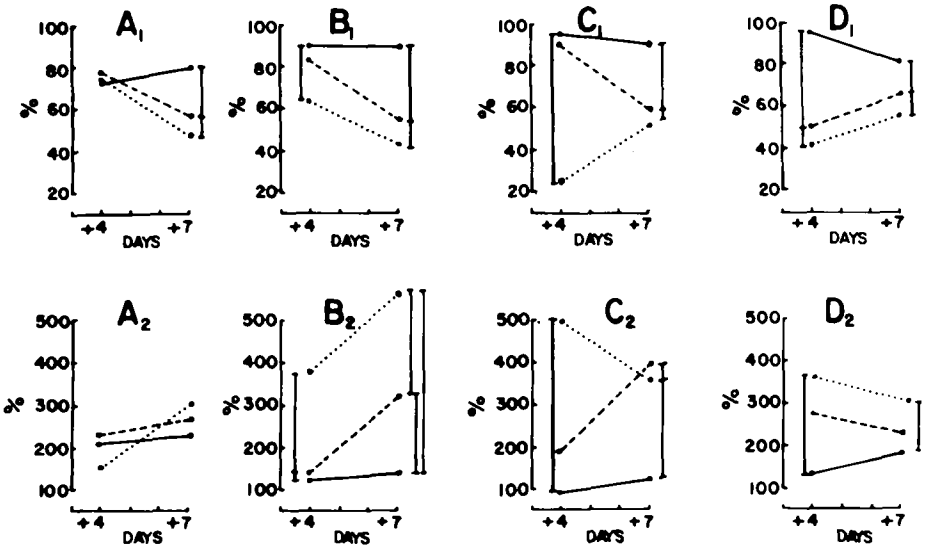

FIG. 5. Effects of clofibrate on pre-β-LP-TG (panels A_1, B_1, C_1, D_1) and β-LP-TG (panels A_2, B_2, C_2, D_2) of Sprague-Dawley rats. Values for control (• —•) and the drug-treated groups (•– – –•), 0.2 mmol/kg/day × 2 and •·····•, 0.4 mmol/kg/day × 2) represent ([pre-β-LP-TG] day +4 or +7/[TG] day +4 or +7) ÷ ([pre-β-LP-TG] day -1/[TG] day -1) × 100 and ([β-LP-TG] day +4 or +7/[TG] day +4 or +7) ÷ ([β-LP-TG] day -1/[TG] day -1) × 100 in panels A_1-D_1 and A_2-D_2, respectively. Each data point is the mean of n = 5-6. The vertical bars indicate that the values were significantly different at the level $0.0001>p<0.05$.

following 7 days of chronic administration (25,27). Because clofibrate or its chlorochroman analog had no reproducible significant effect on liver HMG-CoA reductase activity, these findings support the view that the drug-induced serum total CH lowering is associated with a modification of LP turnover rather than a specific inhibition of the rate-limiting step in the liver CH biosynthetic pathway (17).

Contrary to the decrease in serum total CH concentration following clofibrate treatment, serum total TG concentrations were essentially unaffected by this drug. Similar effects of clofibrate treatment were observed in young rats (3 months old, 200-250 g) whereas in older rats (6 months old, 500 g), clofibrate caused an increase in serum TG concentrations (28). Our findings are in agreement with those of Priego et al. (29) who also found serum total CH lowering but no change in serum TG concentrations of fasted rats given clofibrate for 4 days. While others have reported a lowering of serum TG concentrations in nonfasted, clofibrate-treated rats (30-33), these discrepancies can be explained by relating the dietary status of the animal to the presence or absence of hypotriglyceridemia due to drug treatment. For example, those results showing a serum TG lowering effect of clofibrate were mainly from experiments using nonfasted animals (3,30-34), whereas those using fasted animals (28,29), including the present one, showed no significant changes in serum TG concentrations in the animals treated with clofibrate-related drugs.

Few investigators have described any changes in distribution of serum lipids among LP classes in normolipidemic rats after treatment

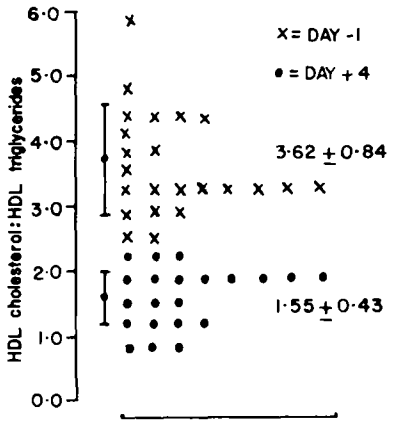

FIG. 6. Effect of clofibrate on the ratio of α-LP-CH (HDL-cholesterol) to α-LP-TG (HDL triglyceride) on day -1 and +4 of treatment with the 0.4 mmol/kg/day × 2 dose for 7 days.

TABLE 2

Effects of Treatment with Clofibrate and Chroman Analogs on Body Weight, Liver/Body Weight, Liver Triglyceride, Cholesterol, Microsomal Protein, Serum Free Cholesterol Concentrations, HMG-CoA Reductase, and LCAT Activities in Male Sprague-Dawley Rats[a]

Drug treatment	Doses (mmol/kg/ day X 2)	Final body wt (% of day -1)	Liver/body wt (%)	Liver triglyceride (mg/g)	Liver cholesterol (mg/g)	Microsomal protein (mg/g)	Serum free cholesterol (% of serum total cholesterol)	Hepatic HMG-CoA reductase (nmol/mg protein/ hr)	LCAT (nmol cholesterol esterified/ml/hr)
Control[b]	.25% methyl cellulose	104.9 ± 0.98	2.82 ± 0.07	2.33 ± 0.25	2.83 ± 0.16	25.7 ± 0.61	18.0 ± 0.64[c]	0.99 ± 0.21	43.5 ± 1.7[c]
Clofibrate[b]	0.2	104.9 ± 1.08	3.58 ± 0.11[d]	2.63 ± 0.32	2.82 ± 0.18	28.4 ± 1.20[e]	20.0 ± 0.98[c]	0.30 ± 0.08[g]	40.7 ± 2.6[c]
	0.4	103.9 ± 1.04	4.31 ± 0.11[d]	3.87 ± 0.71[e]	2.59 ± 0.09	28.1 ± 1.10[e]	24.7 ± 1.62[c,f]	0.26 ± 0.13[g]	34.5 ± 1.3[c,f]
Phenylchroman[h]	0.2	112.6 ± 1.35[i]	2.88 ± 0.06	1.06 ± 0.13	2.93 ± 0.24	32.5 ± 1.60		0.94 ± 0.38	
	0.4	112.0 ± 1.58[i]	2.89 ± 0.05	0.94 ± 0.18	2.51 ± 0.23	32.4 ± 2.90		1.91 ± 0.30	
Cyclohexyl-chroman[h]	0.2	104.7 ± 1.02	2.61 ± 0.06	0.97 ± 0.10	3.47 ± 0.36	9.0 ± 0.43[i]	15.1 ± 0.45	1.06 ± 0.19	19.8 ± 0.98[i]
	0.4	105.5 ± 0.73	2.71 ± 0.05	1.54 ± 0.32	3.82 ± 0.36	15.3 ± 3.3[i]	16.0 ± 0.73	0.93 ± 0.17	24.2 ± 2.32[i]
Chlorochroman[h]	0.2	102.7 ± 0.94	2.54 ± 0.07	3.34 ± 0.36	2.89 ± 0.06	22.5 ± 0.61	18.9 ± 0.57	0.38 ± 0.07	41.5 ± 2.37
	0.4	100.9 ± 1.47	2.63 ± 0.06	3.29 ± 0.33	2.85 ± 0.10	25.7 ± 1.80	18.0 ± 0.65	0.34 ± 0.05	46.0 ± 1.22

[a]Drugs and vehicle were given orally as 1 ml/kg. The rats were fasted overnight after the last dose before sacrifice. Rats were treated for 7 days.

[b]Pooled data of 4 experiments (n = 23-24); values represent mean ± SEM.

[c]Values represent mean ± SEM, n = 12.

[d]Significantly different from the corresponding control group in 4 out of 4 experiments.

[e]Significantly different from the corresponding control group in 1 out of 4 experiments.

[f]Significantly different from the corresponding control group in 2 out of 2 experiments.

[g]Significantly different from the corresponding control group in 2 out of 4 experiments.

[h]Values represent mean ± SEM, n = 5-6.

[i]Significantly different from the corresponding control and clofibrate groups.

Values at the level $0.0001 > p < 0.05$ were considered significantly different.

with hypolipidemic drugs (29). With a unique electrophoretically based assay for the TG distribution among LP, we have shown that only clofibrate shifts TG from pre-β-LP to β-LP.

Although the electrophoretic method for TG distribution showed more variation than that used for distribution of CH on lipoprotein, our observations on effects of clofibrate on TG distribution among the serum lipoproteins are consistent with those reported by Preigo et al. (29). They also observed a decrease in pre-β-LP with a simultaneous increase in β-LP examined using electrophoretic technique. Thus, it is possible that clofibrate administration produces an enhanced catabolism of pre-β-LP-TG and the increase in β-LP-TG could be interpreted as an index of the conversion of pre-β-LP to intermediate density LP.

The decrease in total serum CH produced by clofibrate and chlorochroman II was associated with a parallel decrease in α-LP-CH whereas phenylchroman III increased CH concentration in α-LP without a change in total serum CH. Segal et al. (17) have previously related the CH-lowering action of clofibrate in sucrose-fed rats to an inhibition of HDL synthesis. Further studies on LP catabolism are required to substantiate this mechanism of HDL-CH lowering by clofibrate and chlorochroman II in normal, chow-fed rats. We observed a decrease in CH/TG ratio in α-LP following clofibrate and chlorochroman II administration. This observation provides evidence for the proposal that these drugs induce a change in α-LP composition rather than in the number of circulating α-LP particles.

It is known that LP distribution patterns are markedly different in rat and man, and that α-LP is the major CH-carrying LP in rat (35). Thus, LCAT activity in the rat, but not in man, may be inversely related to CH removal from the serum compartment. Accordingly, we observed a significant reduction in LCAT activity following clofibrate administration which was positively correlated to the decrease in total serum and α-LP-CH. However, both the hypocholesterolemic chloro- and inactive cyclohexyl-chromans (II and IV) did not show a favorable correlation between LCAT activity, serum total and α-LP-CH concentrations. While lowered LCAT activity may be related to an increase in free CH available for exchange and removal from the rat, additional studies will be required to delineate the role of LCAT in LP-CH metabolism in this model.

Only clofibrate was shown to have any inhibitory effects on liver microsomal HMG-CoA reductase activity. With the exception of the effect of clofibrate and cyclohexylchroman IV on liver-to-body wt ratio, none of the other liver parameters were modified. Thus, these liver parameters do not appear to be related to the observed drug-induced hypolipidemic actions.

This investigation and the previous one (2) also revealed that clofibrate and chlorochroman II exhibited a hypocholesterolemic effect irrespective of dietary status (fasted or nonfasted) of the experimental animals. Although phenyl- and cyclohexyl- chromans (III and IV) were shown to be more effective in nonfasted normal rats (1,2) and Triton WR 1339-induced hyperlipidemic rats (3), they showed no effect in the present study with fasted animals. By contrast, only the chloro-substituted clofibrate and chlorochoman II, which have virtually identical log P values (36) and molecular weights, were hypolipidemic in all nonfasted or fasted models tested (1-3). Thus, data presented in this paper clearly indicate that factors such as seasonal variation and the dietary status of animals should be considered when hypolipidemic effects of drugs are evaluated.

ACKNOWLEDGMENT

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Components of Royal Jelly: I. Identification of the Organic Acids

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ABSTRACT

This present work characterizes the fatty acid constituents of the lipid fraction of royal jelly. Among the organic acids found after fractionation by thin layer chromatography of the corresponding methyl esters, the following compounds were identified by combined GC-MS: saturated and unsaturated linear fatty acids, saturated and unsaturated linear and branched dicarboxylic acids, mono- and dihydroxy acids. The most common characteristic of the organic acids was that most contained 8 or 10 carbon atoms, whether saturated or unsaturated, linear or branched.

INTRODUCTION

Royal jelly is a secretion from the hypopharyngeal (cephalic) and mandibular glands of worker bees (*Apis mellifera* L.) between 5 and 15 days old. This secretion is fed only temporarily (up to and no more than 3 days) to the brood of workers and drones but it constitutes the exclusive food of the queen bee for the entire span of both her larval and adult lives.

This secretion is a creamy, whitish, strongly acid material with a slightly pungent odor and taste. Because of its high protein content, it is believed to be synthesized mainly from pollen, with possibly a small addition of honey. Royal jelly is commonly regarded as one of the possible reasons for the considerable morphological and functional differences between queen bees and worker bees. Larvae from fertilized eggs fed on royal jelly evolve with a normal and extremely efficient sexual apparatus. Furthermore, queen bees feed during their whole life on this material and have a longevity of about 4 or 5 years. Worker bees live about 2-6 months and are fed as larvae with pollen and honey. Some researchers suggest that caste differentiation is due to juvenile hormones (1) but many aspects of this phenomenon still remain to be understood.

For 20 years or more, royal jelly has been used by humans, especially in dietetics and cosmetics, and has become a commercial product. The supposed restorative or even therapeutic properties of royal jelly are proclaimed by some and strongly contested by others. Neither of these 2 opinions, however, are supported by reliable experimental data.

In spite of the numerous studies beginning in 1852 (2) dedicated to the chemical composition of royal jelly which have resulted in the identification of the principal classes of components (3-15, with considerable differences among authors, however), knowledge in this field has remained scarce and fragmented, probably because most of the data were obtained without the aid of modern methods of instrumental analysis. Particularly deficient are data on the detailed composition of the various major nutrient classes which make up the substance: proteins, lipids and sugars.

The objective of this study was to characterize the fatty acid components in royal jelly. This has a certain practical importance because the jelly spoils easily and, at present, is not subject to any type of quality control.

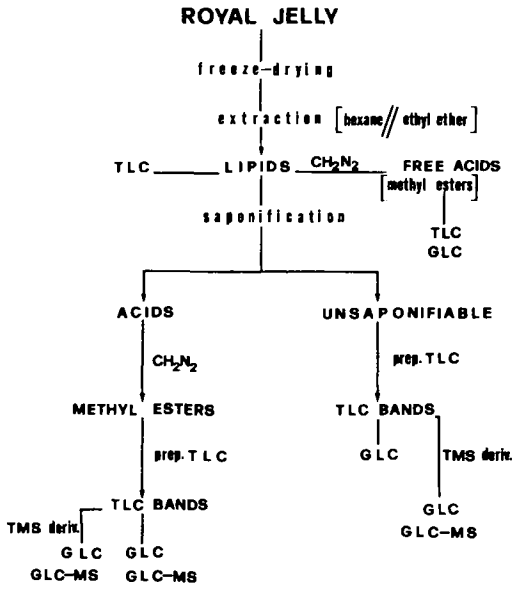
EXPERIMENTAL PROCEDURE

The royal jelly studied was collected from 5 different production areas in northern Italy and during 2 different periods in 1977 for each area.

Fractionation of the constituents was done according to Scheme I, in which the analytical procedures are also briefly indicated (16).

Separations with thin layer chromatography (TLC) were done on 0.25-mm-thick Stratocrom plates (Silica Gel G) with 1.5:1 (v/v) hexane/ethyl ether at 0 C. Analytical plates were sprayed with $H_2SO_4/K_2Cr_2O_7$ and charred for 20 min at 130-140 C; preparative plates were sprayed with 0.2% sodium 2,7-dichlorofluoresceinate in ethanol.

In general, gas liquid chromatography (GLC) was done using a glass column, 2 m × 3 mm id, filled with OV-225 at 1.5% on 100-120 mesh Gas Chrom Q. The injector and detector temperatures were both 250 C and the column temperature was 160 C; the carrier gas was N_2 with a flow rate of 35 ml/min. Comparison of the retention times of the acid components of the royal jelly as methyl esters and standard substances were also done with a metal polar column, 2 m long and 1/8 in. id, filled with



SCHEME I. Fractionation of the constituents of royal jelly.

15% DEGS on 80-100 mesh Gas Chrom P at 200 C (injector and detector temperatures at 250 C) with a nitrogen flow of 25 ml/min.

Infrared (IR) absorption spectra of the distinct bands collected with preparative TLC were obtained with a Perkin Elmer Model 177 in KBr. Preparation of trimethylsilyl derivatives (TMS) was done using the Sweeley et al. method (17).

Mass spectra (MS) were obtained at the Mass Spectrometry Center of the University of Bologna with an LKB Model 9000 GC-mass spectrometer. For each TLC band, mass spectra were recorded at the apices of the GLC peaks with electron beam energy of 70 eV.

Reduction with $LiAlH_4$ was done in dry ethyl ether followed by quenching with ethyl acetate and treatment with acidified water. Microhydrogenation (18) and determination of the position of the double bond (19) were done as described in previous studies. Reduction with $NaBH_4$ was done in methanol in the presence of a small drop of water followed by quenching with acidified water.

Synthesis of methyl 9-hydroxydecanoate was done on a microscale. Three ml of a 2 N ether solution of CH_3MgI was added slowly, under N_2, to 5 ml of an ether solution of about 5 mg of semiazelaic aldehyde, followed by aqueous acid work-up and TLC purification.

RESULTS AND DISCUSSION

The amounts of total lipid in royal jelly determined in this study are reported in Table 1 along with comparable data found in the literature (20-27). The differences observed are probably the result of both variability in the natural product and methods of analysis.

The lipid fraction is composed primarily of polar components; only a small hexane-soluble fraction was obtained (Scheme I). TLC of the total lipid fraction showed the presence of a considerable quantity of free acids. Separation of acidic from neutral substances (about 15% of the total lipids) gave a mixture of free organic acids. After methylation (CH_2N_2), the corresponding esters were determined by GLC with both a polar column (DEGS at 15%) and a nonpolar column (SE 30 at 1% and OV-225 at 1.5%). The latter produced the best separation of this complex mixture of methyl esters. Saponification of the total lipids gave 8-10% nonsaponifiables and an acid fraction that was methylated and analyzed by GLC as already described.

TABLE 1

Gross Composition of Royal Jelly

	Present work		Literature data				
		Mean	(20)	(21)	(22)	(23,24)	(25)
	%						
Moisture	56.6 ÷ 63.6	61.6	–	70.0	60 ÷ 70	66.0	68.4
Lipid extract[a]	3.4 ÷ 13.2	7.6	14.1	16.0	14.2	11.5	10.4
Sugar extract[a]	22.5 ÷ 40.8	29.4	20.0	25.0	27.0	–	40.8
Proteins (n X 6.25)[a]	16.9 ÷ 38.2	23.2	50.2	50.0	41.0	39.8	46.1
Number of samples examined		10	–	–	–	4	3

[a]On dry matter basis.

[b]The sole determination of lipid extract was reported by Townsend and Lucas, 10.0 ÷ 15.0% (26) and Haydak, 13.6 ÷ 16.1% (27).

Figure 1 shows a separation by TLC of the total lipids after methylation of the free acids present. The same conditions were used to separate the methyl esters of the acids obtained after saponification of the total lipids. Methyl esters of both free and total acids showed about the same GLC pattern (Fig. 2A); only peaks 16, 19 and 30 were much smaller.

Individual bands from TLC were eluted and analyzed by GLC. Figure 2, B-D, shows the GLC traces corresponding to single bands having the same functional groups as determined by IR.

Simple Fatty Acids

Using combined GLC-MS, the fatty acid methyl ester band (Fig. 2B) was found to be composed of methyl palmitate (peak 20) and octadecenoate (peak 30) along with small amounts of myristate, stearate and octadecadienoate. The IR spectrum of this band did not show the characteristic absorption of *trans* double bonds.

Dicarboxylic Fatty Acids

Figure 2C shows predominantly peaks 9, 13, 17 and 22, corresponding to methyl esters of dicarboxylic acids. Microhydrogenation (18) of the mixture of the 3 TLC bands, R_f 0.74, 0.69 and 0.60 (Fig. 1), showed that peaks 13 and 22 are due to unsaturated compounds corresponding to peaks 9 and 17, respectively.

The mass spectrum of peak 13 (Fig. 3A) confirms the hypothesis that this peak is produced by the monounsaturated acid of the corresponding peak 9, which was found to have the same mass spectrum as the dimethyl ester of a nonanedioic acid.

In the same way, the spectra of peaks 22 adn 17 (Fig. 3B) show that they originate from dicarboxylic acids having 10 carbon atoms, the first of which is monounsaturated and the second saturated.

A comparison of retention times indicated that peak 9 does not correspond to that of azelaic acid, which is also present (peak 10, Fig. 2C) as based on the behavior of an authentic standard.

The mass spectrum (Fig. 3A) suggests a branched methyl structure (28) for the 2 dicarboxylic acids with 9 carbon atoms. This is based on the presence of the fragments M^+-15 (m/e 199); M^+-15-32 (m/e 167) and M^+-31-18 (m/e 150).

The material for which the GLC trace is shown in Figure 2C is made up of 3 TLC bands (Fig. 1): the one having an R_f of 0.74 contains component 9, that with R_f of 0.60 is composed only of component 22 and that with an R_f of 0.69 is composed of sebacic and nonendioic

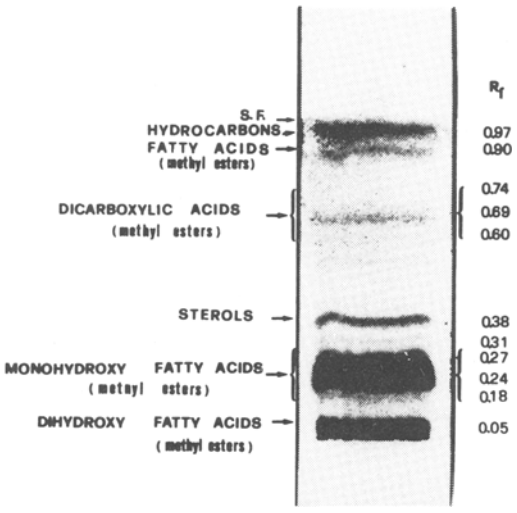


FIG. 1. TLC separation of total lipid extract after methylation with diazomethane: Silica Gel G plate, hexane/ethyl ether (1.5:1 v/v) at 0 C.

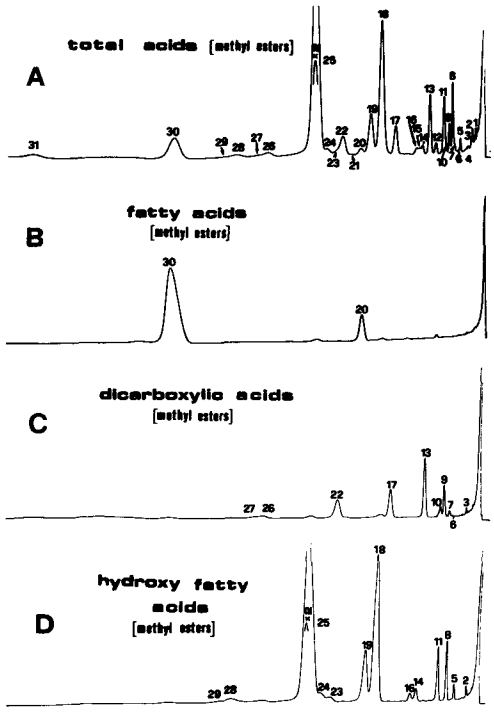


FIG. 2. GLC traces of principal TLC-bands of royal jelly acids shown in Fig. 1, on 2 m 1.5% OV-225 column.

acids (peaks 17 and 13 of Fig. 2C).

IR analysis of the bands with R_f = 0.69 and 0.60, which contain the 2 unsaturated compounds, shows the characteristic absorption of *trans* double bonds near electrophilic groups (10.22 μ).

Hydroxy Fatty Acids

The methyl esters of hydroxy fatty acids (Fig. 1, R_f 0.18 ÷ 0.31) consists of many compounds (Fig. 2D). The compound of peak 19 is transformed into that of peak 16 and the compound of peak 25 into that of peak 18 after microhydrogenation (18). IR spectra of this group of peaks showed absorption at 10.23 μ which indicates that at least part of the unsaturated components present have double bonds in the *trans* configuration. Mass spectra of the trimethylsilyl (TMS) ethers of the components of the mixture substantiated that peaks 19 and 25 are the monounsaturated counterparts of peaks 16 and 18, respectively. The spectra of the unsaturated compounds show fragments in the high mass range which differ from those of the saturated ones (Fig. 4) by 2 mass units. In all the mass spectra, the fragments at higher m/e values correspond to the loss of 15 mass units from the molecular ions, as confirmed by the presence of fragments M^+-31 and M^+-15-32, and by an ever-present intense metastable peak.

The synthesis of some ω-hydroxy esters by partial reduction of the methyl esters of available standard dicarboxylic acids (C_7, C_8, C_9 and C_{10}) with $LiAlH_4$, followed by purification with preparative TLC and comparison

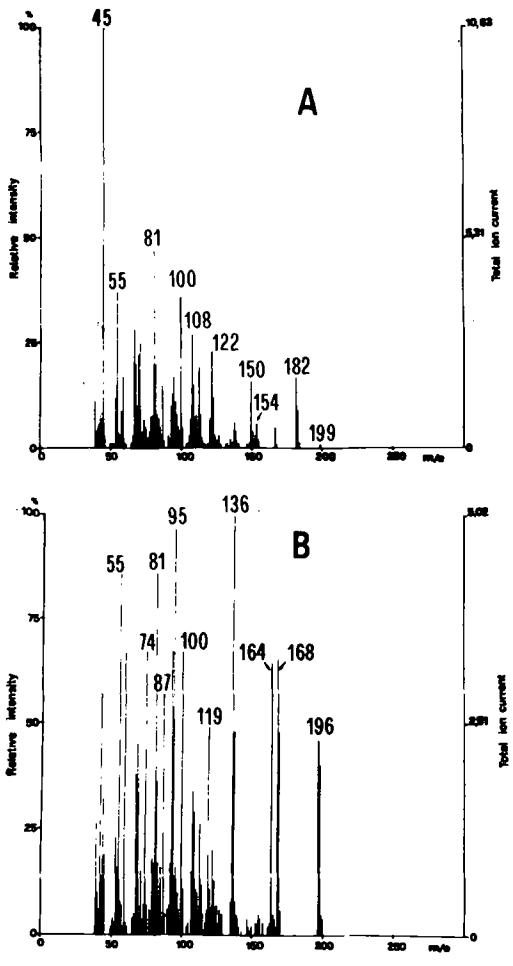


FIG. 3. Mass spectra of unsaturated dioic acids: (A) dimethyl methyloctendioate and (B) dimethyl decendioate.

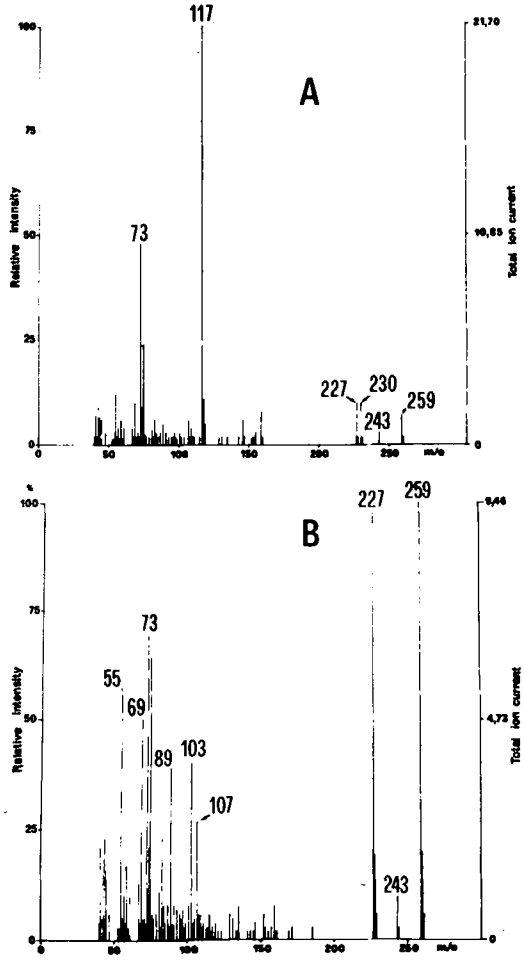
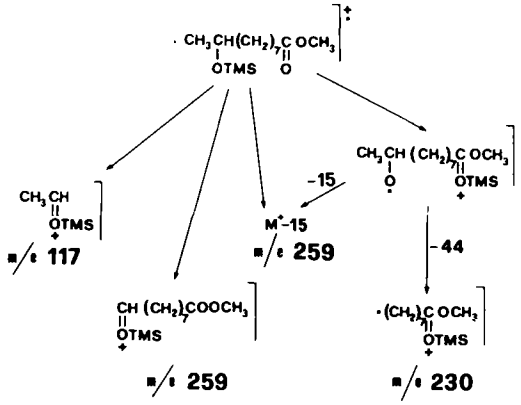


FIG. 4. Mass spectra of saturated hydroxy acids (TMS derivatives): (A) methyl 9-hydroxy decanoate and (B) methyl 10-hydroxydecanoate.



SCHEME II. Characteristic fragmentation shown in mass spectrum of GLC peak 16 of Fig. 2D.

of retention times and mass spectra of their trimethylsilyl derivatives, confirmed that peak 18 corresponds to methyl 10-hydroxy-decanoate and peak 14 to methyl 9-hydroxynonanoate. Consequently, peak 25 is methyl 10-hydroxy-decenoate.

The presence of fragments at m/e 117 and 230 (M^+-44) (Fig. 4A) in the spectra of peaks 16 and 19 of Fig. 2D, in addition to the fragments already considered, support the structure indicated in Scheme II for the saturated compound.

Synthesis of methyl 9-hydroxydecanoate from semiazelaic aldehyde and CH_3MgI, and its retention time and mass spectrum confirmed this compound as corresponding to peak 16 (Fig. 2D). As a result, peak 19 must be methyl 9-hydroxydecenoate.

Mass spectra of the TMS ethers (Table 2) of the diols prepared with the osmiate method (19) from the unsaturated esters corresponding to peaks 19 and 25 of Figure 3C indicate that the position of the double bond is between C_2 and C_3 for both compounds. The mass spectrum of the TMS derivative of the diol from 10-hydroxydecenoic acid, regarding fragmentation, is the same as that reported for the homologous 18-hydroxy-9-octadecenoic acid (29). The same result has been reported by others (3) and is confirmed by the shift of the IR absorption bands due to C=O (stretching) to lower frequencies in α,β-unsaturated esters. The mass spectra of peaks 5 and 11 (Fig. 2D) were similar to those of peaks 16 and 18 (Fig. 4, A and B), respectively. The difference of 28 mass units between the fragments at the highest m/e values, compared to the same fragments observed for the peaks already identified, indicate that the first 2 represent lower homologs of the last 2. On this basis,

TABLE 2

Mass Spectral Data of Some Hydroxy Components Present in Royal Jelly Lipids[a]

	Rrt[b]											Other significant peaks			
Methyl 3,10-dihydroxydecanoate	4.91	75	73	175	89	347	133	109	147	103	159	259	203		
		1000	850	620	470	380	240	238	230	170	130	125	80		
Methyl 3,11-dihydroxydodecanoate	5.33	75	285	117	73	175	89	273	247	44	375				
		1000	200	190	190	150	80	70	60	60	50				
Methyl 3,12-dihydroxydodecanoate	5.73	75	73	65	287	67	175	147	375	285	253				
		1000	180	120	95	90	70	70	60	40	20				
Methyl 3,13-dihydroxytetradecanoate	8.54	75	73	76	117	77	89	175	313	271	147				
		1000	160	78	62	60	52	27	26	20	23				
TMS derivatives of diols of the principal monounsaturated components (methyl esters)															
9-Hydroxydecenoate[c]		78	176	109	129	103	147	289	234	161	377	345	435		
		1000	100	100	90	85	85	80	80	85	20	15	3		
10-Hydroxydecenoate		73	234	78	75	129	65	103	109	289	147	435	303	345	
		1000	330	310	310	290	290	280	280	220	220	20	15	8	

[a]The first line shows the m/e value of the 10 most intense peaks; the second line reports their relative abundance.
[b]On 2 m 1.5% OV-225 column, with Rrt = 1.000 for 1,10-decandioic acid (see Table 3).
[c]m/e 73 (220), 77 (170), 75 (150), 74 (140), 89 (120) are not listed.

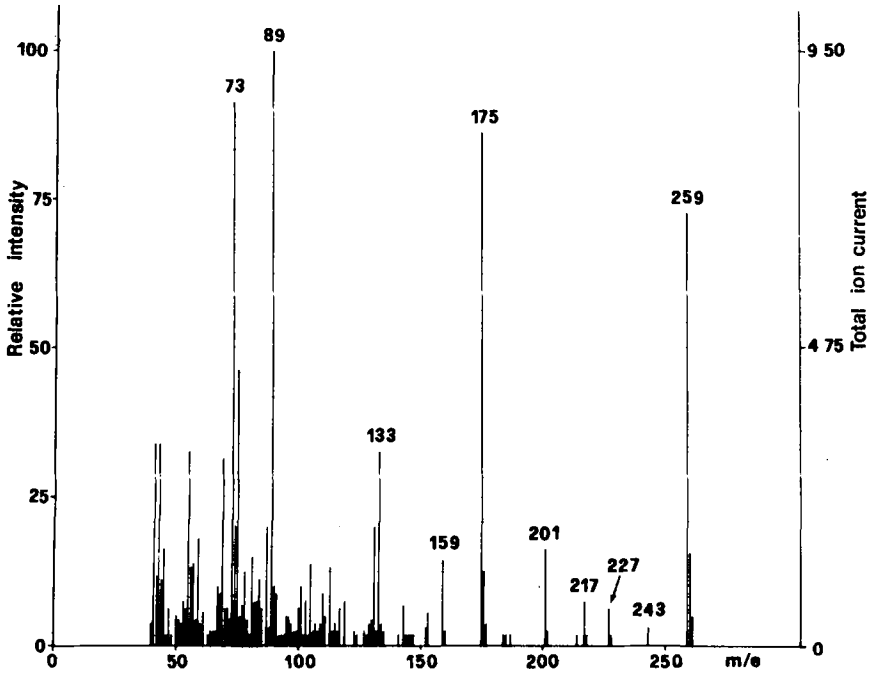


FIG. 5. Mass spectrum of GLC peak 8 of Fig. 2D (TMS derivative): mixture of methyl 3-hydroxydecanoate and methyl 6-hydroxydecanoate.

peak 5 represents methyl 7-hydroxyoctanoate and peak 11 represents methyl 8-hydroxyoctanoate.

In the mass spectrum of peak 11, studied as the TMS derivative, fragments are present at m/e 224, 209, 193 and 177. For an $M^+ = 224$, these correspond to the loss of 15, 31 and 15 + 32 mass units, respectively. The intensity of these fragments at high m/e values and their m/e in relation to the R_f on TLC (0.27), at which the analyzed bands are located, indicate that these fragments arise from a methyl ester of a hydroxy benzoic acid (TMS derivative). Comparison of the retention times of the 3 isomers confirms the presence of methyl *p*-hydroxybenzoate beneath the GLC peak of 8-hydroxyoctanoate, as also found in other studies (8).

The compound represented by peak 8 in Figure 2D has a mass spectrum (Fig. 5) for which the characteristics are similar to that of peak 18 (Fig. 4) but with the presence of fragments at m/e 217, 201, 175 and 159. These fragments could result from the overlapping of the mass spectra (as TMS derivatives) of 2 hydroxy ester isomers both with 10 carbon atoms in which the hydroxyl group is in position 3 for one compound and in position 6 for the other. Considering the fragments at m/e 217 and 175 as representative of the structure which maintains the -OTMS group and the $-COOCH_3$ group, as well as the number of carbon atoms corresponding to each isomer, and considering the fragments with m/e 159 and 201 as representative of the structure with only the -OTMS group, as well as the hydrocarbon chain, it was calculated (19) that the relative amounts of the 2 isomers were ca. 83% for the 3-hydroxydecanoic acid and 17% for the 6-hydroxydecanoic acid. The 3-hydroxydecanoic acid had already been identified by Weaver et al. (14).

The mass spectrum of compound 15 (present only in the methyl esters of the fatty acids obtained after saponification of the total lipids and present in a TLC band at $R_f = 0.42$) showed the same distribution of fragments as that in the spectra of dimethyl tere- and isophthalate. Comparison of retention times with those of authentic compounds indicated that peak 15 corresponds to methyl isophthalate.

The TLC band at $R_f = 0.05$ (Fig. 1) contained a few minor peaks by GLC (the most intense of these was 31 in Fig. 2A) for which the mass spectra, as TMS derivatives, were those of diol esters with 10, 12 and 14 carbon atoms (Table 2). In particular, the GLC peak 31 in Figure 2A was found to contain a small quan-

tity of the isomers 5,10-dihydroxydecanoic acid and 9,10-dihydroxydecanoic acid along with 3,10-dihydroxydecanoic acid, a substance already found in royal jelly by Weaver et al. (14).

The remaining identified peaks of TLC band with R_f 0.05 (Fig. 1) showing GLC retention times greater than that of peak 31 in Figure 2A (Table 2) were those corresponding to methyl 3,11-dihydroxydodecanoate, methyl 3,12-dihydroxydodecanoate and methyl 3,13-dihydroxytetradecanoate.

The results of our study are summarized in Table 3. The majority of the acids identified and present in appreciable quantities are those with 8 or 10 carbon atoms. This leads us to propose that these substances originate by degradation of fatty acids with 18 carbon atoms found in vegetable tissue, including pollen (30-33, and Lercker, unpublished data), rather than by direct biosynthesis. This hypothesis is supported by the almost complete absence of the fatty acids (free or combined) characteristic of pollen in the lipids of royal jelly.

On the basis of the sizes and structures found, we believe, in agreement with hypotheses expressed by others (14), that some acids can be intermediates in the biosynthesis of others. For example, it seems to us that ω-hydroxy acids may not be precursors of the dicarboxylic acids, but are their metabolic products. As a matter of fact, ω-hydroxy acids are the most abundant components of the lipid

TABLE 3

Identification of Acids Present in Royal Jelly

Peak	Rrt (OV-225)	Identification (methyl esters)	Previously reported compounds
1	0.12	Diester[b]	
2	0.15	Hydroxyester[b]	
3	0.16	Methylhexenedioate[d]	
4	0.20	Unknown[c]	
5	0.28	7-Hydroxyoctanoate	
6	0.31	Diester[b]	
7	0.35	Diester[b]	
8	0.37	3-Hydroxydecanoate + 6-hydroxydecanoate	14
9	0.41	Methyloctanedioate	
10	0.44	*n*-Nonanedioate	9[a]
11	0.47	8-Hydroxyoctanoate + *p*-hydroxybenzoate	14,8
12	0.55	Unknown[c]	
13	0.62	Methyloctenedioate[d]	
14	0.71	9-Hydroxynonanoate	
15	0.74	Isophthalate	
16	0.77	9-Hydroxydecanoate	7
17	1.00	*n*-Decanedioate	5
18	1.16	10-Hydroxydecanoate	13
19	1.27	9-Hydroxy,2-decenoate[d]	6
20	1.38	Palmitate	24[a]
21	1.49	Hydroxyester[b]	
22	1.58	*n*-Decenedioate[d]	5
23	1.64	Hydroxyester[b]	
24	1.75	Hydroxyester[b]	
25	1.88	10-Hydroxy,2-decenoate[d]	3
26	2.38	Diester[b]	
27	2.48	Methyltridecenedioate[a]	
28	2.72	11-Hydroxyundecanoate	
29	2.86	11-Hydroxydodecanoate	
30	3.39	Octadecenoate[e]	
31	4.91	3,10-Dihydroxydecanoate + 5,10-dihydroxydecanoate + 9,10-dihydroxydecanoate	14
32	5.33	3,11-Dihydroxydodecanoate	
33	5.73	3,12-Dihydroxydodecanoate	
34	8.54	3,13-Dihydroxytetradecanoate	

[a]Identification based on retention times (GLC).

[b]Identification based on polarity (TLC).

[c]Substances with netural structures present in the GLC trace of the lipid extract, without derivative formation by the diazomethane treatment.

[d]Double bond present, at least in part, in the *trans* form.

[e]Double bond present in the *cis* form.

fraction and it appears that these compounds are essential for the biochemical value of the royal jelly. It is known that ω-hydroxy acids have definite biological functions (34-36). Dicarboxylic acids may also be considered oxidation products of these compounds through the sequence: ω-hydroxy→ω-aldehyde→dicarboxylic compounds. Furthermore, the 3-hydroxy diols may be the precursors of the hydroxy acids with double bond in position 2 (14). These hypotheses must be subjected to experimental verification.

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The Influence of *trans*-Acids on Desaturation and Elongation of Fatty Acids in Developing Brain[1]

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ABSTRACT

trans-Monounsaturated acids account for up to 3% of the total octadecenoic acyl chains of human brain lipids. To investigate the influence of *trans*-acids on desaturation and chain elongation of fatty acids, in vitro and in vivo experiments with rat brain were performed. In the in vitro assays of Δ9 desaturation, Δ6 desaturation and chain elongation, *trans,trans*-dienoic acid was inhibitory, particularly to chain elongation. Slight differences between the inhibitory effects of *trans*-monoenoic acids and their *cis*-isomers were observed. In an in vivo model, unlabeled fatty acid (stearate, oleate, elaidate, linoleate, linoelaidate, arachidonate, or *trans*-monoene from margarine) was injected simultaneously with [1-^{14}C]linoleic acid into the brains of suckling rats. Linoelaidate and oleate inhibited desaturation and elongation of linoleate, whereas elaidate, stearate and *trans*-monoene from margarine were stimulatory. While the demonstration of differences between *cis* and *trans* monoenic isomers required relatively high levels of the test acids, it appears that *trans*-acids can influence desaturation and elongation enzymes that lead to acyl chain modification in the central nervous system.

trans-Unsaturated fatty acids are geometric isomers of the *cis*-acids found abundantly in nature. The double bonds of *trans*-acids are distinct from *cis*-double bonds produced by oxidative desaturation in mammalian and plant enzyme systems and impart different physical properties to the fatty acids. Because fatty acids with *trans*-bonds are not produced by body tissues, *trans*-acids have been described as unnatural, nonphysiological or foreign. However, these terms are not completely accurate as *trans*-acids are produced by rumen microorganisms and by chemical rearrangements during commercial hydrogenation of vegetable oils. Accordingly, *trans*-acids may be consumed through diets containing beef fat, milk fat, margarines and shortenings (1,2).

Ingested *trans*-acids are absorbed and incorporated into body tissues and fluids of experimental animals and humans. *trans*-Octadecenoate has been found in human liver, adipose tissue, heart, and milk at levels of 2-14% of the total fatty acids (1,3,4). The influence of these *trans*-acids on normal metabolism, particularly in humans, remains unclear (1,5). There are suggestions that *trans*-unsaturated fatty acids may not be innocuous in biological systems (6). Much of the evidence is indirect and somewhat controversial with respect to adverse effects that can be attributed directly or uniquely to *trans*-acids. However, studies have indicated that *trans*-acids may alter the activities of enzymes that influence the quality and quantity of complex lipids in the blood, and, in particular, may be associated with increased levels of circulating cholesterol (5). In addition, experimental models have implicated a relationship between levels of *trans*-acids and other physiological alterations (6).

While much attention has been given to the influence of *trans*-fatty acids on the circulatory system and organs such as liver and heart, which are metabolically active with fatty acids (5), little is known about what effects *trans*-acids might have on the developing nervous system. Previously, we reported the incorporation of *trans*-monoenoic (7,8) and *trans*-dienoic isomers (9), relative to their *cis*-isomers, into the complex lipids of rat brain. In this study, the presence of *trans*-acids in human brain is demonstrated. Also, the capacity of brain enzymes to desaturate and elongate *trans*-acids and the effects of *trans*-acids on desaturation and elongation of saturated and *cis*-unsaturated fatty acids by developing brain are described.

MATERIALS AND METHODS

Materials

[1-^{14}C]Stearoyl-CoA (53 mCi/mmol), [1-^{14}C]oleic acid (52 mCi/mmol), [1-^{14}C]*cis,cis*-linoleic acid (50 mCi/mmol), [1-^{14}C]*cis,cis,cis*-linolenic acid (52 mCi/mmol) and [2-^{14}C]-malonyl-CoA (40 mCi/mmol) were from NEN Canada. Lachine, Quebec. [1-^{14}C]Elaidic acid

[1] Portions of this study were presented at the meeting of the American Oil Chemists' Society and the International Society for Fat Research in New York, NY, April 1980.

Abbreviations used: FAME–fatty acid methyl ester; GLC–gas liquid chromatography; HPLC–high pressure liquid chromatography; TLC–thin layer chromatography.

(56 mCi/mmol) and [1-^{14}C] *trans,trans*-linoelaidic acid (15 mCi/mmol) were obtained by special order from Amersham Corp., Oakville, Ontario. [1-^{14}C] *cis,trans*-Octadecadienoic acid (50 mCi/mmol) was a generous gift from NEN Canada. The purity of *trans*-acids, determined using several analytical procedures, was described in detail previously (9). Nonradioactive fatty acids and lipids were obtained from Serdary Research Laboratories, Inc., London, Ontario. Coenzyme A, malonyl-CoA, ATP, NADH, NADPH and defatted bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Diazald (used in preparation of diazomethane), 2-bromoacetophenone, and crown-ether (18-crown-6) were obtained from Aldrich Chemical Co., Metuchen, NJ. All solvents were HPLC grade.

For brain preparations, Wistar rats of either sex from our inbred colony were used. Subcellular fractionation procedures have been described (10,11).

Analyses of Human Brain

Samples of human brain, obtained at autopsy, were stored at -70 C; in some instances, a white matter preparation was crudely dissected prior to freezing. Approximately 1 g of each sample was homogenized in 20 ml $CHCl_3$/CH_3OH (2:1, v/v). Butylated hydroxytoluene (5 μg) and margaric acid (1.2 mg as an internal standard) were added to each sample and the lipids were extracted by the procedure of Folch et al. (12). Lipid extracts were methylated with BF_3/CH_3OH (1:10, w/v) (13). Nonhydroxylated FAME were separated from hydroxylated FAME by TLC on Silica Gel G plates developed in petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v) using authentic standards to identify these 2 fractions. FAME were extracted from the gel with diethyl ether and treated by the method of Farquhar (14) to remove dimethylacetals derived from plasmalogens. Aliquots of the FAME were directly analyzed by capillary GLC.

GLC was performed on a Perkin Elmer Model 900 gas chromatograph equipped with flame ionization detectors. The GLC was coupled to a linear recorder and an automatic digital integrator (Model CR5-208, Infotronics Corp., Austin, TX). A 50 m x 0.25 mm stainless steel capillary column coated with Silar 7-CP liquid phase was used with a helium pressure of 40 psi. The temperature program was 170 C for 20 min followed by an increase to 185 C at 32 C/min. Peaks were identified by comparison with authentic standards. Under these conditions, the relative retention times, expressed as the ratio of peak retention time to that of palmitate (6.2 min), were as follows: 18:0–1.93; *trans*-18:1(n-9)–2.13; *trans*-18:1-(n-7)–2.18; *cis*-18:1(n-9)–2.24; *cis*-18:1(n-6)–2.27; *trans,trans*-18:2(n-6)–2.60; *cis,cis*-18:2-(n-6)–2.74. To further verify the identity of the *trans*-monoene, aliquots of the FAME mixture were fractionated by TLC on Silica Gel H impregnated with 20% $AgNO_3$ (w/w) and activated for 2 hr at 110 C. Plates were developed twice in toluene, and the fractions containing saturated (R_f=0.80), *trans*-monoenoic (R_f=0.76), and *cis*-monoenoic (R_f=0.65) FAME were extracted. Each was analyzed by capillary GLC before and after hydrogenation over palladium chloride catalyst. Hydrogenation totally converted the *trans*-monoenoic fraction to 18:0.

Desaturation and Chain Elongation Assays

Desaturation of [1-^{14}C]labeled fatty acid substrates was determined using incubation conditions previously described (10,11). Briefly, 50 μM [1-^{14}C]substrate, suspended by sonication in 0.05% Triton WR-1339, was incubated in phosphate buffer at pH 7.4 with 200 μM CoA, 2 mM $MgCl_2$, 2 mM ATP, 500 μM NADH and 2.4 mg homogenate protein for 4-day-old rat brains in a final vol of 1.0 ml. After 15 min, the reaction mixture was subjected to saponification and acidification and the fatty acids were extracted.

Aliquots of the fatty acids were derivitized to form bromophenacyl esters (15), and ca. 20,000 cpm of total esters were separated by HPLC. Analysis was on a Spectra Physics 8000 HPLC equipped with a variable wavelength UV detector set at 254 nm. A 25 cm x 4.6 mm column packed with 10 μM LiChrosorb (RP-18) was used with a solvent program of 90% acetonitrile in H_2O to 100% acetonitrile during the first 10 min followed by a period of 30 min at 100% acetonitrile at a flow rate of 1.2 ml/min. Between each run, the column was flushed 5 min with 50% acetonitrile in H_2O and then equilibrated with the initial solvent mixture for an additional 10 min. Fractions were collected at 1-min intervals from 0-30 min and quantitated by scintillation counting in Beckman HP scintillation fluid. Routinely, >95% of the radioactivity applied to the HPLC column was recovered. In most instances, HPLC analyses were confirmed by methylating an aliquot of the fatty acid extract with etherial diazomethane and subjecting the FAME to argentation TLC (9).

Assay conditions for determination of chain elongation of various fatty acids have been described (16, and H.W. Cook, submitted for publication). Briefly, unlabeled acceptor sub-

strates (e.g., 18:3[n-6], 16:0, *cis*-18:1[n-9], *trans*-18:1[n-9], 18:0) suspended in Triton WR 1339, were incubated with [2-^{14}C]malonyl-CoA, essential cofactors and 1.2 mg microsomal protein from the brains of 18-day-old rats. After 20 min of incubation, the reaction mixtures were subjected to saponification and acidification, and fatty acids were extracted. An aliquot of the labeled fatty acids was quantitated by liquid scintillation counting and the remainder was methylated with etherial diazomethane. Labeled FAME were separated by GLC on a 2 m × 0.64 cm column packed with 10% Silar 9-CP on GasChrom Q; the effluent was split and 80% of the separated esters was subjected to analysis using a radioactive flow monitor attached to an integrator and a recorder (17).

For inhibitor studies, each fatty acid was suspended in Triton WR 1339 detergent so that the final detergent concentration was 0.1% in all incubations.

in vivo Alteration of Desaturation and Elongation

For in vivo experiments, 3 μCi [1-^{14}C] *cis,cis*-linoleic acid (50 nmol) was suspended by sonication in 5% defatted bovine serum albumin with 750 nmol of nonradioactive fatty acid. The mixture of substrate and inhibitor was injected intracerebrally into 10-day-old rats. After 24 hr, brain lipids were extracted and the total phospholipid fraction was transesterified in BF_3/CH_3OH (1:10, w/v). Individual FAME were quantitated by radio-GLC. Details of the analytical procedures have been described previously (9).

A *trans*-monoene fraction was obtained from the total FAME of a commercial margarine by 3 sequential chromatographic separations on argentation-TLC using the conditions already outlined for separation of *trans*-monoene of human brain. The *trans*-octadecenoate content of the total margarine extract was 21.6%, and that of the final *trans*-monoene fraction was >94% (the remainder was *cis*-18:1 and 16:0), as determined by capillary-GLC of the FAME. FAME fractions were saponified and converted to the acid form before use.

RESULTS

trans Acids in Human Brain

The *trans*-octadecenoic acid content of human brain was determined for 5 samples obtained at autopsy (Table 1). *trans*-18:1 was a component of the total lipid and white matter lipids of all samples examined. Levels ranged from 0.5-3.0% of the total octadecenoic acid and represented 0.1-0.5% of the total esterified fatty acid. In this sampling, it was not possible to show significant relationships between *trans*-18:1 content and sex, age or white matter vs whole brain lipid. Combined argentation-TLC and capillary GLC techniques indicated that several positional isomers of *trans*-octadecenoate were present.

trans,trans-Octadecadienoic acid or *trans*-acids of chain length greater than 18-carbons were not detected in these samples.

TABLE 1

trans-Octadecenoic Acid Content of Total Lipid and White Matter of Human Brain Samples

Brain sample		*trans*-Octadecenoate	
Age	Sex	Total octadecenoate (%)	Total methyl esters (%)
8 mo	M	0.68	0.12
6 yr	F	2.96	0.46
54 yr	M	1.55 (0.84)[a]	0.32 (0.21)
81 yr	F	0.56	0.15
84 yr	M	1.26	0.29
Mean ± SD		1.40 ± 0.96	0.27 ± 0.14

[a]Value in brackets is for white matter for the same brain.

TABLE 2

Relative Rates of in vitro Desaturation of *cis* and *trans* Fatty Acids by Brain Homogenate of 4-Day-Old Rats

Fatty acid substrate	Relative rate of desaturation
cis,cis-18:2	1.00[a]
cis, trans-18:2	0.27
trans, trans-18:2	0.18
cis-18:1	0.03
trans-18:1	0.03

[a]Desaturation activities are compared to a rate for *cis,cis*-18:2 of 82 pmol product formed/min/mg protein.

Desaturation and Elongation of *trans*-Acid by Brain

The ability for brain homogenates and microsomes from 10-day-old rats to desaturate *cis*- and *trans*-isomers was measured using in vitro conditions. Desaturation of the dienoic isomers containing *trans*-double bonds (Table 2) was less than for *cis,cis*-diene but was clearly detectable with activities for the dienes of 15-25 pmol of product formed/min/mg of protein. The *trans*-monoenic acid, similar to its *cis*-isomer, was a poor substrate for desaturation by rat brain microsomes.

Chain elongation of *trans*- and *cis*-acids was also determined under optimal in vitro condi-

tions established for elongation of γ-linolenic acid. Comparisons with γ-linolenic acid (Fig. 1) indicate that both the *trans*-monoenic and dienoic acids were elongated, but at relatively low levels.

Effect of *trans*-Acids on Desaturation and Elongation

The relative inhibition of Δ9 desaturation of stearoyl-CoA and Δ6 desaturation of α-linolenic acids by various competitor acids in vitro is shown in Table 3. Both Δ9 and Δ6 desaturation were more inhibited by the *trans*-monoene, elaidic acid, than by the *cis*-monoenoic isomer, oleic acid, but the difference was slight. *trans, trans*-Dienoic acids was appreciably more inhibitory than the monoenoic isomers, particularly for desaturation of α-linolenic acid, but was less inhibitory than its *cis,cis*-isomer.

The effects of *cis*- and *trans*-acids on chain elongation of γ-linolenic acids also were investigated in vitro (Table 4). The 2 positional isomers of *trans*-octadecenoic acid, *trans*-18:1(n-9) and *trans*-18:1(n-7), altered elongation to a similar extent, and the inhibition was intermediate to that of their *cis*-isomers. The *trans, trans*-18:2 was considerably more effective as an inhibitor of elongation than was *cis,cis*-18:2.

Using an in vivo model, individual unlabeled fatty acids were injected simultaneously with [1-^{14}C]*cis,cis*-linoleic acid into the brains of 10-day-old rats. After 8 or 24 hr in vitro, little alteration of the normal desaturation and elongation of linolenic acid was seen with 110 nmol competitor-acid (data not shown). With 750 nmol competitor, effects were observed at 24 hr (Fig. 2). The *trans,trans*-18:2 significantly decreased formation of arachidonic and docosapentaenoic acids compared to the control with unlabeled *cis,cis*-18:2 administered at an equivalent level (indicated by the dashed line at 1.0 in Fig. 2). On the other hand, elaidic acid, *trans*-18:1(n-9), significantly stimulated linoleate conversion, behaving more like the saturated acid, stearic acid, than its *cis*-isomer, oleic acid. Similar stimulatory effects on linoleate metabolism were observed with the *trans*-monoene fraction isolated from a commercial margarine.

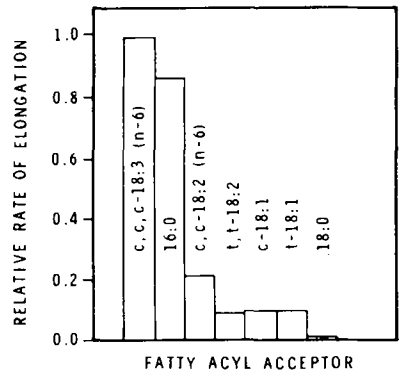

FIG. 1. Comparison of the relative rates of chain elongation of saturated, *cis* and *trans* fatty acids by brain microsomes from 18-day-old rats. Conditions for assay are described in Methods. The rate for 18:3(n-6) was 254 pmol product formed/min/mg protein.

DISCUSSION

Until recently, a conclusion reached by McConnell and Sinclair (18) that *trans*-acids are selectively excluded from brain membrane lipids has been widely accepted. This conclusion was based on their study of the relative extent of incorporation of *trans*-acids into brain, liver and muscle of rats following long-term feeding of diets high in elaidic acid. Kaufmann and Bandyopadhyay (19) concluded from analyses of human brain obtained at autopsy that brain lipids remain free of *trans*-fatty acids; however, their data showed that *trans*-acids actually represented 0.05-0.8% of the total fatty acids of brain lipids. It is clear also from our analyses that *trans*-acids are not excluded from human brain. We found that, in all samples of human brain examined, *trans*-acids accounted for 0.5-3.0% of the total octadecenoic acid in brain lipids.

TABLE 3

Relative Inhibition of Desaturation of Stearoyl-CoA or Linolenic Acid by Various Fatty Acids in Brain Preparations from 4-Day-Old Rats

Fatty acid tested as inhibitor	Concentration that decreased desaturation by 50%[a]	
	Stearoyl-CoA as substrate	Linolenic Acid as substrate
18:0	206	433
cis-18:1(n-9)	282	532
trans-18:1(n-9)	244	526
cis,cis-18:2(n-6)	84	95
trans,trans-18:2(n-6)	137	176
cis,cis,cis-18:3(n-6)	185	96
cis,cis,cis-18:3(n-3)	422	726
Sterculic acid	28	515

[a]The concentration (μM) required to decrease the rate of desaturation to 50% of optimal rate was determined by linear regression analysis of exponential curves of the data for each fatty acid. All values were the average of duplicate determinations and each regression curve included 5 concentrations of inhibitor. Observed values are relevant only to the specific concentrations of substrate used in these experiments, i.e., 50 μM stearoyl-CoA and 25 μM linolenic acid. A lower value indicates greater inhibition.

In the past, some confusion may have been resulted from direct comparisons of brain with other tissues that are generally more active metabolically and exchange fatty acids more readily. It is now apparent that fatty acid metabolism and turnover in brain is generally slower than that in other tissues, particularly in adult animals (20). Also, although the central nervous system may be more selective than other organs in the utilization of circulating fatty acids, this does not indicate total exclusion of dietary *trans*-acids. Such a conclusion is further supported by our previous studies (7-9), and those of Karney and Dhopeshwarkar (21,22), which showed that both monoenoic and dienoic *trans*-acids are not prevented from entering the brain by the blood-brain barrier. They are incorporated into brain lipids at rates similar to their *cis*-isomers.

Rapid metabolism of saturated, monoenoic and essential fatty acids occurs in the central nervous system, particularly at early stages of brain growth and myelination during the first few weeks of life (10,11). As *trans*-acids can be components of brain lipids, they could influence enzyme systems involved in desaturation and chain elongation in this tissue. Our present in vitro experiments and previous in vivo experiments (7-9) with developing rat brain have shown that fatty acids with *trans*-bonds in the same position as the predominant *cis*-acids are relatively poor substrates for desaturation and chain elongation. It seems unlikely that longer or more unsaturated acids derived by desaturation or chain elongation of *trans*-acids in brain will become major quantitative components of brain membranes. On the other hand, all the *trans*-acids tested do associate, to some extent, with the desaturation and elongation enzymes of brain. Our results indicate that, while they are not highly reactive substrates themselves, *trans*-acids, particularly *trans,trans*-octadecadienoic acid, can influence the reaction of other fatty acids and thus effect desaturation and chain elongation of substrates normally used in brain.

This type of interaction by *trans*-acids and possible interference with normal desaturation and chain elongation processes is not without precedent. Previous studies with rats fed diets deficient in essential fatty acids have shown that *trans*-isomers of octadecadienoic acid cannot substitute for the essential fatty acids to prevent development of, or recovery from, the deficiency (23-25). *trans*-Acids appear to enhance signs of deficiency (26), suggesting that *trans*-acids may interfere with metabolism of the decreasingly low levels of essential fatty acids available in diet-restricted animals. Indeed, the inhibition of metabolism of essential fatty acids by a wide variety of *cis*- and *trans*-unsaturated acids has been demonstrated in vivo (27) and with rat liver microsomes in vitro (28-30). The effects of positional isomers of *trans*-octadecenoic acid on the desaturation of palmitate, linoleate and eicosatrienoate by rat liver microsomes have been described (31). As demonstrated in other experimental model (5), the position of the *trans*-double bond in the acyl chain has a marked influence on its ability to interfere with normal metabolic processes. The *trans*-monoenoic acid, elaidic acid, and the total *trans*-monoenoic fraction of margarine were not inhibitory in our in vivo model; nevertheless, in view of the effects in extracerebral systems, it is possible that a more specific examination of other positional isomers of *trans*-acids could reveal those that are more inhibitory than the ones that we have tested.

In conclusion, the *trans*-acids tested in our experimental systems are capable of altering the metabolism of fatty acids, particularly of the essential fatty acids. While the concentrations required to demonstrate effects with all fatty acids (*trans*, *cis* or saturated) were high relative to the substrate concentration, and may not be directly compared to the physiological state, differences between *cis*- and *trans*-isomers were evident. Moreover, in the physiological state, other factors might further amplify their

TABLE 4

Effects of Various Fatty Acids as Competitors of Chain Elongation of 18:3(n-6) by Brain Microsomes from 18-Day-Old Rats

Competitor acid	Concentration that decreased elongation by 50%[a]
18:0	2200
cis-18:1(n-9)	39
cis-18:1(n-7)	525
trans-18:1(n-9)	299
trans-18:1(n-7)	304
cis,cis-18:2(n-6)	278
trans,trans-18:2(n-6)	74
cis,cis,cis-18:3(n-6)	125
cis,cis,cis-18:3(n-3)	88

[a]The concentration (μM) required to decrease the rate of elongation to 50% of optimal rate was determined from linear regression analyses of exponential curves of data for each fatty acid. Each regression curve included 5 concentrations of inhibitor. All values used in regression analyses were means of duplicate determinations except for *cis*-18:1(n-7) and *trans*-18:1(n-9) where 3 separate experiments were done independently and the values from each were averaged before regression analysis. The mean correlation coefficients for these analyses was 0.96. These values are relevant only to 50 μM of the test substrate, 18:3(n-6).

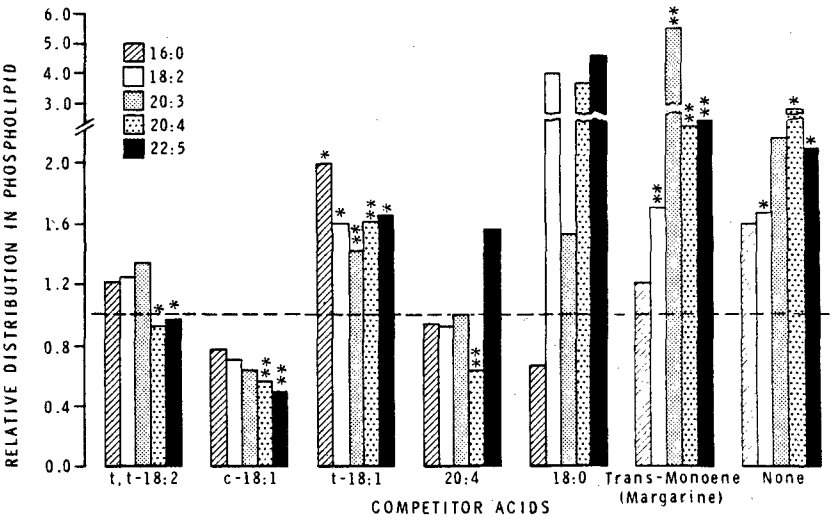

FIG. 2. Effects of competitor-acids on the metabolism of linoleic acid in brain of 10-day-old rats in vivo. The mean value of each competitor-acid is compared to that obtained when nonradioactive *cis,cis*-18:2 was added in the same amount (750 nmol); i.e., the value for each labeled fatty acid produced from [1-^{14}C] linoleic acid under control conditions was set at 1.0 as indicated by the dashed line. Each bar represents the mean value for 4 experiments, except for 18:0 (stearate), for which only 2 separate determinations were done. Significant differences from control values are indicated by * for $p<0.05$ and ** for $p<0.01$.

inhibitory potential. For example, the relative proportion of different positional *trans*-isomers crossing the blood-brain barrier, the extent of their esterification to complex lipids vs further metabolism and degradation, the position of esterification in the phospholipid, and their relative release and turnover from phospholipids may be important factors. At this point, it has not been demonstrated that *trans*-fatty acids are, in fact, significantly deleterious to the development or maintenance of central nervous system composition and function. Nonetheless, it does appear that *trans*-acids have properties that warrant further attention. We should continue to assess the desirability of *trans*-acids as dietary components, particularly with regard to possible subtle effects they may have on the formation and maintenance of crucial membrane structures in such a vital system as the central nervous system.

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METHODS

Rapid Fractionation of Human High Density Apolipoproteins by High Performance Liquid Chromatography

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ABSTRACT

A simple and rapid fractionation procedure (30 min) has been developed for the isolation of the major apoproteins from human serum high density lipoproteins by molecular sieving in a high performance liquid chromatographic column. Apo A-I, apoA-II and the C peptides are quantitatively resolved up to a protein load of 3 mg. The technique has also been successfully applied to the final purification of A apoproteins which had been isolated by conventional chromatographic procedures and as a sensitive analytical tool for assessing apoprotein purity.

ABBREVIATIONS

HDL, high density lipoproteins; apoA-I and apoA-II, 2 major apoproteins of human HDL; C peptides, low-molecular-weight apoproteins of HDL occurring in minor proportions; HPLC, high performance liquid chromatography.

INTRODUCTION

The procedures which are in common use to isolate on a preparative scale the apoproteins of HDL utilize either molecular sieving (1) and/or anion exchange (2,3) column chromatography. Although large quantities of proteins can be isolated, these procedures are time-consuming (2-3 days) and structural alterations of the apoproteins must be carefully monitored. In an attempt to achieve both rapidity of fractionation and good resolution, we have applied the technique of HPLC to separate apoproteins obtained by delipidation of human serum HDL. The results of these studies are the subject of this communication.

MATERIALS AND METHODS

The protein moiety of HDL (apo HDL) was obtained from human serum HDL of d.1.063-1.21 g/ml by delipidation with ethanol/ether (4). Standard apoA-I and apoA-II were prepared by molecular sieve and ion exchange chromatography in 8 M urea as previously described (1-3).

High Performance Liquid Chromatography

The studies were done in a Varian (Varian Instruments Co., Sunnyvale, CA) LC 5000 chromatography unit equipped with a Varian UV-50 variable wavelength detector. Two analytical columns (0.8 × 300 mm) Micro Pak TSK gel 3000 SW, for high performance gel filtration chromatography, manufactured by Toyo Soda Co., Japan, were purchased from Varian. These 2 columns were connected in series and were preceded by a guard column (0.75 × 100 mm), Micro Pak TSK GSWP. The analytical columns were purchased prepacked with a rigid, spherical porous silica gel with a particle size of 10 μm and a molecular weight exclusion limit of 1.5×10^5. The guard column contained the same type of packing material but of a lower molecular weight exclusion limit, about 2×10^4. The mobile phase used throughout this study was a 6 M urea solution adjusted to pH 3.15 with formic acid. Urea was recrystallized before use and then dissolved in deionized and doubly distilled water. All solutions were freshly filtered through a 0.4-μm polyester filter (Nuclepore Co., Pleasanton, CA). The columns were equilibrated with 6 M urea, pH 3.15, for 4 hr at a flow rate of 0.5 ml/min prior to the first protein sample injection. A flow rate of 0.5 ml/min at a pressure of 34 atm and 26 C was used during apoprotein fractionation. Selected fractions were pooled and dialyzed at 4 C against 10 mM NH_4HCO_3. The purity of the isolated protein fractions was ascertained by polyacrylamide gel electro-

phoresis in the presence of sodium dodecyl sulfate and 8 M urea (5) by immunoprecipitin methods using monospecific antibodies against each apoprotein and by amino acid analysis of protein hydrolysates in a Durrum Model 502 amino acid analyzer (Dionex Co., Palo Alto, CA). Protein content was determined by the Lowry procedure (6).

RESULTS AND DISCUSSION

The fractionation of apo HDL is depicted in Figure 1. Essentially 4 peaks were obtained. The first, a bimodal peak, eluted between 9 and 11 ml. Based on electrophoretic analysis (Fig. 1, gel a), this peak was interpreted as being composed of an aggregate of apoA-I and at least 2 additional unidentified proteins. The second and third peaks eluted at 11.8 and 14.3 ml and were identified as apoA-I and apoA-II, respectively. This identification was based on: (a) the elution volumes of standards of apoA-II and apoA-I chromatographed individually on identical HPLC columns (Fig. 2A and B); (b) the appearance of a single immunoprecipitation line when the 11.8- and 14.3-ml eluates were separately reacted against anti-apoA-I and apoA-II, respectively; (c) the amino acid analysis of these 2 fractions which was found to be in accord with published data on apoA-I and apoA-II; and (d) each fraction migrated in the position of apoA-I and apoA-II standards when subjected to electrophoresis on polyacrylamide gels in 8 M urea or 0.1% sodium dodecyl sulfate (Fig. 1, gels b and c). In all samples analyzed, although in different amounts, there was a shoulder in the descending portion of the apoA-II peak that eluted at 15.5 ml. This component was resolved from apoA-II upon rechromatography on the same column (Fig. 1, inset). Currently, this protein is under characterization. The fourth and last peak (Fig. 1) eluted at 17.5 ml and was found to contain the whole of the C peptides based on the electrophoretic behavior of the peak in polyacrylamide gels containing 8 M urea and sodium dodecyl sulfate (Fig. 1, gel e). We are now studying the fractionation of the various components of this peak by HPLC using ion exchange columns. An approach using reversed-phase chromatography has been reported by others (7).

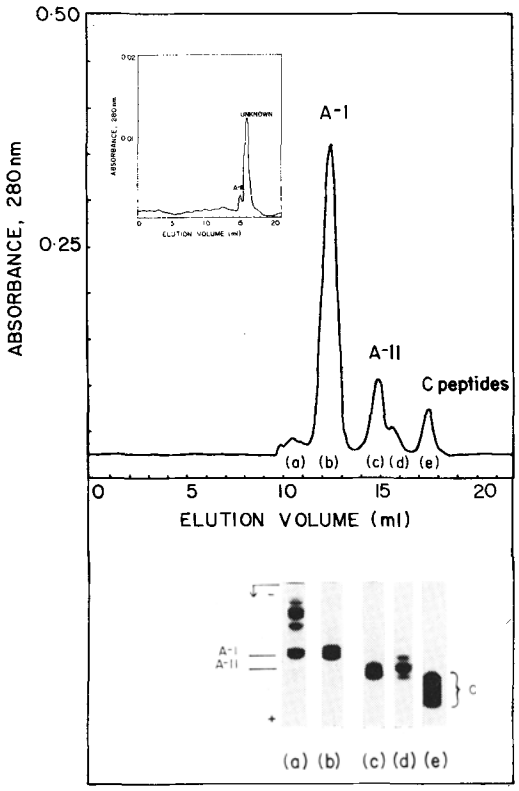

FIG. 1. Top panel: elution pattern from the HPLC column of apo HDL in 6 M urea pH 3.15. Conditions: injection volume, 100 µl at 10 mg/ml of apo HDL; flow rate, 1 ml/min; chart speed, 1 cm/min; 280 nm absorbance range, 0.5. Bottom panel: SDS gel electrophoretic patterns of eluted fractions from a through e. Approximately 0.05 mg of protein was applied. The gels were stained with Coomasie Blue. Inset: elution profile of rechromatographed fraction d (shoulder of apo A-II peak). The chromatographic conditions were essentially the same, except that only 0.02 mg of protein was injected.

In order to correlate protein load with column resolution, apo HDL at a concentration of 10 mg/ml was applied to the column using fixed volume injection loops of 100, 200, 300 and 500 µl. Under our chromatographic conditions, the highest resolving power was accomplished up to a maximal total protein load of 3 mg. A resolution value of 1.7 between the apoA-I and apoA-II peaks was obtained. (The resolution, R, is defined as $R = \Delta t/w_2+w_1$, where Δt is the retention time between 2 peaks and w is the peak width in units of time.) In general, a value of 1.0 or greater is required for a good separation (8). The percentage protein recovery obtained under these conditions was above 90%.

A significant advantage of this method is the ease and rapidity with which apoA-I and apoA-II can be obtained in mg quantities and in high purity. For instance, we find this procedure ideal when we need to prepare fresh, pure antigens for our immunological work. Moreover, the fact that the fractionation is

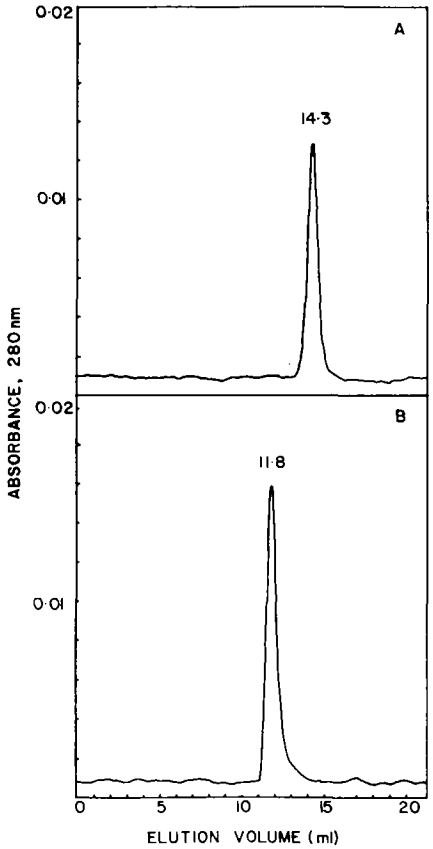

FIG. 2. Elution pattern of apoA-II (A) and apoA-I (B) under identical column conditions as in Fig. 1, except that 0.02 mg of apoproteins was injected and scanned at 0.02 absorbance range. The apoproteins were prepared and purified by conventional procedures using Sephadex and anion exchange chromatography.

done in an acidic buffer (pH 3.15) eliminates urea-related protein changes since cyanate formation and, thus, carbamylation of the lysine residues of proteins is inconsequential at this pH (9). A further advantage of the HPLC method is that it can be applied in the final purification step of apoproteins separated by conventional chromatography. In the example presented in Figure 3, impure apoA-II, obtained by DEAE ion exchange chromatography, upon rechromatography using our HPLC system, eluted as a symmetrical peak in the position of a pure apoA-II standard (14.3 ml). We must add that the sensitivity of the detection system can be significantly improved, e.g., by using the absorbance of the peptide bond at 220 nm. Under these conditions, the purity of

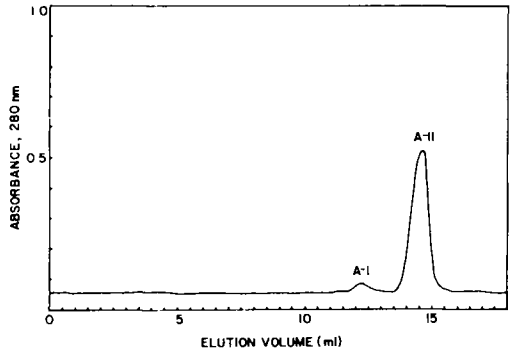

FIG. 3. Repurification by HPLC of apoA-II which was obtained by DEAE anion exchange chromatography. Conditions: 0.7 mg of protein applied; flow rate, 0.5 ml/min; chart speed, 1 cm/min.

apoproteins can be assessed and quantified within a 0.001% limit.

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COMMUNICATIONS

Enzymatic Formation of Cholesteryl Ester from Cholesterol by Gallbladder Mucosa

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ABSTRACT

The formation of cholesteryl ester from cholesterol and acyl CoA catalyzed by the enzyme acyl CoA:cholesterol acyltransferase (EC 2.3.1.26) was studied in guinea pig gallbladder mucosa homogenate and the subcellular fractions. The enzymatic activity was enriched in the microsomal fraction. Highest activity was observed with palmitoyl, stearoyl or oleoyl CoA as substrate. Lowest activity was observed with linoleoyl CoA. These data elucidate one mechanism for the formation of cholesteryl ester from cholesterol by the gallbladder wall.

INTRODUCTION

In cholesterosis of the gallbladder, large quantities of cholesteryl ester accumulate in the gallbladder wall (1-3). One source of this cholesteryl ester is the bile within the gallbladder. Although bile does not contain cholesteryl ester, it does contain large quantities of nonesterified cholesterol (4). Human gallbladder bile is saturated or often supersaturated with cholesterol (5,6) and this cholesterol is thus available for absorption by the gallbladder mucosa. We observed with an animal model that, after instillation of [^{14}C]cholesterol dissolved in bile into the in situ gallbladder, cholesterol was absorbed by the mucosa (7). Further, a portion of this absorbed [^{14}C]cholesterol was converted to [^{14}C]cholesteryl ester (7).

In previous papers (8,9), we reported on the absorption and metabolism by the gallbladder mucosa of lysophosphatidylcholine, a toxic phospholipid found in the bile of patients with acute cholecystitis (10). In this report, the formation of cholesteryl ester catalyzed by the enzyme acyl CoA:cholesterol acyltransferase (EC 2.3.1.26) in the gallbladder mucosa was studied.

MATERIALS AND METHODS

Analytical Procedures

Radioactivity was measured as previously described (8,11). Fatty acids in a lipid were quantified by gas chromatography after direct methylation with 10% boron trichloride/methanol (w/v) using heptadecanoic acid as the internal standard (12,13). Protein concentrations were determined by the procedure of Lowry et al. (14) for insoluble protein with bovine serum albumin as standard. Cholesterol, cholesteryl ester and fatty acid were separated on Silica Gel G with a solvent containing light petroleum ether/diethylether/acetic acid (80:20:1, v/v) (15). Cholesterol and total bile salts were quantified as previously described (7).

Materials

Silica Gel G plates used for analytical thin layer chromatography (TLC) and the cellulose plates used for preparative TLC were purchased from Analtech, Newark, DE. Silica Gel H plates used for preparative TLC were prepared from commercial absorbent (Sigma Chemical Co., St. Louis, MO). All solvents used for preparative TLC were redistilled prior to use. Fatty-acid-free bovine serum albumin was prepared according to the method of Chen (16).

Cholesterol (Sigma Chemical Co.) was recrystallized 3 times from 95% ethanol prior to use (mp 149-150 C [lit. 149 C]) (17). Cholesteryl oleate and all carrier fatty acids were purchased from Applied Science Labs., State College, PA, and cholesterol [1-^{14}C]oleate from New England Nuclear, Boston, MA. Chromatographically pure egg phosphatidylcholine was prepared as previously described (18).

[1-^{14}C]Palmitic, oleic, stearic and linoleic acids were purchased from New England Nuclear. When necessary, the radioactive fatty acids were purified by preparative TLC. The lithium salt of CoA was purchased from PL Biochemical Inc., Milwaukee, WI.

The [1-^{14}C]acyl CoA derivatives were synthesized and purified as previously described

(9). The acyl CoA derivatives used in these experiments had a specific radioactivity of from 4392 to 5123 dpm/nmol.

Enzyme Assay

Guinea pig gallbladder homogenate and subcellular fractions were prepared in 0.25 M sucrose as previously described (7,9). Succinic dehydrogenase, acid phosphatase and glucose-6-phosphatase activities were measured by standard procedures (9). Acyl CoA:cholesterol acyltransferase was measured by determining formation of cholesteryl [1-^{14}C]ester from cholesterol and [1-^{14}C]acyl CoA. A typical incubation consisted of 40 nmol added cholesterol dispersed in 120 nmol egg phosphatidylcholine and 3 mg fatty-acid-free albumin in 1 ml 0.1 M Tris:HCl buffer, pH 7.4. Buffers containing phosphate were also used for the studies on the effect of changes in pH. [1-^{14}C]-Acyl CoA, usually 74 nmol, in 0.1 ml water, pH 7.4, was added to this mixture at 37 C. The enzyme source, 0.1-0.5 mg protein in 0.1 ml 0.25 M sucrose, was then added to make a final incubation vol of 1.2 ml. The reaction mixture was incubated at 37 C with gentle agitation for up to 60 min and was stopped by addition of 5 ml chloroform/methanol (1:4, v/v).

For the blank, 0.1 ml of a boiled gallbladder protein preparation was incubated as already described. The lipids were isolated as previously described (9) and dissolved in 2 ml chloroform/methanol (1:1, v/v) and the radioactivity in 0.1-ml aliquot was measured. The lipids in a second 0.1-ml aliquot were separated by TLC and the spots corresponding to standard cholesteryl ester and fatty acid were visualized with iodine vapor, scraped into counting vials, and then the radioactivity was measured. The proportion of radioactivity incorporated into cholesteryl ester was converted to nmol on the basis of the known amount of [1-^{14}C]acyl CoA added to the incubation.

To test for the presence of a neutral cholesteryl ester hydrolase (EC 3.1.1.13), incubations were performed with 10 nmol [1-^{14}C]-cholesteryl oleate in place of the acyl CoA and cholesterol. All other conditions were the same as already described.

Statistical Analysis

All values are reported as mean ± SD for experiments usually performed in triplicate. For the studies on the effect of time and protein on enzymatic activity, 12 data points from each graph were submitted to linear regression least squares analysis for paired data and the coefficient of correlation (r) is presented.

RESULTS

Subcellular Distribution of Acyl CoA:Cholesterol Acyltransferase

Table 1 presents the distribution of acyl CoA:cholesterol acyltransferase in guinea pig gallbladder mucosa. Although 40% of the protein from the gallbladder homogenate was recovered in the 100,000 × g soluble fraction, only 8% of the enzymatic activity was here. The microsomal fraction had 9% of the protein and 37% of the enzymatic activity. This represents a 4-fold purification of the activity in the homogenate. This distribution of acyl CoA: cholesterol acyltransferase activity was identical to that of our microsomal marker, glucose-6-phosphatase, where we found 30% of the activity in the microsomal fraction. This was also identical to the distribution of acyl CoA: lysophosphatidylcholine acyltransferase (9).

We also tested homogenate and subcellular fractions for the presence of neutral cholesteryl ester hydrolase activity. Under the conditions of our assay, no hydrolysis of cholesteryl

TABLE 1

Distribution of Acyl CoA:Cholesterol Acyltransferase in Guinea Pig Gallbladder Mucosa[a]

	Protein (mg) (%)	Enzyme units (pmol min^{-1}) (%)[b]	Sp act (pmol · min^{-1} · mg^{-1})
Whole homogenate[c]	3.1 ± 0.1 (100%)	10.4 ± 0.4 (100%)	3.4 ± 0.2
Nuclear, debris	0.7 ± 0.1 (21%)	3.1 ± 1.0 (30%)	4.9 ± 1.6
Mitochondria	0.3 ± 0.1 (9%)	1.1 ± 0.5 (10%)	3.5 ± 1.2
Postmitochondria	0.3 ± 0.1 (9%)	2.7 ± 0.6 (26%)	9.4 ± 1.7
Microsomes	0.3 ± 0.1 (9%)	3.8 ± 0.9 (37%)	13.1 ± 2.0
Soluble	1.4 ± 0.2 (46%)	0.8 ± 0.4 (8%)	0.6 ± 0.2

[a]Values represent mean ± SD from 3 separate experiments using 4 animals/experiment (9).

[b]Percentage of enzyme units is based on 100% activity in the gallbladder homogenate.

[c]Values are for 1 gallbladder.

[1-^{14}C] oleate was observed. No measurable quantities of bile acids were found in the homogenate or the subcellular fractions.

Enzymatic Activity in Microsomal Fraction

In Figure 1A, B and C, the data for formation of cholesteryl [1-^{14}C] oleate by guinea pig gallbladder microsomes are presented. The formation of cholesteryl oleate was linear with time up to 60 min of incubation with microsomal protein (Fig. 1A, r=0.995). We also observed linear formation of cholesteryl oleate with protein over a range of 0.1-0.5 mg of microsomal protein (Fig. 1B, r=0.995). The activity of this enzyme was optimal over a range of pH 6-9 (Fig. 1C). Activities above pH 9 could not be accurately measured because the substrate, acyl CoA, spontaneously decomposes to fatty acid and CoA at pH 10 and above.

Effect of Albumin and Bile Salts on Activity

In the absence of added albumin, the activity of this enzyme was 8 pmol • min^{-1} • mg^{-1}. Addition of 1.5-6 mg of fatty-acid free albumin to the incubation mixture increased enzymatic activity to values ranging from 11 to 15 pmol • min^{-1} • mg^{-1}. Addition of 0-1 mM of the bile salts sodium taurocholate or sodium deoxycholate had no effect on the enzymatic activity, whereas 5 mM bile salts abolished enzymatic activity.

Substrate Effect

In Table 2, the effect of different substrates on the activity of acyl CoA:cholesterol acyltransferase in the gallbladder microsomal fraction is presented. For palmitoyl, oleoyl and stearoyl CoA, the same sp act was observed (12-13 pmol • min^{-1} • mg^{-1}); however, with linoleoyl CoA, the activity was considerably less (3.7 ± 1.8 pmol • min^{-1} • mg^{-1}). The activity correlated directly with increasing substrate concentration of each substrate and the concentration of acyl CoA used in these experiments (60 μM) was found to be optimal. Higher concentrations of acyl CoA inhibited the activity.

DISCUSSION

Although acyl CoA:cholesterol acyltransferase activity has been demonstrated in liver (19-21), intestine (22,23) and aorta (24,25), this is the first demonstration of this activity in the gallbladder mucosa. In a preliminary communication, Ravi-Subbiah and Dicke (26) showed that the 1,000 × g supernate of the gallbladder formed cholesteryl ester from cholesterol and [^{14}C] fatty acid. The exact

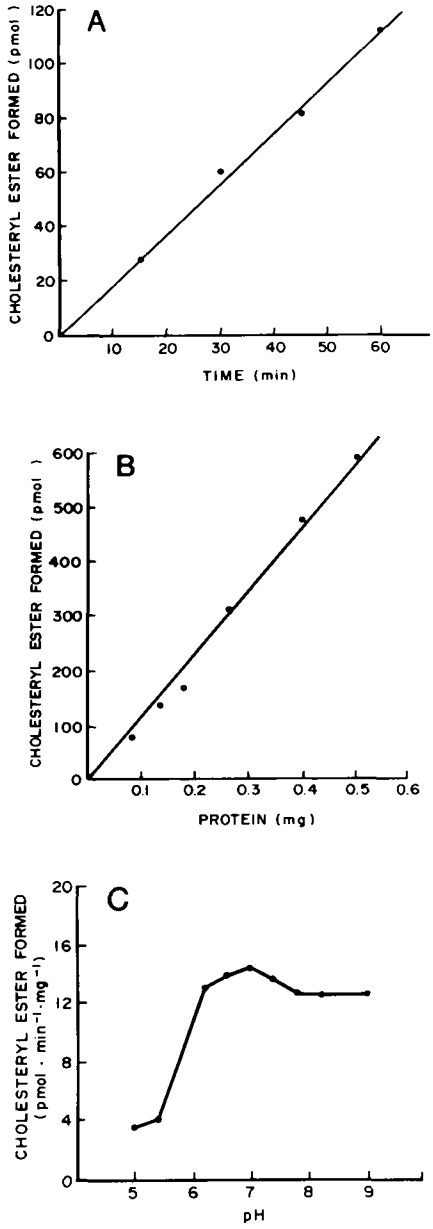

FIG. 1. The formation of cholesteryl oleate by guinea pig gallbladder microsomal protein. Assays contained 40 nmol of cholesterol, 74 nmol [1-^{14}C]-oleoyl CoA, and were incubated at 37 C. Data points are the means obtained from experiments performed in triplicate. (A) Time course for cholesteryl oleate formation. Incubations were done with 0.15 mg microsomal protein at pH 7.4. (B) Effect of protein concentration on cholesteryl oleate formation. Incubations were done at pH 7.4 for 60 min. (C) Effect of pH on cholesteryl oleate formation. Incubations were done with 0.15 mg microsomal protein for 60 min.

TABLE 2

Microsomal Acyl CoA:Cholesterol Acyltransferase Activity with Various Substrates[a]

Substrate	Cholesteryl ester formed (pmol · min^{-1} · mg^{-1})
Palmitoyl CoA	13.3 ± 4.7
Stearoyl CoA	11.9 ± 3.8
Oleoyl CoA	13.1 ± 2.0
Linoleoyl CoA	3.7 ± 1.8

[a]Values are mean ± SD, n=4.

mechanism of this synthesis, however, was not determined. Our data show that the synthesis of cholesteryl ester from exogenous cholesterol absorbed from bile by the gallbladder mucosa can use an acyl CoA:cholesterol acyltransferase activity (EC 2.3.1.26).

We observed that bile salts up to 1 mM had no effect on cholesteryl ester synthesis whereas higher concentrations (5 mM) inhibited the activity. Normally, the intracellular concentration of bile salts for the gallbladder mucosa is minimal; however, in a damaged gallbladder, they can increase. Ostrow found that guinea pig gallbladders with mucosal injury or those from animals with spontaneously diseased gallbladders have a greater permeability to ionized substances, such as bile salts, but not to lipid-soluble substances such as cholesterol (27). Thus, the intracellular concentration of bile salts can increase under these conditions and potentially affect cholesteryl ester synthesis.

We found that the synthesis by the gallbladder mucosa of cholesteryl oleate, palmitate and stearate were essentially the same and that cholesteryl linoleate formation was considerably depressed. Stein et al. (28) also observed the same specificity for this enzyme from human fibroblasts, as did Hashimoto et al. (29) for this enzyme in microsomes of rabbit aorta. This led Hashimoto et al. to suggest that this specificity may explain the dominance of cholesteryl oleate in rabbit atheroma. Analysis of the cholesteryl esters from human gallbladders with cholesterosis show that oleic, palmitic and stearic acids make up 93% of the fatty acids in the cholesteryl esters and that linoleic acid is a minor fatty acid (30). Although the present study demonstrates the presence of an enzyme which synthesizes these cholesteryl esters in the gallbladder mucosa, it does not show that this enzyme is responsible for cholesteryl ester accumulation in the diseased state. Other factors, such as the reversal of cholesteryl ester synthesis or the transfer of cholesteryl ester may also be involved in the etiology of this disorder.

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Effect of Buffer Constituents on Rat Liver 3-Hydroxy-3-methyl Glutaryl Coenzyme A Reductase

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ABSTRACT

Rat liver microsomes prepared in Tris buffer exhibited 3 to 10 times higher 3-hydroxy-3-methyl glutaryl CoA reductase specific activity than microsomes prepared with potassium phosphate buffer. This higher activity was observed in rats killed during mid-light cycle, but microsomes from rats killed during mid-dark cycle showed no significant difference in enzyme activity between buffers. When microsomes prepared in the 2 different buffers were preincubated with ATP and MG^{++}, enzyme activity was inhibited to the same extent. The cytosol fraction in each of the 2 different buffer preparations possessed similar phosphatase activity. The higher 3-hydroxy-3-methyl reductase activity in Tris buffer, therefore, does not appear to be due to differences in phosphorylation or dephosphorylation activity.

INTRODUCTION

The role of 3-hydroxy-3-methyl glutaryl CoA (HMG CoA) reductase as the major regulatory enzyme in the biosynthesis of cholesterol has been documented by many investigators (1-4). Regulation of this enzyme in rat liver occurs in several different ways: diurnal rhythm (3,5), hormonal control (5-7), and phosphorylation and dephosphorylation of the enzyme itself (8). Microsomal HMG-CoA reductase is extensively inactivated in vitro in the presence of Mg^{++} and ATP, and by a protein present in microsomes and cytosol (8-13). This inactivation was shown to be due to phosphorylation of the enzyme by incubating with [^{32}P]ATP and Mg^{++}, followed by precipitation of the radioactive enzyme with antiserum to HMG-CoA reductase (14,15). Lee and coworkers showed that the ATP and Mg^{++}-inactivated microsomal reductase could be reactivated by incubating the inactive enzyme with liver phosphoprotein phosphatase (16,17). The incorporation of acetate into cholesterol has been shown to be higher if the rat liver homogenate is prepared in Tris buffer rather than phosphate buffer (18). The present report deals with investigations of HMG-CoA reductase activity in Tris and phosphate buffers under varying conditions.

MATERIALS AND METHODS

DL-[3-^{14}C]HMG-CoA (sp act 18.5 μCi/μmol) was purchased from New England Nuclear Corp. Unlabeled HMG-CoA (from PL Biochemicals, Milwaukee, WI) was added to the radioactive substrate to give a sp act of 0.8 μCi/μmol, and this substrate was used in all the experiments. DL-[4-^{3}H]mevalonic acid was purchased from Amersham/Searle. Unlabeled mevalonic acid was obtained from the Sigma Chemical Co.

After light ether anesthesia, rats were killed by decapitation during the midpoints of the dark phase or the light phase. Livers were divided into 2 portions and homogenized with a Polytron using either 0.04 M potassium phosphate buffer, pH 7.2, or 0.04 M Tris HCl buffer, pH 7.2, containing 0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA and 0.01 M dithioerythritol. Microsomes were prepared by the method of Srikantaiah et al. (19), and HMG-CoA reductase activity was assayed according to the method of Ackerman et al. (20).

To study the inhibition by ATP and Mg^{++}, the microsomes were prepared in the different buffers as just described, except that EDTA was excluded. Microsomes were then preincubated with 2 mM ATP and 4 mM $MgCl_2$. The reaction was stopped by adding 0.03 M EDTA containing 4 mM NADPH. After addition of 75 nmol of [^{14}C]HMG-CoA (300 μM final concentration), the remainder of the procedure was done as described by Ackerman et al. (20). Enzyme activity was calculated as pmol mevalonolactone formed/mg protein/min.

Phosphatase activity was assayed in the cytosol fraction of the rat liver preparations in the 2 different buffers at pH 7.2 using *p*-nitrophenyl phosphate as the substrate according to the method of Bessey et al. (21).

Microsomal and cytosol protein contents were determined by the method of Bradford (22).

RESULTS AND DISCUSSION

HMG-CoA reductase activity in microsomes prepared in Tris buffer from the rats killed during midpoint of the light cycle was substantially higher than that in microsomes prepared in phosphate buffer (Table 1). No significant

TABLE 1

HMG-CoA Reductase and Phosphatase Activity in Tris or Phosphate Buffer

Experiment	HMG-CoA reductase (pmol/min/mg protein)		Phosphatase (μmol/min/mg protein)	
	Phosphate	Tris	Phosphate	Tris
Rats killed at midpoint of dark cycle	242 ± 103 (6.5 ± 1.1)[a]	279 ± 134 (4.3 ± 0.6)[a]	5.0 ± 1.0	4.0 ± 0.05
Rats killed at midpoint of light cycle	35.8 ± 23 (4.7 ± 0.3)[a]	212 ± 125 (3.05 ± 0.5)[a]	3.2 ± 0.4	4.0 ± 0.30

[a]Protein/ml of microsomal suspension.

All values are means ± standard deviations, calculated from 4 different experiments.

Assay conditions are described in Methods for HMG-CoA reductase. Phosphatase activity was tested at pH 7.2 in the appropriate buffer using 0.1 ml cytosol and a 10 mM *p*-nitrophenyl phosphate substrate.

TABLE 2

Inhibition of HMG-CoA Reductase Activity by ATP and Mg^{++}

Experiment		HMG-CoA reductase activity (pmol/min/mg protein)	
		Phosphate buffer	Tris buffer
Rats killed at midpoint of dark cycle	Control	286 ± 21.4	319 ± 22.1
	ATP (2 mM) and Mg^{++} (4 mM)	98.3 ± 19.1	91.3 ± 17.2
Rats killed at midpoint of light cycle	Control	44.6 ± 8.1	182.6 ± 23.7
	ATP (2 mM) and Mg (4 mM)	20.3 ± 2.1	55.0 ± 6.2

Assay conditions are defined in Methods.

All values are means ± standard deviations calculated from 4 different experiments.

difference was observed in microsomes from rats killed during midpoint of the dark cycle. The difference in enzyme activity in the microsomes from light-cycle rats could not be explained by any alteration in phosphorylation regulation of the enzyme in the different buffers, because inhibition by ATP and MG^{++} was comparable for microsomes prepared either in phosphate or Tris buffer (Table 2). The phosphatase activity in the cytosol of the 2 different buffer preparations as tested using the *p*-nitrophenyl phosphate substrate showed similar activities, indicating unchanged dephosphorylation (Table 1). Possible inhibition of HMG-CoA reductase by phosphate or activation by Tris were ruled out by observing no changes in activity using mixed buffer assays. (Microsomes from light-cycle rats, which gave a sp act of 150 units in Tris buffer, remained the same with phosphate buffer added up to 0.07 M.) The increase in HMG-CoA reductase activity can be partly ascribed to an observed 25% reduction in total protein content in the microsomal preparations in Tris buffer (presumably without a reduction in enzyme protein). It is, at present, unknown what effect Tris hydroxy methyl amino methane has on the membrane enzymes. The reduction in total protein content for microsomes prepared in Tris buffer suggests the absence of a protein enzyme inhibitor in this preparation. Tris (0.02 M) does not interfere with the protein determination (22).

The only previous report on the effect of buffer on cholesterol biosynthesis is the abstract by Hotta (18), who demonstrated that rat liver homogenates prepared in Tris buffer alone incorporate more acetate and mevalonate into cholesterol than if phosphate is added to the buffer.

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Exchange of Phosphatidylcholine between Rabbit Erythrocytes and Plasma in vivo

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ABSTRACT

Phosphatidylcholine exchange between rabbit erythrocytes and plasma was studied in vivo. The erythrocyte phosphatidylcholine was labeled by exchange in vivo with [^{32}P] phosphatidylcholine and in vitro by acylation with [^{3}H] 16:0 and [^{14}C] 18:2. The erythrocytes were then injected into rabbits and the loss of labeled phosphatidylcholine from the cells by exchange was followed. The rate constants for the exchange of [^{32}P]-, [^{3}H] 16:0-, and [^{14}C] 18:2-phosphatidylcholine were .0131 ± .0010, .0093 ± .0014 and .0074 ± .0013 h^{-1}, and the exchange rates of the labels relative to that of [^{32}P] were 1.0, 0.71 ± .16, and 0.56 ± .14, respectively. These results confirm our earlier in vitro findings and represent the first in vivo demonstration of the dependency of the exchange rate of erythrocyte phosphatdylcholines on their metabolic prehistory.

INTRODUCTION

The exchange of phospholipid in vitro between mammalian erythrocytes and various acceptors such as whole plasma (1-4), isolated lipoproteins (3,5) phospholipid vesicles (6,7) and liver microsomes (8,9) has been investigated intensively for many years. In some of these studies, intracellular phospholipid exchange proteins from various organs were used to facilitate the transfer of phospholipid between donor and acceptor (6-9). The exchange of phosphatidylcholine (PC) between the outside of the erythrocyte membrane and the acceptor in the medium is rapid only in the presence of added intracellular (from heart or liver) phospholipid exchange protein (7-9). In other more physiological experimental systems, such as those in which only whole serum or plasma is used as the medium, the rate of exchange is slow, i.e., about 1%/hr (2,3). Thus, while phospholipid exchange protein is useful as a model system probe, it has little physiological significance in relation to the erythrocyte. Indeed, with few exceptions (2), the physiological importance of all of the in vitro investigations remain to be demonstrated in vivo. Earlier we showed in vitro that the rates of exchange of PC between rabbit erythrocytes and serum depended on the origin of the PC. Those PC formed in the cell by acylation of 9,12-octadecadienoic (linoleic) and hexadecanoic (palmitic) acids with endogenous lysophosphatidylcholine exchanged at rates only 53 and 64%, respectively, of the rate of ^{32}P-labeled PC which originally entered the erythrocyte by exchange (3). In this report, we provide results of a corresponding in vivo study of these phenomena.

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MATERIALS AND METHODS

[9,10-^{3}H] Palmitic acid, [1-^{14}C] linoleic acid and $^{51}CrO_4$ were obtained from New England Nuclear Corp., Boston, MA, and $Na_3{}^{32}PO_4$ was from Charles E. Frosst and Co., Montreal, Quebec. New Zealand White rabbits were supplied by Canadian Breeding Farms, St. Constant, Quebec.

The PC of rabbit erythrocytes were labeled in vivo by exchange with [^{32}P] PC from the plasma, and in vitro by acylation of erythrocyte lysophosphatidylcholine with [^{3}H] palmitic acid and [^{14}C] linoleic acid. The methods used have been described in detail before (3), but are outlined briefly here. $Na_3{}^{32}PO_4$ was administered intravenously or intraperitoneally to donor rabbits and, after 3-4 days, the blood was collected. The ^{32}P-labeled erythrocytes, freed of the buffy coat and repeatedly washed, were incubated with a mixture of [9,10-^{3}H] palmitic acid and [1-^{14}C] linoleic acid in Krebs-Ringer bicarbonate buffer for 2 hr at 37 C. During the last half hour of this acylation incubation, the erythrocytes were additionally labeled with $^{51}CrO_4$ by the method of Mager et al. (10) in order to allow correction for loss of radioactivity due to removal of erythrocytes from the circulation. Two μCi of $^{51}CrO_4$/ml incubation mixture was added to the incubation medium. Incorporation of radioactive chromate was stopped 5 min before the end of the incubation by addition of ascorbic acid to a final concentration of 1 mg/ml. The erythrocytes were then separated from the medium and washed as before (3). Thus, all the erythrocytes prepared for infusion had been subjected to the same labeling conditions and were tagged with all 4 isotopes.

Fifteen to 20 ml of the labeled erythrocytes suspended in saline at a hematocrit of 75-80%

was infused over a period of a few minutes into recipient rabbits via the marginal ear vein. The first sample was taken 3/4 hr later, in order to allow sufficient time for mixing of the labeled cells in the circulation, and was considered the "zero time" control. The samples were taken via a femoral catheter or by cardiac puncture. An aliquot of each blood sample was taken for the γ-counting of ^{51}Cr radioactivity. The erythrocytes and plasma of the remainder of each sample were then separated and the cells washed as before (3). Hemoglobin determinations on the aliquots of each sample were performed by the method of Brownstone and Denstedt (11). Using procedures described earlier (3), the lipids of the cells and plasma were extracted, separated by column and thin layer chromatography, and the PC fraction recovered and quantitated. Decay of ^{32}P radioactivity over the experimental period was corrected back to the time of infusion of the labeled erythrocytes into the animals.

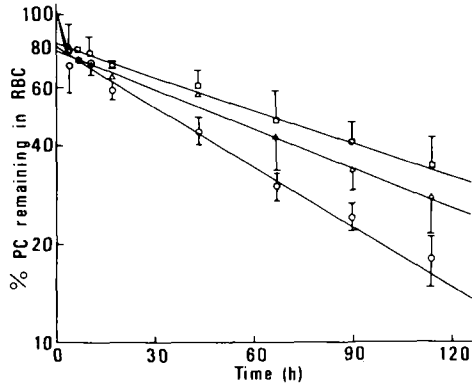

FIG. 1. Exchange of labeled phosphatidylcholine (PC) from rabbit erythrocytes in vivo. Data points are the means from 3 rabbits ± SEM: □, [^{14}C]linoleoyl PC; △, [^{3}H]palmitoyl PC; ○, [^{32}P]PC. Labeled erythrocytes were injected into rabbits and samples of blood were taken as shown. The data were corrected for erythrocyte loss from the circulation and for reverse-exchange from the plasma.

RESULTS AND DISCUSSION

The loss of labeled erythrocytes from the circulation following infusion of the cells into the rabbits as monitored by ^{51}Cr/mg Hb was found to be logarithmic for each animal over the course of the experiments, but varied from one animal to another. Therefore, for each sample, fractional corrections for the corresponding losses of PC radioactivity were made by calculation, respectively, of $^{3}H/^{51}Cr$, $^{14}C/^{51}Cr$, and $^{32}P/Cr$. Appropriate correction, based on PC concentrations in cell and plasma, was also made for back exchange from the low levels of radioactive PC appearing in the plasma during the experiment (12). The data for each sample were normalized to 100% for the "zero time" control of each animal. The means of the normalized results from 3 rabbits are shown in Figure 1. With each label, there appeared to be an initial rapid, and as yet unexplained, drop in radioactivity between the first and second time points. After that, the decay of radioactivity was exponential for the remainder of the experiment.

From the linear portion of the curves, the rate constants for the exchange of the labeled PC were determined and the relative exchange rates of the acyl-labeled PC were calculated. These are compared in Table 1 with the corresponding data from our previous in vitro investigation (3). The rate constants for the exchange of the PC in vivo are similar to those calculated from the in vitro data. The exchange rates of the acyl-labeled PC relative to the ^{32}P-labeled PC are also in very good agreement. Both the in vivo and in vitro data show that the rates of exchange of erythrocyte PC derived originally from the acylation of fatty acid with endogenous lysophosphatidylcholine are significantly lower than that of PC which entered the erythrocyte membrane originally by exchange. Also, in both studies, the PC labeled with [^{14}C]linoleate exchanged at a rate which was

TABLE 1

Comparison of the Rate of Exchange of Acyl- and ^{32}P-Labeled Phosphatidylcholines between Erythrocytes and Plasma in vitro (3) and in vivo

Radioactive label in phosphatidylcholine	Rate constant X 10^3 (h^{-1})		Relative exchange rates	
	in vivo	in vitro	in vivo	in vitro
[^{32}P]	13.1 ± 1.0 (3)[a]	16.6 ± .64 (14)	1.0	1.0
[^{3}H]Palmitate	9.33 ± 1.4 (3)	9.95 ± .17 (10)	.71 ± .16	.64 ± .12
[^{14}C]Linoleate	7.37 ± 1.3 (3)	8.78 ± .10 (10)	.56 ± .14	.53 ± .05

[a]Mean ± SEM (n).

somewhat lower than that labeled with [^{3}H]-palmitate. The differences observed in the exchange rates for the different labeled PC probably is related to variation in their location in the erythrocyte membrane, which probably arose from differences in their individual metabolic origins. The palmitate- and linoleate-labeled PC probably were generated, in part, in the interior side of the plasma membrane and may have occupied slightly different pools in the membrane. On the other hand, the [^{32}P]PC probably resided mainly on the external side of the erythrocyte membrane.

This in vivo study therefore confirms our earlier in vitro conclusions that the rate of exchange of erythrocyte PC varies with the metabolic origin of the molecular species of the phospholipid being exchanged. Furthermore, it provides the first in vivo evidence of this dependency of the rate of exchange of PC on the acylation reaction in the erythrocyte and probably on the molecular species of PC thus formed.

ACKNOWLEDGMENTS

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Distribution of Long Chain Polyenoic Acids among Phospholipids of Mouse Testis

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ABSTRACT

Fatty acid composition of phospholipid (PL) classes was measured in mouse testis. Among the long-chain polyenoic acids (LCPA), 22:6 was found in highest concentration in phosphatidylethanolamine (PE), whereas percentages of 20:4 and 22:5 were not different in PE than in phosphatidylcholine. Each PL class had a unique fatty acid composition which was also different from that of triglycerides and cholesteryl esters. Differential metabolisms of 22:5 and 22:6 suggest different roles for these fatty acids in mouse testis. Tissue-specific functions of LCPA in mouse spermatogenesis may be divided between 22:5 and 22:6.

Evidence is growing that 22-carbon LCPA of the linoleic (n-6) and linolenic (n-3) acid families play a specific and obligate role in spermatogenesis in many species (1). LCPA metabolism of mouse testis is of particular interest due to relatively high concentrations of 3 LCPA, 20:4(n-6), 22:5(n-6) and 22:6(n-3) (2), which are disproportionately distributed among the various cell types and lipid classes of adult mouse testis (3). For example, the highest percentages of 20:4 occur in PL of nongerminal cells or germinal cells which are in the earliest stages of differentiation. On the other hand, highest percentages of 22:5 occur in triglycerides of germinal cells in relatively late stages of spermatogenesis. Highest percentages of 22:6 also occur in late stages of spermatogenesis, but in phospholipid rather than triglyceride. Moreover, percentages of 22:6 are much less variable among cell types and stages of spermatogenesis than the LCPA derived from linoleic acid. We report here further evidence for the highly specific differential metabolism of these LCPA in PL of mouse testis.

METHODS

Fatty Acid Analysis of Lipid Classes

Total lipids were extracted from testes of 60-day-old mice by the modified Folch procedure of Bridges and Coniglio (4). Extracts were dried under a stream of N_2, redissolved in $CHCl_3$ and separated on Whatman LHP-K silica gel thin layer plates developed with petroleum ether/ethyl ether/HOAc (80:20:1). Bands of PL, triglyceride and cholesteryl ester were visualized with Rhodamine 6G spray (Supelco) under ultraviolet (UV) light and scraped into screw-capped vials for methylation by transesterification with methanolic sodium methoxide as described previously (3). Methyl esters were extracted with petroleum ether and quantitated on a Packard 427 gas chromatograph equipped with dual flame ionization detectors and a 10% SP-2340 (Supelco) column, temperature-programmed from 165-240 C at 4 C/min. Methyl pentadecanoate was used for internal standardization. Methyl esters were identified by comparisons of retention times with those of commercial standards and biological samples of previously determined composition.

Phospholipid Class Analysis

PL classes were quantitated by a modification of the procedure used by Beckman et al. (5). PL classes were separated by 2-dimensional thin layer chromatography (TLC) on Whatman LHP-K silica gel plates using tetrahydrofuran/MeOH/NH_4OH (10:10:1) in the first development and $CHCl_3$/MeOH/acetone/HOAc/H_2O (6:8:2:2:1) in the second dimension. For phosphorus analysis, plates were sprayed lightly with fluorescamine reagent (Supelco) and illuminated by UV light to locate ethanolamine and serine-containing lipids. Plates were then sprayed with 40% H_2SO_4 and charred lightly. Spots corresponding to PL classes were scraped from the plates and analyzed for total phosphorus as described by Bartlett (6).

For fatty acid analysis, plates were scraped first with fluorescamine to locate PE and PS and then developed with Phospray (Supelco) to locate other PL. Spots were scraped from the plates and methylated for gas chromatographic (GC) analysis of fatty acids as already

Abbreviations: LCPA = long chain polyenoic acids; 20:4(n-6) = all *cis*-5,8,11,14-eicosatetraenoic (arachidonic) acid; 22:5(n-6) = all *cis*-4,7,10,13,16-docosapentaenoic acid; 22:6(n-3) = all *cis*-4,7,10,13,16,19-docosahexaenoic acid; PL = phospholipid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; LPC = lysophosphatidylcholine; CL = cardiolipin.

TABLE 1

Phospholipid Composition of Mouse Testis

Phospholipid class	N	Phospholipid (mol %)[a] By phosphate[b]	By fatty acid
Phosphatidylcholine	3	48 ± 1	54 ± 4
Lysophosphatidylcholine	3	10 ± 0.5 $p<.005$	4 ± 1
PC + LPC[c]	6	54 ± 2	55 ± 3
Phosphatidylethanolamine	6	24 ± 1	23 ± 3
Phosphatidylserine	6	5 ± 0.7	5 ± 0.8
Phosphatidylinositol	6	5 ± 0.4	9 ± 2
Cardiolipin	6	3 ± 0.6	3 ± 1
Sphingomyelin	6	9 ± 1	5 ± 2

[a]23.8 ± 0.8 μg phospholipid/mg testis (wet wt).

[b]Percentage of 32 ± 0.6 nmol lipid phosphorus/mg testis (wet wt).

[c]Phosphatidyl- and lysophosphatidylcholine combined for analysis.

described and in Grogan et al. (3).

PL class composition was calculated as a mol % from both phosphate analyses and quantitative fatty acid analyses using stoichiometric correction factors based on the molar ratios of phosphorus or individual fatty acids to the respective PL.

RESULTS AND DISCUSSION

PL class compositions were calculated from both lipid phosphorus analyses and quantitative GC analyses of component fatty acids (Table 1). The values obtained with the 2 methods differ significantly only in mol % LPC and are quite similar to those reported previously for mouse testis (3).

There are substantial differences in the distributions of the various fatty acids among the various PL classes (Table 2). The fatty acid composition of PC is quite similar to that of the total PL, of which PC comprises 50%. LPC has the highest percentage of 16:0 among PL classes and only trace amounts of LCPA, suggesting that this component is almost entirely the 2-LPC (7). As previously reported for other tissues in various species (8), the highest levels of 22:6 are found in PE. However, the percentage of 22:5 is about the same in PE and PC. In contrast, Beckman and Coniglio (9) have found a higher percentage of 22:5 in PE than in PC of rat testis, but no comparable differences in 22:6.

PS and PI are 2-fold higher in percentage of 18:0 than any of the other PL classes. However, PS is indistinguishable from PC in LCPA composition, whereas PI is different from PS in percentages of both 20:4 and 22:5. CL is the only PL class to contain as much as 10% 18:2, but has relatively low levels of LCPA. In like manner, each other lipid class examined had a unique fatty acid composition.

Thus, no 2 PL classes have sufficiently similar fatty acid compositions to suggest that they

TABLE 2

Fatty Acid Composition of Phospholipid Classes of Mouse Testis

Lipid class	Fatty acid (mol %)[a] 16:0	18:0	18:1	18:2	20:4	22:5	22:6
Phospholipid	44 ± 2	12 ± 2	16 ± 2	3 ± 0.3	12 ± 2	8 ± 0.6	4 ± 0.5
Phosphatidylcholine	45 ± 5	11 ± 1	11 ± 1	2 ± 0.6	10 ± 2	9 ± 1	6 ± 2
Lysophosphatidylcholine	59 ± 4	11 ± 1	20 ± 1	2 ± 0.4	trace	trace	trace
Phosphatidylethanolamine	33 ± 4	13 ± 1	9 ± 0.2	3 ± 2	15 ± 2	10 ± 1	11 ± 1
Phosphatidylserine	36 ± 3	24 ± 2	8 ± 1	3 ± 1	8 ± 1	8 ± 2	4 ± 1
Phosphatidylinositol	37 ± 1	28 ± 3	6 ± 2	2 ± 0.4	13 ± 2	3 ± 0.5	4 ± 0.6
Cardiolipin	57 ± 6	2 ± 0.4	12 ± 1	11 ± 2	3 ± 1	4 ± 0.4	3 ± 0.4
Sphingomyelin	38 ± 5	10 ± 1	15 ± 5	6 ± 2	7 ± 2	3 ± 0.7	3 ± 0.5
Triacylglycerol	31 ± 1	9 ± 0.4	26 ± 2	5 ± 0.5	3 ± 0.2	7 ± 1	3 ± 0.7
Cholesteryl esters	20 ± 2	10 ± 0.5	41 ± 2	2 ± 0.3	6 ± 2	–	2 ± 0.6

[a]Means of 6 values ± standard errors of the means; hydroxy fatty acids not determined for sphingomyelin. Trace indicates less than 1%.

are interconverted or derived from a common phosphatidic acid without modification of the acyl groups of the glycerol moiety. This specificity may be imposed at the level of biosynthesis by deacylation-reacylation activities or by selective turnover with respect to fatty acid composition (8). Whatever the responsible mechanism, it is apparently able to discriminate among 20:4(n-6), 22:5(n-6) and 22:6(n-3) in "formulating" the various classes of acylglycerols.

The differential metabolisms of 22:5 and 22:6 seen here and in the earlier report (3) suggest different roles for these LCPA in mouse testis. Although the pattern of distribution with respect to lipid class and cell type for each of these fatty acids resembles, in some respects, the pattern for 22:5 in rat testis (1,5,9), neither 22:5 or 22:6 alone has an entirely analogous distribution in mouse testis. It is possible that specialized functions of 22:5 in the rat testis may be divided between 22:5 and 22:6 in the mouse.

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Lethal Effect of *cis*- but not *trans*-22-Dehydrocholesterol on Mouse Fibroblast Cells

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ABSTRACT

cis- and *trans*-22-dehydrocholesterol were incorporated into the culture medium for mouse fibroblast cells. Although neither isomer had an effect on sterol biosynthesis, the *cis* isomer inhibited cell growth and viability and increased Rb^+ efflux from the cells. The *trans* isomer had no effect on growth and could replace exogenous cholesterol for growth of cells for which sterol biosynthesis was blocked by 25-hydroxycholesterol.

Sterol side-chain unsaturation at Δ^{22} in the ergostane, stigmastane and poriferastane series is widespread in nature, e.g., *trans*-Δ^{22} derivatives brassicasterol, ergosterol, stigmasterol, spinasterol, poriferasterol and chondrillasterol. It is encountered much less frequently in the cholestane series and essentially only in marine organisms (1-4). Both *cis*- and *trans*-22-dehydrocholesterol (*cis*-DHC and *trans*-DHC, Fig. 1) have been prepared (5,6) and recently tested with 2 organisms that do not synthesize sterols. *Tetrahymena pyriformis* stops tetrahymenol biosynthesis in the presence of exogenous cholesterol and metabolizes the latter to *trans*-5,7,22-cholestatrienol. Addition of either *cis*- or *trans*-DHC to its medium also blocked tetrahymenol biosynthesis, but neither isomer inhibited multiplication of the prootozoan (7). *Phytophthora cactorum*, a fungus which grows vegetatively in the absence of sterols but requires them for sexual reproduction, formed oospores when *trans*-, but not when *cis*-DHC, was added to its medium (8). In this communication, we describe the results of incubation of the 2 isomers with mammalian cells.

MATERIALS AND METHODS

cis- and *trans*-DHC were prepared by hydrolysis of their acetates (6), *cis*-DHC mp 140-140.5 C; lit (5) 137-9 C, *trans*-DHC mp 138.3-139 C; lit (5) 133-5 C.

Cell Growth

Mouse fibroblast cells (L-cells) were plated in 25-cm^2 disposable Corning flasks in 5 ml of Waymouth's serum-free 752/1 medium (5-6 × 10^5 cells/inoculum) and allowed to attach to the surface (9). Sterol solutions in EtOH were mixed with 10 vol of 5% BSA in the 752/1 medium and this mixture was added to 10 vol of medium in the culture flasks. After appropriate times, the medium was decanted and the cells washed 3 times with phosphate-buffered saline (pH 7.6). Cell growth was measured by treatment with 1.67% SDS in 1 ml 0.33 N NaOH and subsequent determination of protein content (10). An inoculum of 10 × 10^5 cells was used for the experiment with 4 μg/ml *cis* plus 0-50 μg/ml cholesterol.

cis-DHC Uptake

L-cells (60 × 10^5), that had been incubated in 75-cm^2 flasks for 1 and 2 days with 20 ml medium containing *cis*-DHC (2 and 4 μg/ml) were washed 3 times with Hanks' balanced salt solution and pelleted by centrifugation. They were resuspended in 0.5 ml H_2O, an aliquot (20-μl) was taken for protein determination (10) and an internal standard (30 μg coprostanol) added. The lipids were extracted (11) and analyzed for phosphorus (12) and for free desmosterol and *cis*-DHC by GLC (3% AN 600,255 C) after silation with HDMS and TMCS in pyridine.

Cell Properties

HMG-CoA reductase activity, $^{86}Rb^+$ efflux, and capacity for fatty acid and sterol biosynthesis from [^{14}C]acetate by cells incubated with *cis*- or *trans*-DHC were measured by previously published methods (13-15).

RESULTS AND DISCUSSION

Results from the addition of cholesterol, *cis*-DHC or *trans*-DHC (20 μg/ml) to the medium of L-cells are shown in Figure 1, in which the deleterious effect of the *cis* isomer on cell multiplication is clearly apparent. While *trans*-DHC could replace cholesterol by allowing growth of cells whose sterol biosynthesis has been blocked by 25-hydroxycholesterol (16),

Abbreviations: BSA, bovine serum albumin; SDS, sodium dodecyl sulfonate; HDMS, hexamethyldisilazane; TMCS, trimethylchlorosilane, HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

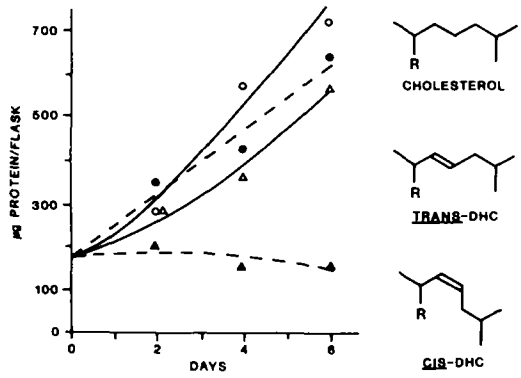

FIG. 1. Growth of L-cells in control medium (○—○) and in the presence of 20 µg/ml cholesterol (△—△), *trans*-DHC (●---●) and *cis*-DHC (▲---▲). Side chains of sterols–R is the tetracyclic Δ^5-3β-OH nucleus.

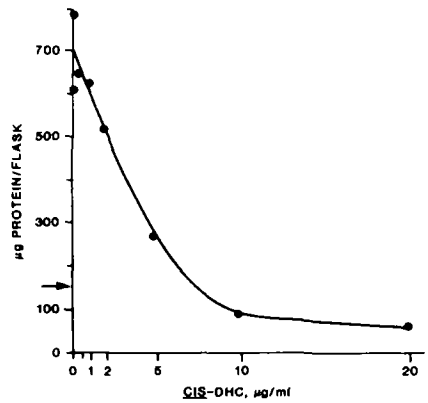

FIG. 2. Four-day growth of L-cells in media containing 0-20 µg/ml *cis*-DHC. Arrow indicates original inoculum.

low concentrations of the *cis* isomer inhibit cell growth, even in the absence of 25-hydroxycholesterol (Fig. 2). Addition of as much as 50 µg/ml cholesterol did not completely overcome the growth inhibition of L-cells incubated with 4 µg/ml *cis*-DHC over a 4-day period (µg protein/flask, average of 2 replicates): control–1160, 50 µg/ml cholesterol–1230, 4 µg/ml *cis*-DHC-300, plus 10 µg/ml cholesterol–580, plus 20 µg/ml cholesterol–790, plus 50 µg/ml cholesterol–920.

Uptake of *cis*-DHC by the cells is shown in Table 1. Even with the low concentrations in the medium, the cells replaced about 40% of their endogenous desmosterol with the *cis*-isomer after 2 days.

Neither isomer had an appreciable effect on the cells' HMG-CoA reductase activity (pmol/min/mg protein): controls–338,445, *cis*-DHC 5 µg/ml–266, 20 µg/ml–354, *trans*-DHC 5 µg/ml–435, 20 µg/ml-330. *cis*-DHC also did not significantly affect the metabolism of [^{14}C]acetate by the cells (dpm CO_2, fatty acids, sterols, all per µg DNA): controls–1040, 79,55; 860,52,56; *cis*-DHC 2 µg/ml–940,64,54; 6 µg/ml–1066,82,46; 20 µg/ml–980,70,43.

When cells were incubated in control or in *cis*-DHC-containing media overnight, washed, then suspended in ^{86}RbCl solution for 2 hr, washed again, and radioactivity measured over a 2.5-hr period in isotope-free medium, those which had been incubated in *cis*-DHC medium had a greater rate of $^{86}Rb^+$ efflux than the controls (Fig. 3). This increased permeability of the cells to Rb^+, coupled with the lack of metabolic changes induced by *cis*-DHC (HMG-CoA reductase, [^{14}C]acetate to CO_2, fatty acids, sterols), indicates that this isomer changes the cells' membrane properties. *trans*-DHC appears to be equivalent to cholesterol in its effect on normal L-cells or on cells treated to block sterol synthesis.

The extended side chain of *trans*-DHC resembles that of cholesterol whereas the

TABLE 1

Sterol Composition of L-cells Incubated with 2 and 4 µg/ml *cis*-DHC for 1 and 2 Days

Sample	Days	Protein (mg)	All values/mg protein: Lipid phosphorus (µg)	Desmosterol (µg)	*cis*-DHC (µg)
Control	1	1.7	7.5	16	–
	2	3.8	6.3	15	–
cis-DHC, 2 µg/ml	1	2.0	6.1	14	5.2
	2	3.3	7.6	13	5.4
cis-DHC, 4 µg/ml	1	2.0	6.5	13	9.4
	2	3.4	8.5	11	8.9

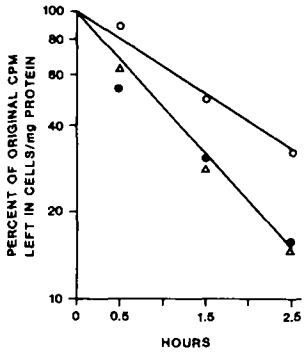

FIG. 3. Efflux of $^{86}Rb^{+}$ from L-cells preincubated 18 hr in control (○—○) and 4 μg/ml (△—△), and 10 μg/ml (●—●) *cis*-DHC media.

Δ^{22} bond in the *cis* isomer produces a kink in the chain (Fig. 1) and perhaps renders it less suitable for interdigitation between phospholipid acyl chains in membranes (17). Consequently, membrane permeability is altered, cell replication is blocked, and with sufficient *cis*-DHC in the medium, the cells used to initiate the culture die after several days due to the exchange of their membrane sterols with exogenous *cis*-DHC.

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LETTER TO THE EDITOR

An Improved Procedure for the Isolation of Ghost Membranes from Human Red Blood Cells

Sir:

In following the published procedures for the preparation of ghosts from human erythrocytes (1,2), we have consistently been troubled by the persistence through successive washes, of a small volume of somewhat more dense, red-colored material. Prolonged standing of the ghost membranes in cold, hypotonic buffer (1-12 hr), or the addition of EDTA, or change in the pH of the buffer did not improve the situation. Unfortunately, we could not simply discard this red material because we ultimately wished to perform a quantitative extraction of the lipids from the ghost membranes.

We have found that the problem is caused by washing the initially produced ghost material with the same buffer solution used in the initial preparation. By a simple modification of the published procedure so that washing is performed with buffers of successively weaker concentration, *a high yield of white ghosts is obtained after only 2 washes.*

Our procedure follows the method of Steck and Kant up to the initial preparation of the ghosts (2). Thus, red blood cells freshly donated or obtained from the Red Cross Blood Bank are washed 3 times in phosphate-buffered saline and a sample of the washed, packed, red blood cells is hemolyzed by rapid and thorough mixing with chilled 5 mM phosphate buffer, pH 8.0. The ghost pellet obtained after centrifugation and aspiration of the supernatant is washed by resuspending it in 2.5 mM phosphate buffer, pH 8.0, and centrifuging as before. The ghosts are washed a second time by an identical procedure using 1.25 mM phosphate buffer, pH 8.0.

At this point, the ghosts are free of all visible red material. In order to minimize the loss of ghost cells, we do not attempt to remove the small, hard button of contaminating proteases at the bottom of each tube (2). This is not important for the recovery of lipids.

The lipids of the intact red blood cells and their ghost membranes were extracted by a modification of the Folch-type method as used by Nelson (3). The lipid residue was taken up in *n*-octane and was analyzed for fatty acids, cholesterol and phosphorus. The results, presented in Table 1, indicate a high yield of ghosts from the parent red cells.

It is realized that the lower buffer concentrations used in our wash procedure might lead to some fragmentation of the ghost membranes (4). This, however, does not cause loss of lipid material. It should also be pointed out that the choice of a dilution factor of 2 was somewhat arbitrary and it is possible that smaller reductions in buffer concentration may be just as effective.

TABLE 1

Comparison of Fatty Acid, Cholesterol and Phosphorus Values of Lipid Extracts of Intact, Washed Red Blood Cells and Their Ghost Membranes for 3 Different Blood Samples[a]

Sample	Fatty acid[b]	Cholesterol[c]	Phosphorus
1 Intact cells	833	1382	65
Ghosts	938	1360	81
2 Intact cells	928	1182	93
Ghosts	914	992	112
3 Intact cells	928	1146	95
Ghosts	961	944	100

[a]All units in μg/ml of packed red blood cells.

[b]Total amount of fatty acid determined relative to heptadecanoic acid by GC after hydrolysis by anhydrous NaOMe/MeOH (0.5 M).

[c]Determined by GC relative to cholestane added after the hydrolysis.

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